Oxidative stability of echium crude oil during the cold-press extraction process

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ORIGINAL ARTICLE

Abstract

Crude oil extracted from *Echium plantagineum* L. is a promising sustainable and economical source of omega-3 fatty acids (n-3 FA) for human consumption. However, it is highly susceptible to oxidation during seed processing and oil storage. The objective of this study was to enhance the oxidative stability of crude echium oil by applying antioxidants or thermal treatment to inactivate potential lipoxygenase activity. Echium seeds were subjected to thermal treatment (100°C for 15 min) to inhibit enzymatic oxidation or mixed with tocopherols (300 ppm, oil-based) to mitigate autoxidation during seed crushing. Our analysis revealed no detectable lipoxygenase activity in echium seeds. Interestingly, both thermal treatment (ECH) and the addition of a tocopherol mixture (TOC) increased hydroperoxide concentrations to 10.37 ± 0.56 mmol and12.69 ± 0.49 mmol, respectively, compared to 7.81 ± 0.18 mmol in the untreated seeds (CONT). Similarly, volatile compound levels rose significantly, reaching a ratio of 1.15 ± 0.04 (ECH) and 2.16 ± 0.33 (TOC), compared to 0.44 ± 0.12 in the CONT. In addition, these treatments also reduced the total tocopherol content, with values of 53.69 ± 0.22 mg/100 g (ECH) and 29.62 ± 0.21 mg/100 g (TOC) compared to 62.47 ± 0.71 mg/100 g in the CONT. These findings suggest that oxidation of crude echium oil is not catalyzed by lipoxygenases and that the tocopherol-based antioxidant strategy used in this study, at the tested dose and protocol, did not improve oxidative stability. Future research should explore alternative approaches, such as CO_2 supercritical extraction, to enhance the preservation of crude echium oil.

Keywords: Echium, oxidation, lipoxygenase, volatile, sensory

Introduction

The guidelines of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS) for the management of dyslipidemias recommend the intake of omega-3 fatty acids (n-3 FA) for individuals with high levels of triacylglycerol, which helps to prevent atherosclerosis, a major cause of death worldwide (Mach *et al*., 2019; Roth *et al*., 2020). Other studies have also suggested that n-3 FA, particularly eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), could help resolve inflammation, reducing the residual

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risk for patients on medication for cardiovascular disease (Sherratt *et al*., 2023; So *et al*., 2021).

Traditional sources of n-3 FA for humans have been fish (e.g. sardine, mackerel, and anchovy), algae, and krill oils (Burri *et al*., 2012; Takic *et al*., 2022). However, the Western countries typically have a low intake of n-3 FA because of relatively high prices, concerns about environmental contaminants (mercury and polychlorinated biphenyls), taste preferences, and dietary choices (Olsen, 2011). Moreover, the global supply of EPA and DHA from all traditional sources is insufficient to meet human nutritional requirements (Tocher *et al*., 2019). In addition, n-3 FA supplements sold in drugstores or on online platforms are expensive. This prohibitive cost limits access to these supplements for vulnerable subgroups of the population, who are most affected by cardiac mortality (Minhas *et al*., 2023).

Among the alternative sources for EPA and DHA are microalgae and genetically modified oils. However, have limitations, including high cost and low scale of production (Tocher *et al*., 2019). Edible vegetable oils, such as soybean, rapeseed, flaxseed, and chia, contain α-linolenic acid (ALA; C18:3 n-3), the primary source of plant-based n-3 FA, which can be endogenously converted into EPA and DHA (USDA, 2024; Anderson and Ma, 2009). However, in the cascade of elongation and desaturation, the presence of high amounts of omega-6 fatty acids, such as linoleic acid (LNA; C18:2 n-6), typical in Western diets, hinders the metabolic transformation of ALA to EPA and DHA, leading to the production of high amounts of arachidonic acid (AA; C20:4 n-6) (Takic *et al*., 2022). Thus, another option to produce EPA and DHA are seeds that contain stearidonic acid (SDA; C18:4 n-3), as SDA bypasses the Δ6-desaturase rate-limiting step of the conversion and, therefore, the conversion to EPA is improved from SDA in comparison to ALA (Prasad *et al*., 2021).

Echium plantagineum L., a natural plant from the *Boraginaceae* family, contains about 10–15% of SDA, making its seeds an excellent alternative to marine sources (Carlini *et al*., 2021). Moreover, the physiological effects of the intake of echium oil have been evaluated in the context of atherosclerosis and inflammation (Botelho *et al*., 2013; Botelho *et al*., 2015). However, because of its high level of unsaturation, echium oil is oxidized, forming several volatile compounds that impart an unpleasant odor to both the crude and refined oil (Roschel *et al*., 2021; Nogueira *et al*., 2019; Grosshagauer *et al*., 2019). The n-3 FA oxidation can occur by enzymatic catalysis, autooxidation, or photooxidation. It must be avoided as it leads to a nutritional loss and lower sensory acceptance (Jacobsen *et al*., 2010). The off-odor of echium oil, attributed to lipid oxidation, becomes apparent immediately after the seeds are crushed and persists in the refined oil during storage. However, the specific mechanisms underlying this oxidation remain unclear.

Lipoxygenases (LOX), naturally occurring enzymes, are widely distributed in plants and animals (Singh *et al*., 2022). They catalyze the biooxygenation of polyunsaturated fatty acid (PUFA) containing a cis,cis-1,4-pentadiene unit to form conjugated hydroperoxydienoic acids (Baysal and Demirdöven, 2007). LOX participate in germination of seeds, ripening of fruits, senescence, and many other physiological and biochemical processes. LOX also plays crucial roles in defense responses against biotic and abiotic stress (Singh *et al*., 2022). The simultaneous presence of LOX, lipases, and triacylglycerol is necessary for the formation of flavor, and the type and amount of volatile flavor substances are related to the composition of the lipid substrates (Tian and Hua, 2021). The negative effect of LOX has been extensively studied in soybean products, and the three identified isoforms (LOX-1, LOX-2, and LOX-3) could be inactivated through heat treatment at 100°C for 5 min (Garssen *et al*., 1971; Matoba *et al*., 1985; Hayward *et al*., 2016).

Moreover, unsaturated fatty acids, particularly those containing several bis-allylic hydrogen atoms, are prone to rapid oxidation, and their instability increases with the number of double bonds (Hammer and Schieberle, 2013). Thus, the extracted oil can undergo autoxidation, leading to the formation of various volatile compounds (Nogueira *et al*., 2019), some of which are responsible for unpleasant aromas often described as "fishy" (Hammer and Schieberle, 2013). To avoid autooxidation, natural antioxidants, such as α-tocopherol, a chain-breaking antioxidant, can scavenger free radicals being able to delay, retard, or prevent the development of rancidity and flavor deterioration due to lipid oxidation (Gulçin, 2005).

Understanding the oxidative mechanism is essential to define the best strategy to inhibit oxidation and obtain a palatable oil that is acceptable to consumers. Our group has evaluated different alternatives to improve the oxidative stability of crude and refined echium oil and their emulsions (Roschel *et al*., 2021; da Silveira *et al*., 2020; Comunian *et al*., 2019; Comunian *et al*., 2018; Espinosa *et al*., 2015). However, all these strategies have been applied to the oil rather than the seeds. It was hypothesized that the unpleasant odor of crude echium oil immediately after extraction from the seeds could be caused by volatile compounds formed from the enzymatic and/or nonenzymatic oxidation of PUFA. Thus, the objective of this study was to determine the LOX activity in recently crushed echium seeds and to evaluate the influence of LOX thermal inactivation and the addition of a mix of tocopherols in volatile compounds released during oil extraction.

Material and Methods

Materials

Echium seeds were purchased from De Wit Specialty Oils (ED De Wall, Netherlands). Soybean seeds (Glycine max (L.) Merr.) were bought at a local market. A commercial mix of tocopherols (CAFORT®) was supplied by KEMIN Industries, Inc. (Valinhos, Brazil). 1,1,3,3-Tetraethoxypropane (TEP), tricosanoic acid methyl ester (C23:0), cumene hydroperoxide, 4-methyl-2-pentanone (MIBIK), hexanal (CAT.11,560-6), and (E,E)-2,4–heptadienal (W316407) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Experimental design

The experimental design is summarized in Figure 1. Whole echium seeds (ECH) and soybeans (SOY) (35 g) were heated in a water bath (100°C for 15 min) and dried (40°C for 19 h) in a forced ventilation greenhouse (SOC FABBE Ltda., Guaporé, SP, Brazil), based on a study that inactivated LOX in soybeans (Chong *et al*., 2019). Soybeans were used only as a process control. Untreated seeds were used as the control (CONT). To inhibit autoxidation, 1.3 kg of echium seeds were mixed with 866.67 mL of an aqueous ethanolic solution (70:30 v/v ethanol/water) containing 0.20 mg/mL of a commercial tocopherol mixture (78.95 ± 2.89 g/100 g; composed of approximately 20%, 65%, and 15% δ-, γ-, and α-tocopherol, respectively) and left at room temperature overnight (18 h) to allow for ethanol evaporation (TOC). Oil was extracted from the treatments ECH, SOY, CONT, and TOC by cold pressing using an automatic screw press machine (Tomshin LTP333, Shenzhen, China) at a temperature not exceeding 60°C and centrifuged (25°C, 6,000 × g, 15 min) using the Centrifuge 5804 R (Eppendorf, Hamburg, Germany) to remove any residue of seeds. Then, they were stored at –80°C until oxidative analysis to evaluate the effect of the intervention on the final echium oil obtained. The chemical composition of the echium and soybeans seeds was determined according to AOAC methods (AOAC, 2012).

Determination of LOX activity in the seeds

Treated and untreated echium seeds and soybeans (100 mg) were ground with 500 µL of 60 mM Tris-HCl buffer pH 8.2 and centrifuged at 13,200 × g for 15 min. The supernatant (crude protein extract, 10 µL) was used to determine LOX activity according to the classical method proposed by Axelrod *et al*. (1981). Briefly, 10 µL of a 0.28% (v/v) sodium linoleate water solution containing 0.28% (v/v) Tween 20 was added to 500 µL of 0.2 M sodium phosphate buffer, adjusted to the specific pH for each enzyme (Ben-Aziz *et al*., 1970). Then, 10 µL of crude protein extract was added, and the absorbance was measured in kinetic mode for 300 s using a Shimadzu UV1240 spectrophotometer (Shimadzu Corporation, Tokyo, Japan) with the Shimadzu Program Pack Kinetics version 1.00 software (Shimadzu Corporation, Tokyo, Japan). The following pH and absorbance settings were used: LOX-1 (pH 9.0, 234 nm), LOX-2 (pH 6.1, 238 nm), and LOX-3 (pH 6.5, 280 nm). For this analysis, soybeans were included as a control. All results were obtained in triplicate and expressed as ΔOD (abs) x min^{-1} × mg $protein^{-1}$.

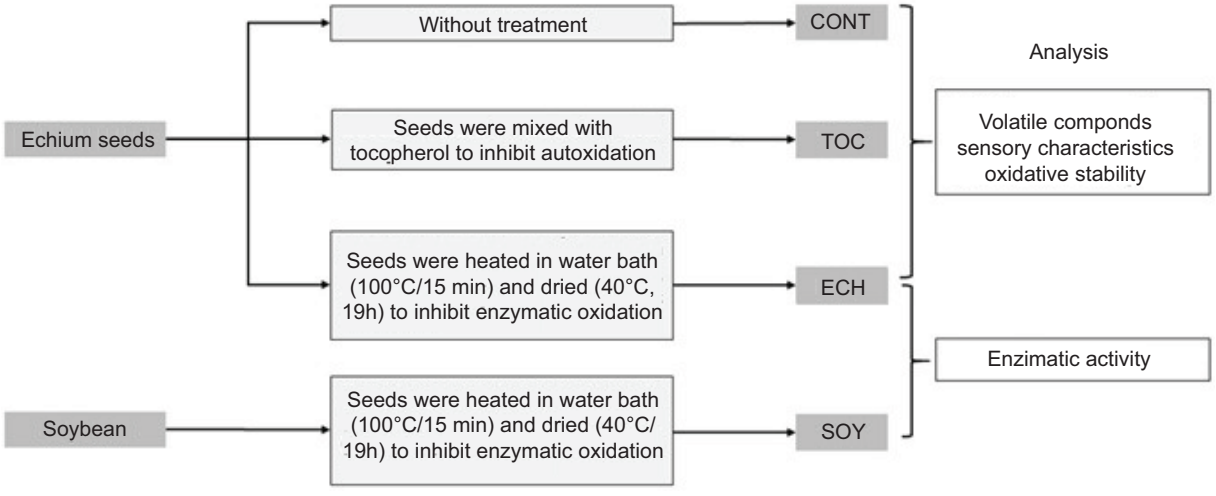

Figure 1. Experimental design.

Fatty acid composition of the oil

Oil samples were diluted in hexane, and aliquots containing 1.5 mg oil were added to tubes containing 1 mg of IS (Tricosanoic Acid Methyl Ester), 50 µL 0.5% BHT, and 1 mL 0.5 M methanolic NaOH. The solution was vortexed for 15 s and heated in a water bath at 100°C for 5 min. After cooling, samples were mixed with 2 mL 14% BF_3 in methanol, vortexed, and heated in a water bath at 100°C for another 5 min. After cooling, 1 mL of isooctane was added. The tubes were vigorously shaken for 30 s, 5 mL of saturated NaCl solution was added, and the tubes were gently homogenized. After centrifugation at 13,000 × g for 5 min, the organic phase was transferred to a new vessel and dried under a nitrogen stream. The recovered lipids were reconstituted in 0.5 mL isooctane. Fatty acids were quantified using gas chromatography-mass spectrometry (GC/MS) (Agilent 7890 A GC System, Agilent G3243A MS detector, Agilent Technologies Inc., Santa Clara, USA). Samples (1µL) were injected into a fused silica capillary column (J&W DB-23 Agilent 122–236; 60 m × 250 mm inner diameter) for separation. High-purity helium was used as the carrier gas at a flow rate of 1.3 mL/min with a split injection of 30:1. The oven temperature was programmed from 80°C to 175°C at a rate of 5°C/min, followed by another gradient of 3°C/min to 230°C, and kept at this temperature for 5 min. The inlet and transfer line temperatures were 230°C and 280°C, respectively. GC-MS was performed using 70 eV EI in scan acquisition mode and quantified by TIC. Fatty acids were identified using the NIST 17 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD, USA). All mass spectra were acquired over the m/z range of 40–500. Samples were analyzed in triplicate, and results were expressed as g/100 g oil.

Determination of volatile compounds in the seeds by headspace solid phase microextraction for gas chromatography-mass spectrometry (HS-SPME-GC-MS)

Oil samples (2 mL) with 1 µL of internal standard (4-methyl-2-pentanone, MBIK) were sealed in a glass vial with 20 mL headspace. A Combi PAL autosampler was used for automated SPME analysis. The vials were agitated (250 rpm) for 5 min at 50°C, then a 50/30 µm Stableflex 23Ga fiber (DVB/CAR/PDMS–57298-U; Supelco; Bellefonte, PA, USA) was inserted into the headspace, and the vial was agitated for 60 min at 50°C. The fiber was injected into the GC-MS (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara, CA, USA) at 250°C for 5 min using a splitless mode. Before each sample, the SPME fiber was thermally desorbed at 250°C for 5 min. Blank vials were inserted after each oil sample. The stationary phase used in this analysis was a 5% phenyl-methylpolysiloxane capillary column (19091S-433UI HP-5 ms; Agilent Technologies, Santa Clara, CA). The ion source and quadrupole temperatures were 230°C and 150°C, respectively. Ultrapure helium was used as the carrier gas at a constant flow of 1.0 mL/min. The oven temperature started at 40°C for 3 min, increased to 185°C at 3°C/min, and then to 250°C at 8°C/min. All mass spectra were acquired in the electron-impact (EI) mode with an ionization voltage of 70 eV, adopting a mass range of m/z 35–350. Compounds were tentatively identified using the NIST17 Library and expressed as peak area/IS peak area. Hexanal and (E,E)-2,4-heptadienal were quantified using a standard curve. Standard volatiles were diluted in methanol and added to 2 mL of MCT containing 60% C8:0 and 40% C10:0 (ERA Com. Imp. de Produtos Naturais, São Paulo). Samples were analyzed in duplicate, and results were expressed as peak area/IS peak area or ng/g oil.

Oxidative stability determination

The oxidative stability of the oil extracted from the seeds was determined using lipid hydroperoxide (LOOH) and malonaldehyde (MDA) concentration according to Shantha and Decker (1994) and Hong *et al.* (2000), respectively. LOOH absorbance was read at 510 nm in a spectrophotometer (Sinergy HT, BioTek, Winooski, Vermont, USA) equipped with Gen5TM v.1.06 software. A cumene hydroperoxide standard curve was prepared to quantify LOOH concentration. Samples were analyzed in duplicate, and results were expressed as mmol. MDA was quantified using an HPLC system (Agilent Technologies 1200 Series, Santa Clara, California, USA) in a reverse-phase C18 analytical column (250 mm × 4.6 mm; 5 mm; Phenomenex) set at 40°C with an LC8-D8 pre-column (Phenomenex AJ0-1287) and fluorometrically quantified at an excitation of 515 nm and emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 40% PBS (10 mmol, pH 7.4) + 60% methanol for 5 min and 60% PBS + 40% methanol for another 5 min, at a flow rate of 1.0 mL/min. A standard curve was prepared using TEP. Samples were analyzed in duplicate, and results were expressed as µmol.

Sensory analysis

A panel of three professional oil tasters, consisting of one woman and two men, was recruited to identify the main olfactory descriptors present in the seeds during manual crushing. Each taster smelled the samples to analyze olfactory perceptions. The main descriptors that characterized the odor of the samples (CONT, LOX, and TOC) were discussed by the panel. For sensory analysis, samples were coded with three randomized digits (CONT=238, LOX=987, and TOC=521) and pressed using a mortar and

pestle. The study was reviewed and approved by the Faculty of Pharmaceutical Science (CAAE: 77272723.5.0000.0067), and informed consent was obtained from each subject prior to their participation in the study.

Tocopherol analysis

The tocopherol content was determined using a high-performance liquid chromatograph (HPLC) (Agilent Technologies 1200 series, Santa Clara, CA, USA), equipped with a Zorbax Eclipse Reverse-phase C18 column (150 mm × 4.6 mm; 5 µm) with a pre-column LC8-D8 (Phenomenex AJ0-1287, Torrance, CA, USA). Briefly, from 0.5 to 1.0 g of oil was diluted in 10 mL of 2-propanol, vortexed, filtered, and injected onto the HPLC column. The mobile phase was composed of 50% acetonitrile and 50% methanol. The injection volume was 20 µL at a flow rate of 1 mL/min. The eluate was detected with a fluorescent detector set at an emission wavelength of 325 nm and excitation at 295 nm. The column temperature was maintained at 35°C. Curves were prepared using corresponding standards from Sigma-Aldrich (Alpha-47783, Delta-47784, and Gamma tocopherol-47785). This method does not separate gamma from beta-tocopherols. Samples were analyzed in duplicate, and results were expressed as mg/100 g oil.

Statistical analysis

All results were expressed as means ± standard deviation. Values observed before and after the thermal treatment were compared by a *T*-test for dependent variables. The three treatments (CONT, ECH, and TOC) were compared by ANOVA followed by the Tukey's test. Equivalent nonparametric tests were applied when data did not exhibit normality (Shapiro–Wilk test) or variance homogeneity (Hartley's test). The significance level was set at $p < 0.05$. All analyses were performed using STATISTICA software version 9.0 (Stat-Soft, Inc., Tulsa, OK, USA), and graphs were created using GraphPad Prism software version 9.5.1 (GraphPad Software, Boston, MA, USA).

Results

Chemical characterization of echium seeds

Table 1 presents the chemical composition of seeds and the fatty acid profile observed in oil. It was observed that a significant proportion of the fatty acids contain two or more double bonds, featuring the cis, cis-1,4-pentadiene structure, which serves as the substrate for all LOX isoforms to catalyze fatty acid oxidation. The moisture content of the samples before pressing was as follows: CONT = 7.48 ± 0.03%, TOC = 8.21 ± 0.02%, LOX = 4.48 ± 0.27%, and SOY = 8.10 ± 0.29%.

Table 1. The chemical composition of echium seeds and the proportion of fatty acids.

Composition (g/100g)[1]	Echium seed
Moisture	7.48 ± 0.03
Lipid	33.72 ± 0.09
Protein	19.16 ± 0.29
Ash	19.92 ± 0.23
Carbohydrate[2]	27.20
Fatty acids (g/100 g oil)	
C16:0	8.17 ± 0.09
C16:1 n-7	0.08 ± 0.00
C17:0	0.08 ± 0.00
C18:0	4.20 ± 0.12
C18:1 n-9 cis	15.40 ± 0.09
C18:2 n-6 (LNA)	13.39 ± 0.25
C18:3 n-6 (GLA)	10.01 ± 0.15
C18:3 n-3 (ALA)	32.68 ± 0.14
C18:4 n-3 (SDA)	15.46 ± 0.23
C20:0	0.05 ± 0.01
C20:1 n-9	0.48 ± 0.01
Total	100.00
SFA	12.51
MUFA	15.96
PUFA	71.53
n-3 FA	48.14
n-6 FA	23.40
n-3/n-6 FA	2.06

[1]Values were expressed as mean ± SD (n=3).
[2]Carbohydrate was determined by difference.

LOX activity

LOX activity in soybeans before and after thermal treatment is shown in Figure 2. Echium seeds did not show any activity for the three isoforms of LOX (Figure 2A–2C), while thermal treatment reduced LOX 1, 2, and 3 activities in soybeans (Figure 2A–2C). The absence of LOX activity in echium seeds indicates that, despite the presence of LOX isoforms, no catalytic activity was observed.

Oxidative stability and sensory analysis

The oxidative stability of echium oil extracted from the seeds is shown in Figure 3. The concentration of

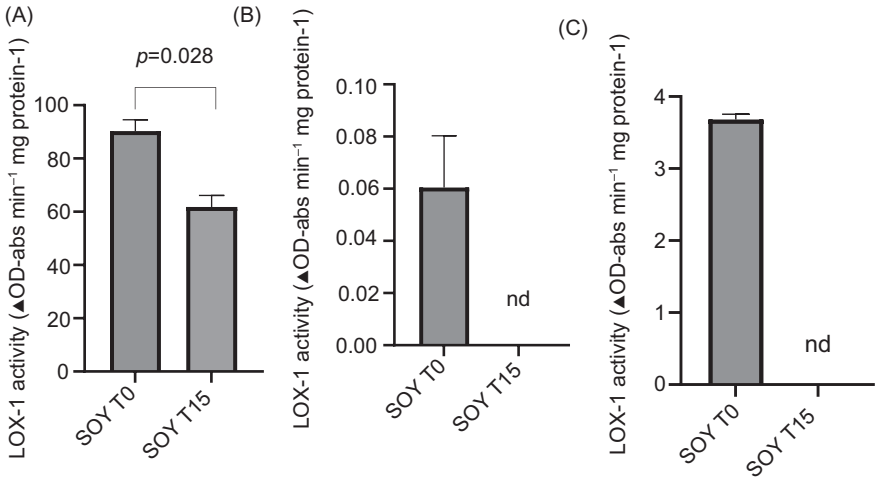

Figure 2. LOX activity determined in the soybeans before (T0) and after (T15) the thermal treatment (100°C/15 min). (A) LOX-1 activity (pH=9.0; λ=234 nm); (B) LOX-2 activity (pH=6.1; λ=238 nm); and (C) LOX-3 activity (pH=6.5; λ=280 nm). Bars represent mean ± SD (n=3). Abbreviations: crude soybean seeds (SOY T0) and soybean seeds after thermal treatment (SOY T15).

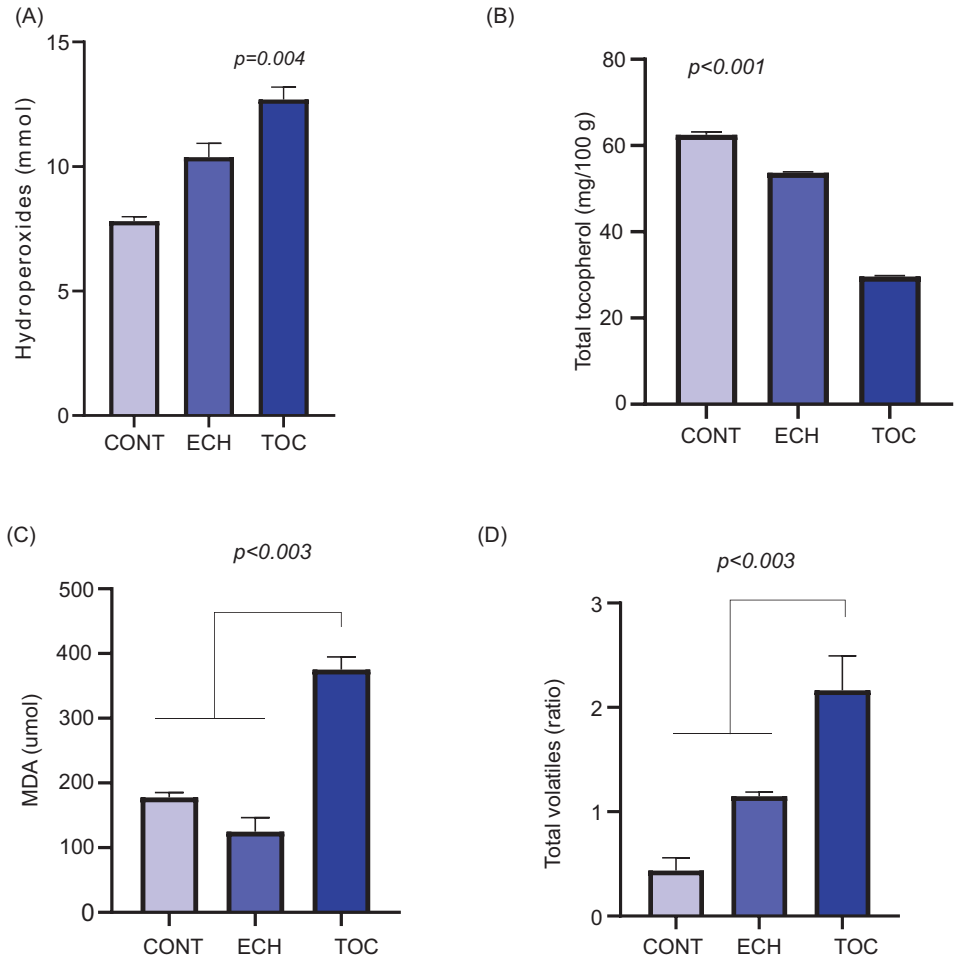

Figure 3. Oxidative stability of the echium oil extracted from the seeds. (A) Hydroperoxides; (B) total tocopherol; (C) malondialdheyde; (D) total volatiles. Bars represent mean ± SD (n=2). Abbreviations: crude echium seeds (CONT), echium seeds after thermal treatment (ECH), and echium seeds treated with tocopherol (TOC).

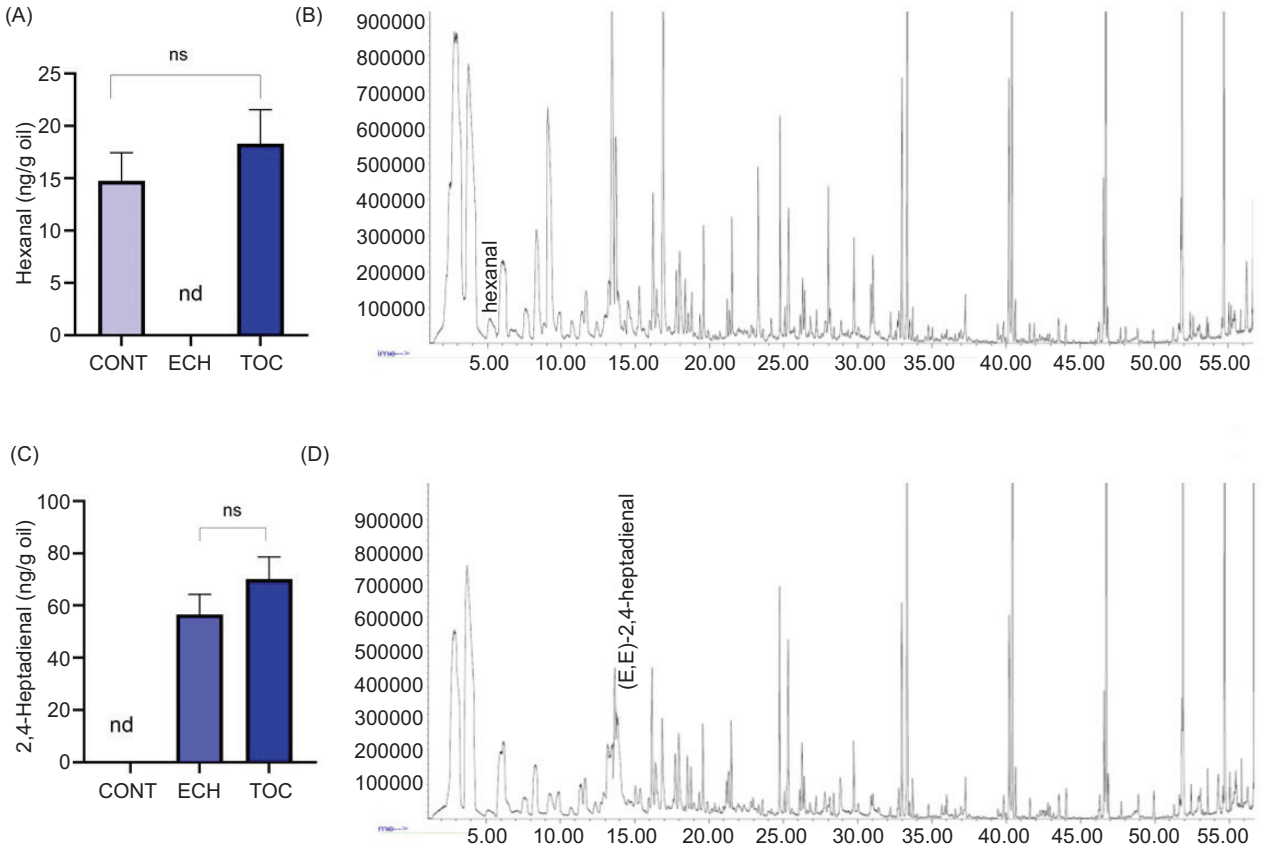

Figure 4. Hexanal and 2,4-heptadienal content of the samples. (A) Hexanal; (B) chromatogram of the TOC sample; (C) (E,E) 2,4 Heptadienal; and (D) Chromatogram of the LOX sample. Bars represent mean ± SD (n=2). Abbreviations: crude echium seeds (CONT), echium seeds after thermal treatment (ECH), echium seeds treated with tocopherol (TOC), nd: not detected.

hydroperoxides (Figure 3A) increased from CONT to ECH and TOC, while the total tocopherol content showed the opposite trend (Figure 3B). Table S1 presents the tocopherol isomers (α, γ, and δ tocopherol) found in the samples. Regarding secondary products of fatty acid oxidation, the TOC sample showed the highest concentration of MDA (Figure 3C) and the highest ratio of total volatile compounds (Figure 3D). Figure 4 presents the concentrations of hexanal and 2,4-heptadienal, both secondary products commonly found in oxidized echium oil. Hexanal was observed in the TOC and CONT samples (Figures 4A and 4B), while 2,4-heptadienal was found in TOC and LOX samples (Figures 4C and 4D). The other main volatile compounds found in the samples are presented in Table S2. As the volatile content was measured in the oil samples, we do not have the volatile profile prior to oil extraction. Overall, these results suggest that both strategies decreased the oxidative stability of the samples. The unpleasant "fishy" odor was present at a low intensity in all three samples. CONT and LOX were characterized by "dried hay" and "fresh hay" odors, respectively, while the TOC sample presented a strong odor of "fermented fruit".

Discussion

In plants, LNA and linolenic acid act as LOX substrates (Singh *et al*., 2022). The fatty acid composition (Table 1) showed that there was enough substrate for LOX activity. LOXs are dioxygenases that catalyze the addition of O_2 to PUFA containing cis, cis-1,4-pentadiene structures, leading to the production of oxylipins as a consequence of mechanical damage (Tian and Hua, 2021; Singh *et al*., 2022). This mechanical damage occurs, for example, during the crushing of seeds for the extraction of oil. This finding supports our hypothesis. However, no activity was observed for all three evaluated LOX isomers (Figure2). LOX is one of the most widely studied enzymes in plant and animal kingdoms, and it is found in more than 60 species (Baysal and Demirdöven, 2007). For this reason, we did not evaluate the enzyme concentration, focusing instead on its activity, which can be determined after extraction at a specific pH. The extract is placed in contact with fatty acids containing a *cis, cis*-1,4-pentadiene structure (e.g., LNA) and O_2, leading to the formation of conjugated dienes, as observed in soybeans (Figure 2). However, the extract obtained from echium seeds did

not show any activity under the same conditions as those observed for soybeans. LOXs can be thermally inactivated above 60°C, which improves the shelf life of foods. In our study, seeds were heated at 100°C for 5 min. However, heating also increases nonenzymatic oxidation, which may exceed the rate of oxidation caused by LOX (Baysal and Demirdöven, 2007). Our results suggest that thermal treatment applied to inactivate LOX actually contributed to autoxidation, which follows the Arrhenius model (Mihaylova *et al*., 2020). Most of the volatile compounds found in LOX samples were also reported in another study that evaluated LOXs and PUFA, including (*E, E*)-2,4-heptadienal (Tian and Hua, 2021).

Regarding the treatment with tocopherol before seed crushing, an increase in all primary and secondary products of fatty acid oxidation was observed, followed by a decrease in tocopherol concentration. In our study, a mixture containing α-, γ-, and δ-tocopherol (300 ppm oil-based) was added to the seeds and left at room temperature overnight to allow the ethanol to evaporate. Typically, tocopherols protect lipids from oxidation by donating hydrogen from their phenolic group on the chromanol ring to peroxy radicals during the propagation step. The optimal concentrations of α-, γ-, and δ-tocopherol to increase the oxidative stability of oil are 100, 250 to 500, and 500 to 1000 ppm, respectively (Kim *et al*., 2007). Therefore, the dose (300 ppm) applied in our study is within the normal range reported by other studies. Martin-Rubio *et al*. (2018) evaluated the effect of α-tocopherol in proportions ranging from 0.02 to 5% by weight on the oxidative stability of soybean oil and observed that hydroperoxide concentration increased at a higher rate as tocopherol levels rose. Despite being considered healthier (Grosshagauer *et al*., 2019), crude bulk oils contain minor constituents that influence their physical and chemical properties, including diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, water, minerals, tocopherols, pigments, and sterols (Chen *et al*., 2011). The tocopherol peroxy radical (TOO·), tocopherol oxy radical (TO·), α-tocopherolquinone oxy radical, α-tocopherolquinone peroxy radical, alkoxy radical (RO·), hydroxy radical (OH·), and singlet oxygen ($1O_2$) formed from tocopherol oxidation are pro-oxidants (Kim *et al*., 2007), and they may have contributed to the oil oxidation during the crushing of seeds because of the temperature of the process (60°C) and the presence of prooxidant minor compounds. Conversely, bleaching uses clay or charcoal to remove many minor impurities like color compounds, oxidation products (peroxides), trace metals, phospholipid remains, and soaps from neutralized oils, improving oxidative stability and sensory qualities (Vaisali *et al*., 2014). This difference may help explain the anti- or pro-oxidant response after the addition of tocopherol in refined and crude oils. However, it is important to note that additional assays, such as a control without tocopherol addition, are necessary to better clarify the effects of tocopherol on oil stability in the treatment applied in our study.

In terms of volatiles, Hammer and Schieberle (2013) identified (*E, E*)-2,4-heptadienal as an indicator of the oxidative status of the oil, while two isomers of decatrienal elicited a smell reminiscent of "oxidized fish oil." The authors also suggested that (Z)-1,5-octadien-3-one was involved in the formation of fishy odors. (*E, E*)-2,4-heptadienal and 3,5-octadien-2-one were observed in our samples. In addition, the sensory panel detected a "fishy odor" in all three samples of echium oil. Thus, it can be suggested that these two volatile compounds should be reduced to improve the sensory acceptability of oil.

Conclusion

These results suggest that the thermal treatment of seeds (100°C for 15 min) did not prevent the oxidation of crude echium oil, and the application of tocopherols (300 ppm, oil-based) to the seeds prior to crushing failed to enhance the oxidative stability of the oil. Consequently, alternative strategies, such as CO_2 supercritical fluid extraction, should be explored in future studies.

Author Contributions

Conceptualization, M.C and I.A.C; methodology and formal analysis, M.C., L.D.C., A.S.G. and H.J.O.R.; resources, I.A.C; data curation, I.A.C.; writing – original draft preparation, M.C.; writing – review and editing, M.C., P.J.G-M. and I.A.C. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors have no financial or proprietary interests in any material discussed in this article.

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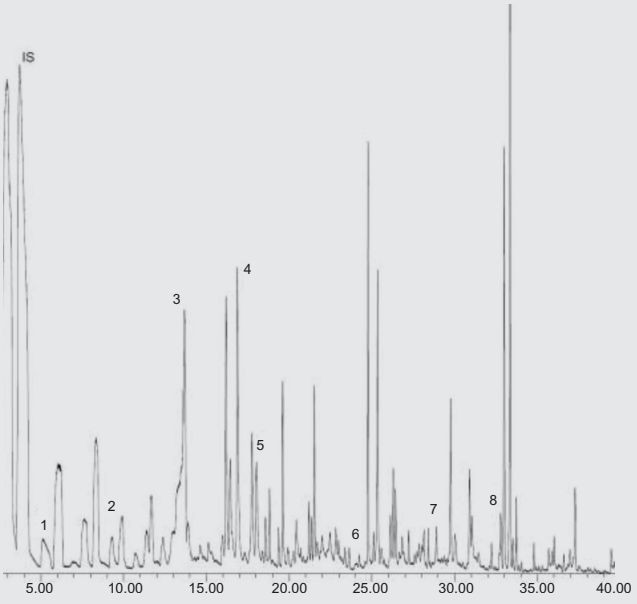
Supplementary

Table S1. Tocopherol isomers observed in the samples.

Isomer[1]	CONT	ECH	TOC	P[2]
α-tocopherol	5.01 ± 0.78	-	-	-
γ-tocopherol	57.46 ± 0.07[a]	53.69 ± 0.22[b]	27.74 ± 0.07[c]	<0.001
δ-tocopherol	-	-	1.88 ± 0.14	-

[1]Values are expressed as mean ± SD (n=2). Values followed by the same upper script letter do not differ (p<0.05).
[2] Probability value.

Table S2. Main volatile compounds identified in the echium oil samples.

Number	Compound
	
IS	IS: internal standard (MBIK)
	Acetic acid
	Oxime-, methoxy-phenyl-
	2-Butenal, 2- ethyl-
1	Hexanal
	Benzeneacetaldehyde
5	Nonanal
7	Tridecane
8	Tetradecane
4	3,5-Octadien-2-one, (E,E)-
	2(3H)-Furanone, 5-ethyldihydro-
	2(3H)-Furanone, dihydro-5-pentyl-
2	2,4-Hexadienal (E,E)-
3	2,4-Heptadienal (E,E)-
6	7-Hexadecenal, (Z)-
	1,4-Butanediol
	9,12,15 – Octadecatrienal
	2,6-Nonadienal, (E,Z)-