Vol. 15, No. 1, 2024
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Optimization and Bioassay Guided Comparative Techniques for Efficient Extraction of Lutein Esters from *Tagetes erecta* (Var. Pusa Narangi Genda) Flowers

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Received July 23, 2023, Revised December 11, 2023, Accepted January 8, 2024
First published on the web March 31, 2024; DOI: 10.5478/MSL.2024.15.1.40

Abstract : Capacity of the analytical/quantitative evaluation techniques to satisfy both qualitative and quantitative considerations for effective extraction of marigold oleoresins/xanthophylls and their potential as anti-mycotic and antioxidant activity was assessed. Accelerated solvent extraction (ASE), Soxhlet extraction (SE), Supercritical fluid extraction (SCFE), Cold extraction (CE), and ultrasonically assisted extraction (USE) techniques were evaluated for extraction of oleoresin/xanthophyll content from *Tagetes erecta* (Var. Pusa Narangi Genda) with respect to solvent consumption, extraction time, reproducibility, and yield. Followed by the antifungal and antioxidant activity evaluation. The overall yield of Tagetes oleoresin was higher in ASE (64.5 g/kg) followed by SE (57.3 g/kg), USE (50.7 g/kg), SCFE (45.3 g/kg) and CE (31.6 g/kg). The lutein esters represented more than 80% of the constituents. Further, xanthophyll/ lutein content in oleoresin was found to be quite higher in HPLC ($r^2 = 0.996$) analysis than in the AOAC recommended UV spectrophotometer analysis. The oleoresin exhibited moderate antioxidant activity (DPPH assay) and antifungal activity against three phytopathogenic fungi. Based on the various parameters, the reproducibility of ASE was better (0.3–8.0%) than that of SE (0.5–12.9%), SCFE (0.2–9.4%), USE (0.3–12.4%) and CE (0.8–15.3%). ASE with (RSD 1.6%) is preferred being faster, reproducible, uses less solvent, robust and automation allows sequential extraction of the sample in less time.

Keywords : Tagetes erecta, Accelerated solvent extraction, Super-critical fluid extraction, Lutein, Antifungal activity, Xanthophyll, Antioxidant, Fatty acid

Introduction

Tagetes genus comprise of more than 50 species. Of these, *Tagetes erecta L*. (African marigold, Aztec marigold) flower petals are a rich source of xanthophylls. It has a much higher concentration of the oleoresin (9.91 g/kg) compared to other plant materials.[1] Marigold is grown extensively in India as well as in several other countries. Lutein the main coloring component of marigold naturally occurs in the ester form. Lutein palmitate is the major ester in the flower. The other lutein esters include dimyristate, myristate palmitate, palmitate sterate, and distearate. The lutein-ester concentration in fresh marigold flowers varies from 4 mg/kg in greenish yellow flowers to 800 mg/kg in orange-brown flowers. Dark-coloured flowers contain about 200 times more lutein esters than the light-coloured flowers.[2] Lutein and zeaxanthin, the major pigment in marigold flower reduces age related macular degeneration.[3] A purified marigold petal extract mainly containing xanthophyll dipalmitate is marketed as an ophthalmologic agent.[4] Lutein is stable in pH range of 3 to 9 and at extreme pH, in the presence of light, lutein undergoes isomerization and/or degradation resulting in color loss.[5]

Bioactive compounds from plant materials are often extracted using the solvent extraction method. Many factors such as solvent composition, solvent to solid ratio,

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extraction temperature, extraction time, etc. has significant influence on the extraction yield.[6] Lutein is conventionally extracted from marigold flowers by solvent extraction,[7] which is rather time-consuming, and the solvent residues can potentially pollute the environment. Extraction of petals and total flower with hexane showed that only the flower petals contain more of xanthophylls.[1]

Additionally, different methods of extraction like solvent extraction, Soxhlet extraction, ultrasonically assisted solvent extraction have been also employed but these are time consuming and require large volumes of organic solvent for extraction. One of the recently introduced techniques[8] namely accelerated solvent extraction (ASE) requires less solvent allows faster extraction and can be performed at different temperatures, time, and pressure to keep solvent in liquid state even at high temperatures. ASE is considered as a suitable alternative to Soxhlet extraction for extracting polycyclic aromatic hydrocarbons (PAHs), lipids, pesticide residues, food additives and medicinal components, biological and plant materials.[9–14]

Furthermore, there has been a growing interest in alternative technologies compared with solvent extraction such as supercritical fluid extraction (SCFE) which is environment friendly.[15] A process has been described for efficient extraction of lutein esters from chlorella by SC-CO_2 employing 50% ethanol as co-solvent.[16] SCFE was also used to extract lutein esters from marigold (T. erecta L.) flowers.[17] Ultrasonic irradiation in combination with supercritical CO_2 extraction has yielded lutein esters (690 mg/100 g) for a particle size fraction of 0.245–0.350 mm.[18] Medium-chain triglycerides (MCTs), sunflower seed oil, soybean oil, rapeseed oil and n-hexane were also evaluated as co-solvents to promote supercritical carbon dioxide extraction of lutein esters from marigold (*Tagetes erecta* L); maximum yield of lutein esters was 1263.62 mg/100 g marigold at an extraction pressure of 46.8 MPa and a temperature of 65.9°C.[18,19]

Tagetes erecta (Var. Pusa Narangi Genda) is a popular variety known for its distinct color and flower size. It has been developed from the cross of Cracker Jack and Golden Jubilee varieties and released in 1995 for commercial use in India. It produces large size deep orange flowers with ruffled florets within 125–135 days after sowing. It has an average yield of 25–30 tons / hectare of fresh flowers with 100–125 kg/ ha of planted seed.[20] Since limited work has been done on chemical composition and biological properties of Pusa Narangi Genda variety of T. erecta therefore, an attempt has been made in this study not only to optimize the consumption of organic solvents, but also to obtain an extract that is as pure as possible, which can be directly injectable and analyzable with various detection systems. Overall, the present study aims at evaluating different methods for their extraction efficiency and evaluation of the xanthophylls of T. erecta (Pusa Narangi Genda) for antioxidant and antifungal activity.

Experimental

Chemicals and reagents

Solvents for extraction (hexane, isopropanol, acetone, n-heptane, toluene, and ethanol) and HPLC analysis (Methanol, Tertiary Butyl Methyl Ether) (TBME), were procured from (Merck India Ltd.). Chemicals such as sodium sulfate, potassium hydroxide, boron trifluoride, quercetin, gallic acid, 1,1-di-phenyl-2-picrylhydrazyl (DPPH) were locally procured and lutein standard (70%) was purchased from (Sigma Aldrich).

Plant material

Tagetes erecta (Var. Pusan Narangi Genda) full blown flower were obtained from Indian Agricultural Research Institute (IARI) farm. The Tagetes crop raised at IARI farm was maintained by Seed Production Unit of the Division of Seed Science & Technology, IARI, New Delhi.

Fungal Cultures: Fungal culture of R. solani (ITCC 4502), S. rolfsii (ITCC 6263) and M. phaseolina (ITCC 6267) was obtained from the Indian type of culture collection, Division of Mycology and Plant Pathology, (IARI), New Delhi, India.

Cold extraction (Solvent extraction)

The lyophilized dried flower powder (50 g) of *T. erecta* (var. Pusa Narangi Genda) was kept soaked in hexane (500 mL) in a conical flask for time intervals ranging from 2 to 10 hr at ambient temperature. The plant material was occasionally stirred with a mechanical stirrer and then filtered through Buckner funnel under vacuo. The resulting plant material after filtration was extracted two more times with hexane (2 × 500 mL) and the combined extract was concentrated under reduced pressure at 40°C to obtain dark yellow viscous residue. The extracts were stored in the dark at -20°C until further analysis.

Soxhlet extraction (SE)

The lyophilized marigold flower powder (50 g) was contained in a thimble of Soxhlet apparatus and extracted with 300 mL hexane. The extraction was continued for 10 h and the extract collected at different time intervals. The extracts were filtered and concentrated under vacuum to obtain a final dark yellow colored oleoresin. The extraction was performed under light protection and the extracts concentrate were kept in the dark at -20°C until analysis.

Ultra-sonic extraction (USE)

Ultra-sonic extraction was performed with a 750 W Sonics Vibra Cell™ ultrasonic instrument. In the first experimental condition, dry marigold flower powder (50 g) contained in a 500 mL glass beaker was suspended in hexane (250 mL) and exposed to ultrasonic waves for predefined time (5, 10, 20, 30 & 40 min). In the second experiment equal quantities of material were exposed to ultra-

sound waves for 30 minutes of pre-defined power (30, 40, 50, 80 watt). Both the extractions were performed at 25 ± 5°C. Afterwards, the suspension was filtered, and the solvent was removed under reduced pressure at 40°C to obtain dark yellow viscous residue. All the operations were performed under light protection and in triplicates. The extracts were kept in the dark at -20°C until analysis.

Accelerated solvent extraction (ASE)

The extraction was carried out using a Dionex ASE 300 (Sunnyvale, CA, USA) accelerated solvent extractor. A 50 g portion of the dried flower powder of marigold was packed tightly in 100 mL stainless steel vessel and extracted with hexane at different temperatures (40, 50, 60, 70, 80°C). The extractions were performed under pressure at 10.34 MPa, with 5 min equilibration, 5 min static time, 60% purge volume and a 60 sec purge for a total of three cycles. About 80–100 mL of solvent was collected in each extraction. The solvent was removed under reduced pressure at 40°C to obtain dark yellow colored viscous residue. All the operations were repeated three times under light protection conditions. The extracts were kept in the dark at -20°C until analysis.

Supercritical fluid extraction (SCFE)

The SCFE of T. erecta flower powder was performed on Thar® SCFE equipment using the following extraction conditions: Pressure range (15-40 MPa) temperature range (40–70°C), CO_2 flow rate (50 g/min) and hexane-ethanol (95:5) as a co-solvent at 5–10% level, and dynamic time range (60–300) min. The optimized condition for which we obtained maximum yield was 40 MPa/70°C and 240 min. All the extraction was performed three times and the extracts were combined and stored in the dark at -20°C until analysis.

Analysis of pigment

The dry marigold flower powder and the oleoresin extracted in all the extracts was analyzed by AOAC Official Method 970.64 with little modification as stated below and by HPLC. About 0.05 g of marigold flower powder/oleoresin was transferred into 100 mL amber colored volumetric flask and 30 mL of extractant comprising of hexane (10 mL), acetone (7 mL) absolute alcohol (6 mL) and toluene (7 mL) was added. To this 2 mL of 40% methanolic KOH was added and the mixture shaken for one minute. The flask was then heated in a water bath (56°C) for 20 minutes, cooled and kept in the dark for an hour. To this solution 30 mL of hexane was added and the mixture shaken for 1 minute. It was then diluted with 10% Na_2SO_4 solution and kept in dark for an hour. The upper organic phase (50 mL) containing xanthophyll was separated and stored at -20°C prior to analysis.

Spectrophotometric method

A volume of 0.5 mL of the upper phase was transferred into a 50 mL flask and made up to mark with hexane. Absorbance was measured at 474 nm using hexane as reference. Xanthophyll content was determined as per the following formula:

$$\text{Total xanthophylls} = \frac{(A_{474} \times D)}{(W \times 236)}\text{; Where,}$$

A_{474} = Absorbance at 474 nm
D = Dilution factor
W= Sample weight
236= Specific absorptivity of trans- lutein (g/L)

Saponification of lutein esters

The Tagetes oleoresin (1 g) contained in a round bottom flask was saponified with 40% methanolic potassium hydroxide (50 mL). The solution was refluxed for 2 hr, cooled and partitioned with hexane (100 × 3 mL). The hexane portion of the extract was evaporated under reduced pressure at 40°C. The saponified lutein RB was then kept in ice bath and 50 mL THF was added over 20 min. The mixture was washed with 1:1 ethanol and water mixture (50 × 3 mL) and the content were centrifuged (10,000 rpm, 20°C, 20 min) until, pH of the supernatant aqueous solution was neutral. The leftover solid in the centrifuge tube was dried under vacuum to get orange powder lutein (30 mg, 70% pure).

High performance liquid chromatography (HPLC) analysis

Analytical reverse phase HPLC (Waters® e2695) fitted with quaternary gradient pump, auto-sampler, controller Empower 2® software, Waters 2998 PDA detector and YMCTM (250×4.6 mm) C-30 carotenoid column was used for the analysis of lutein and its esters. Lutein esters were separated under gradient elution at a flow rate of 0.80 mL min^{-1} using a mobile phase of Methanol: TBME (80:20) and a run time of 55 min. A 20 µL volume of the sample was injected each time and peaks were detected at λ_{max} 450 nm. The retention time (Rt) for each compound was recorded. Water used for the HPLC analysis was purified using Millipore (USA) water purifier system with resistivity of 18.2 MΩ cm.

Fatty acid analysis

Preparation of fatty acid methyl esters (FAMEs): Tagetes oleoresin (0.1 g) was taken in a 50 mL conical flask and mixed with 15 mL sodium hydroxide–methanol solution (0.5 mol L^{-1}). The flask was then placed in a water bath at 80°C for saponification for 1 h. At the end of saponification, 15 mL boron trifluoride–methanol solution (0.15 g mL^{-1}) was added and allowed to react for 5 min. Then 6 mL n-heptane was added, and the mixture was cooled to room temperature and finally treated with saturated salt solution. The mixture was vigorously shaken, the separated organic phase was concentrated under vacuo and diluted to a fixed volume before analysis by GC-MS.[21]

Gas chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis of FAMEs was performed in Agilent 7890A GC system interfaced to an Agilent 5975C inert XI EI/CI MSD with triple axis qudrapole mass spectrometer fitted with HP-5 (30 m × 0.25 mm i.d., 0.25 μm) capillary columns. Helium at a flow rate of 1 mL/min was used as a carrier gas. The column temperature was initially set at 40°C (held for 0.5 min) and then increased to 195°C at 25°C min^{-1}; increased to 205°C at 3°C min^{-1}; increased to 230°C at 8°C min^{-1} and kept at this temperature for 4 min; and finally increased to 240°C at a rate of 5°C min^{-1} (held for 5 min).[21] Injector and interface temperatures were 230°C and 255°C, respectively. EI mass spectra were recorded at 70 eV ionization voltage over the mass range 40–400 amu.

Antioxidant Activity

The antioxidant activity of marigold xanthophylls was assessed by radical scavenging effect of 1, 1-diphenyl-2-picrylhydrazyl (DPPH).[22] The diluted working solution of the test extracts and the standards quercetin and gallic acid (1-100 μg/mL) were prepared in methanol. 1 mL of DPPH (0.002%) solution in methanol was mixed separately with 1 mL of the sample solution and the standard solution. Different concentrations (50, 100, 500 and 1000 μg/mL) of xanthophyll were pipetted to the test tubes and volume adjusted to 3 mL with methanol. 1 mL of alcoholic DPPH (0.002%) solution was added to each sample and the samples were vortexed and then incubated in dark at room temperature for 30 min. Methanol (1 mL) with DPPH solution (0.002%, 1 mL) was used as blank. The spectrophotometric measurements at 517 nm against blank samples were made with a pairs of matched quartz cuvettes using Analyticjena UV-Vis spectrophotometer (SPCORD 200). The optical density was recorded, and radical scavenging activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation:

$$\text{Inhibition (\%)} = \left[\text{Absorbance of control} - \frac{\text{Absorbance of sample}}{\text{Absorband of control}}\right] \times 100$$

Extract concentration providing 50% inhibition (IC50) was calculated from the graph plotted between inhibition percentages against extract concentration with the help of statistical package (GW BASIC).

Antifungal activity

Antifungal assay was carried out by poisoned food technique using potato-dextrose-agar (4% PDA) medium[23] against three phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii* and *Macrophomina phaseolina* at concentrations ranging from 1000 mg.L^{-1} to 31.25 mg L^{-1}. 100 mg and 75 mg of test compounds was accurately weighed and dissolved in 2 mL of methanol. One mL of each solution was added to 50 mL of PDA medium in two separate flasks and mixed properly to give 1000 mg L^{-1} and 750 mg L^{-1} concentrations. The medium was then poured into two Petri plates under aseptic conditions in a laminar flow chamber. In the same way other concentrations (750 mg L^{-1}, 500 mg L^{-1}, 250 mg L^{-1}, 125 mg L^{-1}, 62.5 mg L^{-1} and 31.25 mg L^{-1}) were prepared by serial dilution and poured in Petri plates separately. One mL of methanol was mixed properly with 50 mL of medium and poured in two Petri plates which served as control.

A 5 mm thick disc of fungus (spore and mycelium) was put at the centre of the medium in the test Petri plate and the plates were kept in BOD incubator at 28 ± 1°C till the fungal growth in the control dishes was completed (6-10 days). The mycelial growth (cm) in both treated (T) and control (C) Petriplates were measured diametrically in three different directions. From the mean growth of above readings, percentage inhibition of growth (I) was calculated by using the following equation:

$$\text{Percent growth inhibition (\%I)} = \frac{(T-C)}{C} \times 100$$

EC_{50} (effective concentration for 50% inhibition of mycelial growth) was calculated from the percent inhibition (IC) as per the following equations:

$$\text{IC} = \frac{(\%\text{I} - \text{C.F.})}{(100 - \text{C.F.})} \times 100$$

$$\text{C.F. (Correction Factor)} = \left(\frac{90-C}{C}\right) \times 100$$

Where, 90 is the diameter of the Petri dishes in (mm) and C is the growth of the fungus (mm) in control. EC_{50} (mg L^{-1}) was calculated from the concentration (mg L^{-1}) and corresponding IC of each compound with the help of statistical package (GW BASIC).

Results and Discussion

Comparision among Extraction Methods Efficiencies

We performed cold extraction which is usually done at ambient temperature to isolate heat labile bioactive natural products. In the present study we found that besides being time consuming, extraction efficiency of cold extraction was the lowest even after 10 hours of extraction as indicated by its high range of RSD% (8.2%) (Table 1).

Extraction efficiency of SE was significantly higher than CE and the yield increased with time. Statistically, the results were incoherent as the RSD% in SE showed wide variation (0.65–10.6%) (Table 1). USE leads to enhancement in extraction efficiency. As compared to CE and SE, USE is more time efficient and provided maximum yield at the power level of 80 watt and 30 min sonication time. Moreover, the increasing power level and time of sonication has a positive effect on the yield (Table 1) but after reaching its threshold level the yield was relatively constant

Table 1. Yield[a] of marigold oleoresin by different techniques.[b]

Method	Time (min)	Temperature (°C)	Pressure (MPa)	Yield (g/kg) (% RSD)
CE	120	25 ± 5	-	11.2 (3.6)
	240	25 ± 5	-	15.6 (8.4)
	360	25 ± 5	-	21 (2.9)
	480	25 ± 5	-	31.6 (8.2)
SE	120	60 ± 5	-	18.6 (10.6)
	240	60 ± 5	-	31 (0.65)
	360	60 ± 5	-	49.5 (1.2)
	480	60 ± 5	-	57.3 (10.5)
USE	5	25 ± 5	-	24 (5.2)
	10	25 ± 5	-	32 (8.2)
	20	25 ± 5	-	42.7 (1.2)
	30	25 ± 5	-	50.7 (0.82)
ASE	20	30	10.34	41.3 (4.6)
	20	40	10.34	49.3 (5.3)
	20	50	10.34	54.3 (1.3)
	20	70	10.34	64.5 (1.6)
SCFE	60	40	15	22.7 (5.4)
	120	50	25	32.7 (4.0)
	180	60	30	40.7 (4.4)
	240	70	40	45.3 (1.1)

[a] Data represents values of three independent experiments; Values in parentheses indicates Percent Relative Standard Deviation (RSD, n=3). [b] Cold Extraction (CE), Soxhlet Extraction (SE), Ultrasonically assisted Extraction (USE), Accelerated Solvent Extraction (ASE), Supercritical Fluid Extraction (SCFE).

even at 40 min (100 W). The results for USE were statistically more consistent with RSD% of only 0.82%, for the maximum yield value after 30 minutes of extraction time (Table 1).

SE (57.3 g/kg) and USE (50.7 g/kg) have similar extraction efficiency in terms of total yield but is less than ASE (64.5 g/kg) (Table 1). The yield of *T. erecta* oleoresin was higher with accelerated solvent extraction. Since, in the case of ASE, temperature also plays a critical role in enhancing extraction efficiency. When, ASE was performed at 70°C with 2 static cycles of 5 minute each, oleoresin content was the highest in the first extraction cycle followed by the second and third cycle in which the yield was significantly decreased. Even at 80°C, the yield of oleoresin was constant (64.5 g/kg) after 20 minutes of extraction time (Figure 1). Statistically, also the method was more robust as compared to all other methods with RSD% varying from (1.6-5.3%) (Table 1). The possible reasons because of which the automated system of ASE yield was high could be the use of solvents at high temperatures and pressures that has led to greater solubility of the analytes; reduced matrix effects; more rapid diffusion of the analytes from the matrix to the solvent; less viscous solvent, allowing for the greater penetration in the matrix; and increased pressure that keeps the extracting solvent in a liquid state during the process.

Further, unlike conventional solvent extraction, supercritical CO_2 is non-flammable and environment friendly. However, SCFE being more environmentally benign was less efficient than ASE, SE and USE in terms of total yield but was better than CE, as evident from the increased oleoresin yield (22.7 to 45.3 g/kg) with an increase in temperature from 40–50°C. Statistical congruence was also observed in this case as the RSD% varied from (1.1–5.4%) (Table 1). Moreover, the product quality was better as evident from the HPLC chromatogram with each of the lutein esters peaks clearly resolved in SCFE as compared to other methods (Figure 1) but SCFE method has the disadvantages of high equipment investment and production cost. Even though the solubility of lutein esters increases with a co-solvent, the direct extraction without any co-solvent is considered safe and economically viable. In the present study, the flow rate of CO_2 had incremental effects on lutein ester yield till 50 g/min along with the use of hexane-ethanol (95:5) as a co-solvent at 5–10% level. Our results were found to be in agreement with those reported by Gao et al.[18] The overall yield of *Tagetes* oleoresin was higher in ASE (64.5 g/kg) followed by SE (57.3 g/kg), USE (50.7 g/kg), SCFE (45.3 g/kg) and CE (31.6 g/kg) (Table 1), and the

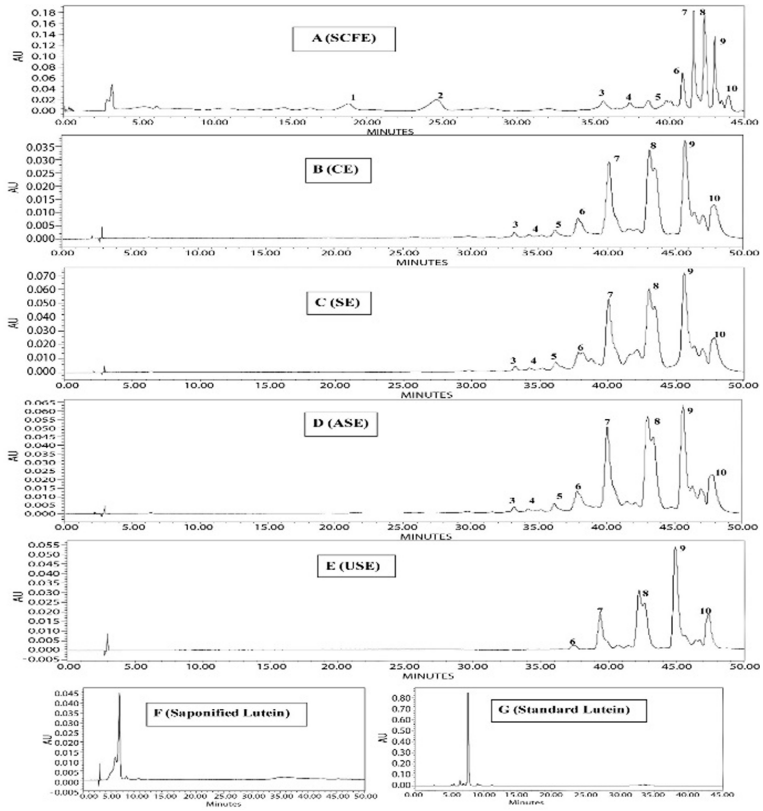

Figure 1. Typical HPLC profile of a marigold extract- (A) SCFE; (B) CE; (C) SE; (D) ASE; (E) USE; (F) Saponified Lutein; (G) Standard Lutein. Peaks: (1) unknown, (2) monomyristate, (3) monopalmitate, (4) monostearate, (5) myristate–palmitate (6) dipalmitate, (7) palmitate–stearate (8) myrisate- stearate (9) distearate, (10) linolenate. HPLC conditions: C30 column, gradient solvent system of Methanol: TBME (80:20, v/v) from 80% Methanol to 20% of TBME with a linear gradient over 50 min, flow rate of 0.8 mL/min and detection at 450 nm.

overall purity was better in SCFE extracted sample. However, due to higher automation, the reproducibility of ASE was better (0.3–8.0%) than that of SE (0.5–12.9%), SCFE (0.2–9.4%), USE (0.3–12.4%) and CE (0.8–15.3%) and also the overall yield% was higher in case of ASE.

Identification of compounds through GC and HPLC analyses

The identification of fatty acids along with dominant hydrocarbons was achieved by gas chromatography after hydrolysis and derivatization to fatty acid methyl esters (FAMEs). The FAMEs compounds were identified by

Table 2. General structure of lutein esters in *T. erecta* (Var. Pusa Narangi Genda)

S.No	Lutein esters	R1	R2	(Rt)* min	MW
1	Unknown	-	-	18.87	-
2	Monomyristate	OH	$OCO(CH_2)_{12}CH_3$	24.86	227.4
3	Monopalmitate	OH	$OCO(CH_2)_{14}CH_3$	35.89	255.4
4	Monostearate	OH	$OCO(CH_2)_{16}CH_3$	37.84	283.5
5	Myrisate palmitate	$OCO(CH_2)_{12}CH_3$	$OCO(CH_2)_{14}CH_3$	39.86	481.8
6	Dipalmitate	$OCO(CH_2)_{14}CH_3$	$OCO(CH_2)_{14}CH_3$	41.10	510.8
7	Palmitate stearate	$OCO(CH_2)_{14}CH_3$	$OCO(CH_2)_{16}CH_3$	41.95	538.9
8	Myrisate stearate	$OCO(CH_2)_{12}CH_3$	$OCO(CH_2)_{16}CH_3$	42.35	510.8
9	Distearate	$OCO(CH_2)_{16}CH_3$	$OCO(CH_2)_{16}CH_3$	44.10	566.9
10	Linolenate	OH	$OCO(CH_2)_{13}CH_3$	44.97	277.4

Retention time

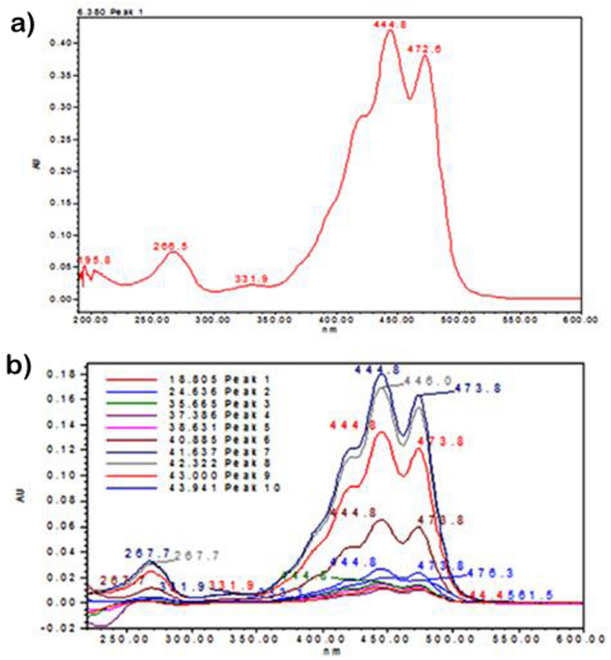

Figure 2. The figure is shown with a low resolution, and some number could be difficult to read. Please replace the figure with high resolution.

matching their mass spectra. NIST (National Institute of Standards and Technologies) mass spectra library was used as a reference for identifying the essential components (Table 2). The different fatty acid moieties attached to lutein in different esters were identified as methyl palmitate (3.81%), methyl stearate (3.68%) and linolenate (1.16%). Among the various hydrocarbon constituents n-eicosane was most abundant (42.8%) followed by n-tricosane (9.5%) and 4-ethyl decane (7.16%). However, the lutein esters represented more than 80% of the constituents. Since lutein is not symmetrical, two structural isomers existed for both myristate–palmitate and palmitate–stearate.

The HPLC profile of marigold flower extract is depicted in (Figure 1). The peaks in the chromatogram were identified as lutein (Figure 2a) and lutein esters (Figure 2b) by considering that acylation does not affect the spectral characteristics and that both lutein and lutein esters showed the same absorbance spectrum (maxima at 444.8 and 472.6 nm) (Figure 2). The identification of each single component monomyristate, monopalmitate, monostearate, myristate–palmitate dipalmitate, palmitate–stearate, myrisate–stearate, distearate and linolenate (Table 3) was achieved by comparing their retention times with those reported in literature and obtained with the similar HPLC conditions.[24] However, in our case the separation of lutein esters was better by employing C-30 carotenoid column in HPLC. The presence of free lutein was confirmed by comparison of the retention time and absorbance spectrum with the standard lutein; as we found the free lutein was absent in all the extracted samples.

Further, calibration curves were obtained using the chromatographic peak areas of all trans- lutein measured at seven different concentrations ranging from 0.005 mg/ mL to 0.1 mg/ mL. Good linearity was observed with a linear correlation equation of A = 19002L – 35140, (r^2 = 0.996) (Data not shown) Where, A is the peak area (AU) and L is all-trans-lutein (mg/ mL). Xanthophyll/ lutein content in the marigold dry flower powder was found to be higher in HPLC analysis (13724 ± 0.03 mg/kg) than in the Association of Official Analytical Chemist (AOAC) recommended U.V. spectrophotometer analysis (11630 ± 0.02 mg/kg) (Table 4), which was further increased when the extracted oleoresin

Table 3. Identification of Fatty acids and hydrocarbons in marigold oleoresin

S.No	Compound	Rt (min)	Area %	Mol Wt
1	4-Ethyl-Decane	4.02	7.16	170.3
2	Methyl Undecane	4.30	4.01	170.3
3	n-Dodecane	4.90	0.60	170.3
4	n-Docosane	5.38	1.08	310.6
5	n-Tridecane	5.49	1.47	184.4
6	n-Tetradecane	6.04	0.62	198.4
7	Neophytadiene	9.36	1.82	278.5
8	Tetratriacontane	10.23	3.12	478.9
9	Methyl palmitate	10.31	3.81	270.5
10	Methyl stereate	12.29	3.68	298.5
11	Methyl linolenate	12.38	1.16	294.5
12	n - Tricosane	15.09	9.52	324.6
13	n-Eicosane	22.29	42.83	282.6

Table 4. Yield* of xanthophyll /lutein content in *T. erecta* (Var. Pusa Narangi Genda).

Sample	Xanthophyll (mg/kg) (AOAC method)	Lutein content (mg/kg) (HPLC method)
Marigold (dry powder)	11630 ± 0.02	13724 ± 0.03
Oleoresin	23840 ± 0.05	26142 ± 0.08

* Data based on average of three replicates

was analyzed for all trans-lutein (26142 ± 0.08 mg/kg, HPLC) as compared to (23840 ± 0.05 mg/kg, AOAC) by the above two methods respectively.

Antioxidant Activity

The *Tagetus* oleoresin which was found to be rich in xanthophylls/lutein esters was further analSyzed for their antioxidant activity by DPPH assay which is based on the reduction of DPPH in the presence of a hydrogen donating antioxidant to its non radical DPPH-H form. The dark color of DPPH radical solution in the presence of xanthophyll turns lighter and the absorbance becomes lower.[25] The DPPH radical scavenging activity is dose dependent and positively correlated with increasing concentration (Figure 3).

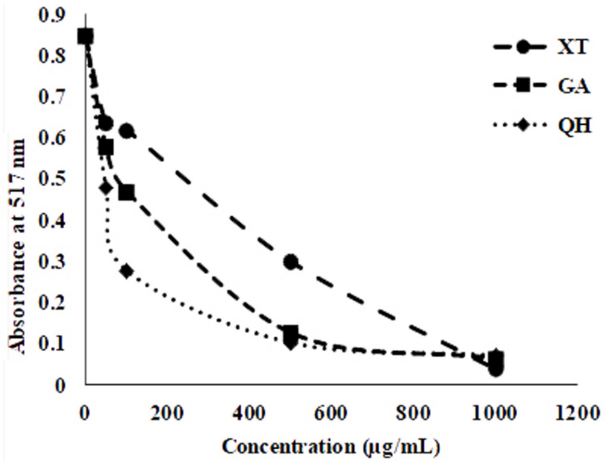

Figure 3. DPPH assay of marigold flower revealed Xanthophylls (XT), Gallic acid (GA), and Querctin hydrate (QH), respectively

DPPH assay revealed that xanthophylls/lutein is a good antioxidant with IC_{50} value of 213.52 ± 0.07 µg/mL however, the antioxidant activity of xanthophyll was found to be lower than that of standards used Gallic acid (GA- 11.26 ± 0.03 µg/mL) and Querctin hydrate (QH- 4.95 ± 0.05 µg/mL) (Table 5). The antioxidant activity of Xanthophylls/lutein and lutein esters can be attributed to the saturated or unsaturated nature of fatty acids linked with the xanthophyll/lutein molecule. However, when polyunsaturated fatty acids were esterified to xanthophylls, the antioxidant action is diminished because the fatty acids propagate the radical chain, increasing the pro-oxidant reactions synergistically.[26]

Antifungal Activity

Further, marigold oleoresin exhibited moderate to significant antifungal activity against *R. solani* (EC_{50} 344.81 mg L^{-1}), *S. rolfsii* (EC_{50} 438.54 mg L^{-1}) and *M. phaseolina* (EC_{50} 663.43 mg L^{-1}) (Table 6). The activity was comparable to control azadirachtin [*R. solani* (EC_{50} 153.81 mg L^{-1}), *S. rolfsii* (EC_{50} 187.56 mg L^{-1}) and *M. phaseolina* (EC_{50} 127.55mg L^{-1})]. The activity was however, far less than the standard commercial fungicide Bavistin (Data not shown). The results of our study showed the importance of *T. erecta* oleoresin as an antimycotic agent.

Conclusions

The present study shows that the overall yield of Tagetes oleoresin was higher in ASE followed by SE, USE, SCFE and CE and the overall purity was better in SCFE extracted sample. However, due to higher automation, the reproduc-

Table 5. IC_{50}* values of marigold xanthophyll and standard antioxidants.

IC_{50} (µg/ mL)	GA	QH	Xanthophyll
DPPH radical scavenging activity	11.26 ± 0.03	4.95 ± 0.05	213.52 ± 0.07

* Data based on average of three replicates

Table 6. Antifungal activity* of marigold xanthophyll against three phytopathogenic fungi.

Test fungi	DF	χ^2	Regression equation	EC_{50} (ppm)	Fiducial limits	
					Higher	Lower
Rhizoctonia solani	3	4.97	Y = 1.81 + 1.2552X	344.81	419.77	283.24
Sclerotium rolfsii	3	0.98	Y = 1.99 + 1.1392X	438.54	545.12	352.81
Macrophomina phaseolina	3	3.15	Y = 2.28 + 0.9611X	663.43	884.14	497.81

* All data based on average of three replicates; Degree of freedom (DF)

ibility of ASE was better than that of SE, USE, SCFE and CE. The experimental conditions, including the mobile phase composition, column type and flow-rate, were optimized to provide high-resolution and reproducible peaks. The method of saponification was also optimized to obtain maximum possible yield of lutein. To the best of our knowledge this is the first such comparative study undertaken to judge the efficiency of modern day by far available techniques for marigold oleoresin extraction in T. erecta (Pusa Narangi Genda) variety. However, ASE seems to be a cost-effective process at laboratory scale as this system involves the use of limited quantities of organic solvent, reducing analysis times (20–25 min.), allowing for total automation, and finally, leading to considerable savings in costs and improved safety and the possibility to improve the number of daily analyses, but a precise economic evaluation will need additional experiments for establishing large-scale units.

Acknowledgments

The authors express their gratitude to Ministry of Agriculture & Farmers Welfare, Haryana; Amity University Noida and Amity University Rajasthan; and VM also wants to acknowledge his appreciation to the SERB, New Delhi for TAR/2022/000673 for providing the necessary facilities.Authors can acknowledge people or financial support.

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