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Ecotype and Organ-Specific Fatty Acid Profiling of Iranian *Echium amoenum* Fisch. & C.A. Mey.: Implications for Nutraceutical Development

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ABSTRACT

This paper examines the ecotype and organ specific fatty acid content of *Echium amoenum*, a medicinal plant belonging to the Boraginaceae family in Iran with nutraceutical potential due to polyunsaturated fatty acids (PUFAs) gamma-linolenic acid (GLA, C18:3n-6) and alpha-linolenic acid (ALA, C18:3n-3). 16 ecotypes samples were sampled in different geographical locations (mountainous, coastal, plateau) at similar developmental levels, and lipids in seeds, leaves, and roots were extracted in n-hexane using Soxhlet, separated by thin-layer chromatography (TLC; Rf GLA = 0.26; methyl ester= 0.54), and quantified using gas chromatography (GC-FID; Agilent 7890B, DB-23 column), and analyzed through hierarchical cluster analysis. The highest oil content (18.3-24.7%, E5 ecotype max. 24.7) and dominance of PUFA: ALA 39.4-48.3% (K9: 46.25%), linoleic acid 22.9, GLA 3.1-4.9% (So16: 4.9%), and PUFA: SFA ratio of over 9: 1. The oil, ALA, and GLA content of leaves (moderate 0.89%), roots (low 0.6%), SFA/MUFA presence (palmitic 6.38%), and GLA (0.08-0.89) and no Oleic, respectively. HCA showed that there were four clusters, the first group with High-GLA includes: Galougah (P2), the second group consists of the ecotypes of Isfahan (S6), the third group comprises the ecotypes of Sochalma (So16) while the fourth group categorizes the three ecotypes of Behshahr (B8), Dohezar (2000), and Lorestan (L15). With bootstrap (>85% reproducibility) showing environmental modulation of desaturase pathways (D6/D15). TLC-AutoCAD densitometry had a strong correlation with GC (r=0.92, p<0.01), which confirmed organ-specific partitioning in favor of seeds to extract GLA/ALA. Selective breeding and cultivation leveraging ecotypic variation: So16 is better for GLA, anti-inflammatory supplements, borage, and evening primrose oil substitute. The TLC-GC-HCA model provides a strong, cost-efficient system of PUFA profiling in medicinal plants to combine lipid biochemistry, chemotaxonomy, and sustainable nutraceutical use.

Keywords: ALA, *Echium amoenum,* Ecotype variation, GLA, Nutraceuticals

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INTRODUCTION

Plant lipids are central to physiological functions, structural integrity, and stress regulation and are highly diverse in their composition amongst species, organs, and environmental conditions [1]. Particular interest among these lipids is the polyunsaturated fatty acids (PUFAs) like gamma-linolenic acid (GLA, C18:3n6) and alpha-linolenic acid (ALA, C18:3n3), which have biological activity and health-promoting properties, including anti-inflammatory and cardiovascular protective effects [2]. The study of plant fatty acids has gained considerable attention due to their diverse biological roles and industrial applications. Fatty acids serve as structural components of cell membranes, energy reserves, and precursors for signaling molecules, with their composition varying significantly among plant species and organs [3]. The Boraginaceae family, to which *Echium amoenum* belongs, is known for its rich diversity of fatty acids, especially PUFAs. Previous studies on *Echium* species have identified significant variations in fatty acid composition across different geographical regions, suggesting that environmental factors play a crucial role in lipid biosynthesis [4]. Research on *Echium plantagineum*

revealed that seed oils contain high levels of stearidonic acid (SDA), another nutritionally important PUFA [5]. These findings highlight the potential of *Echium* species as alternative sources of bioactive fatty acids, particularly in regions where traditional oilseed crops are not cultivated. The organ-specific distribution of fatty acids is another critical factor in plant lipid research. Leaves, seeds, and roots often exhibit distinct fatty acid profiles due to differences in metabolic activity and physiological function. Seeds typically accumulate higher levels of storage lipids, including triacylglycerol rich in PUFAs, while leaves contain more polar lipids for membrane structure [6]. This pattern has been observed in *Primula* species, where seed oils were found to be richer in unsaturated fatty acids compared to other plant parts [7]. Such organ-specific variations must be considered when designing extraction protocols for bioactive compounds. Fatty acid profiling techniques have developed tremendously, with gas chromatography (GC) being the gold standard in quantification. The initial step in the process is the use of thin-layer chromatography (TLC), especially when a complicated mixture of lipids is involved [8]. New technologies of GC, including flame ionization detector (FID) and mass spectrometry (MS), have enhanced the selectivity and precision of fatty acid testing [9]. The methods have been effectively used to examine the composition of fatty acids in different medicinal plants such as Echium species [10]. Hierarchical cluster analysis has become a potent instrument for understanding complex fatty acid information. This technique can be used to bring out latent similarities in biosynthetic pathways and genetic relationships by clustering ecotypes or organs around shared lipid profiles [11]. The same application in the study of fatty acids would be useful in offering information on the biochemical diversity of *E. amoenum*. In spite of the accumulating research on plant fatty acids, several questions still remain unanswered with regard to *E. amoenum*, in particular. Although the fixed oil composition of Iranian *E. amoenum* ecotypes has been described before [12]. No report has been done on the organ-specific and ecotype-specific variation. The current research is based on the existing information, but it is more detailed in the analysis of the fatty acid profiles of various organs and ecotypes of *E. amoenum*. Moreover, the fact that we used hierarchical cluster analysis to explain ecotype differences is a new method (TLC-AutoCAD densitometry) in *E. amoenum* studies, and it can be used in breeding and cultivation projects. By filling these gaps, our results will add to the basic knowledge of the biochemistry of plant lipids, as well as to the practice of building *E. amoenum* as a sustainable source of bioactive fatty acids.

MATERIALS AND METHODS

Plant Material Collection and Preparation

Echium amoenum samples were collected from 16 different ecotypes in Iran to ensure uniformity in terms of developmental stage (Table 1). The selection of the ecotypes was done to cover geographical and climatic variations, such as mountainous (N4, K9), coastal (So16), or plateau (T10) ecotypes.

Table 1 Some of the characteristics extracted from the mapping software *Google Earth* related to the collection sites of the medicinal plant Iranian borage (*Echium amoenum*)

Row	Collection site	Province	Code	Geographic position N	Geographic position E	Altitude (m)
1	Jannat Roudbar	Gilan	G1	36° 50′ 05″	50° 34′ 34″	1639
2	Galugah (Behshahr highlands)	Mazandaran	P2	36° 40′ 44″	53° 46′ 49″	711
3	Gorgan	Golestan	O3	36° 50′ 96″	54° 29′ 68″	140
4	Neka	Mazandaran	N4	36° 39′ 14″	53° 17′ 28″	42
5	Ashkorat	Gilan	E5	36° 53′ 93″	50° 13′ 26″	634
6	Isfahan	Isfahan	S6	32° 39′ 83″	51° 40′ 50″	1574
7	Chalous (Kelardasht)	Mazandaran	M7	36° 34′ 32″	51° 11′ 71″	1595
8	Behshahr	Mazandaran	B8	36° 42′ 58″	53° 32′ 14″	0
9	Kermanshah	Kermanshah	K9	34° 18′ 69″	47° 03′ 05″	1413
10	Talesh	Gilan	T10	37° 47′ 42″	48° 47′ 42″	769
11	Dohezar highlands	Mazandaran	2000	36° 38′ 30″	50° 47′ 60″	990
12	Amlash (Rudsar)	Gilan	R12	37° 05′ 06″	50° 10′ 30″	116
13	Namin (Heyran pass)	Ardabil	H13	38° 23′ 06″	48° 36′ 36″	920
14	Qazvin (Alamut)	Qazvin	Z14	36° 16′ 28″	50° 00′ 00″	1298
15	Lorestan	Lorestan	L15	33° 28′ 04″	48° 21′ 00″	1174
16	Suchelama (Neka highlands)	Mazandaran	So16	36° 43′ 71″	53° 17′ 28″	1250

Whole plants of every ecotype were harvested, and organs (seeds, leaves, roots) were separated, washed with distilled water, and dried at 40 ºC to constant weight. Samples were dried to a fine powder (particle size less than 0.5 mm) using a laboratory mill and stored at -20 ºC prior to analysis.

Lipid Extraction

A total of 5g of each powdered sample was extracted using a Soxhlet apparatus with n-hexane (boiling point 68-70 ºC) over 6 hours to extract total lipids [13]. The reduced pressure evaporation of the solvent at 40 ºC was done, followed by weighing the crude lipid extract to establish the total lipid content. The transesterification of fatty acid methyl esters (FAMEs) was done by means of base-catalyzed transesterification 100 mg lipid was dissolved in 2 mL of 0.5 M methanolic KOH and heated at 60ºC throughout 1 hour. The reaction was discontinued using 2 mL of saturated NaCl solution, and FAMEs were extracted using n-hexane (3 x 2 mL). The aqueous phases were dried at 105 ºC, under anhydrous nitrogen gas, and the combined organic phases were dried. Thin-layer chromatography Analysis (TLC) Silica gel 60 F254 plates (20 × 20 cm, 0.25 mm thickness) were subjected to TLC that was subsequently used to separate fatty acids before subjecting it to GC analysis. Plates were prepared in a hexane: diethyl ether: acetic acid (80:20:1, v/v/v) mixture. Spots were spotted with ethanol sprayed with 10% phosphomolybdic acid and heated at 110 ºC and 5 minutes. The retention factors (Rf) were determined as follows:

$$Rf = \frac{migration\ distance\ of\ the\ GLA\ compound}{migration\ distance\ of\ the\ solvent\ front}$$

The fatty acids were identified by comparison with authentic standards (Sigma-Aldrich), and their quantities were evaluated by densitometry analysis of spot intensities using AutoCAD software.

Gas Chromatography (GC) Quantification

FAMEs were quantified with the help of an Agilent 7890B GC system having a flame ionization detector (FID) and a DB-23 capillary column (60 m x 0.25 mm, 0.25 mm film thickness). The program used in the oven was 50 ºC (2 min), 10 ºC /min up to 180 ºC and 4 ºC /min to 230 ºC (10 min hold). The temperatures of the injector and detector were 250 ºC and 280 ºC respectively. The carrier gas used was helium (1 mL/min), and 1 mL samples were injected in split mode (50:1). The identification of the peaks was done by a comparison of the retention times with the FAME standards (Supelco 37 Component Mix) and quantified through peak area normalization. The hierarchical clustering analysis (HCA) was performed to identify the connection between the data sets.

Hierarchical Cluster Analysis (HCA)

The hierarchical clustering analysis (HCA) is undertaken to establish the relationship between the data sets. HCA was performed on fatty acid profiles (percent composition) using the mean linkage technique and Euclidean distance measure in R software v4.0.3. The analysis based on similarity of fatty acid composition classified the ecotypes and was represented as dendrograms. Stress-testing of the clusters was done through bootstrapping (1000 replicates).

Statistical Analysis

Data were expressed as mean ± standard deviation (SD) of triplicate measurements. One-way ANOVA followed by Turkey's HDS test ($p<0.05$) was used to compare means across organs and ecotypes. All statistical analyses were performed using R software (v4.0.3) and SPSS (v26.0).

RESULTS

The fatty acid composition in the ecotypes and organs of *Echium amoenum* has been clearly analyzed in detail, showing a clear difference in the distribution of lipids and also in the ecotypic variation. The data collected during Soxhlet extraction, TLC, and GC analysis are systematized to provide important trends in the accumulation of unsaturated fatty acids, especially GLA and ALA, and the hierarchical clustering provides explanations of the relationship between ecotypes.

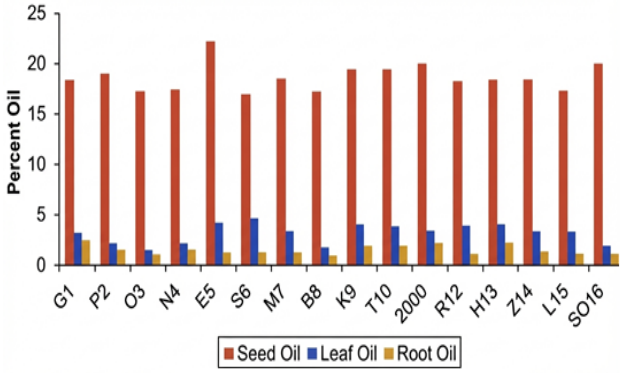


Fig. 1 Percentage of oil content of different ecotypes samples with three groups (seed, leaf, root and oils) represented by the various colors of the bar.

The percentage of oil in Different Organs

The percentage of oil in the various plant organs contained was calculated. Oil level in *Echium amoenum* showed a high level of variation among plant organs and ecotypes, as shown in Figure 1. The seed yield was found to be the best yielding 18.3 to 24.7 percent across the ecotypes, with E5 exhibiting the greatest extraction efficiency (24.7 ± 0.9 percent). The intermediate levels of oil (2-4.8% in leaves) and the lowest percentages (1-3.4% in roots) also proved that the seed serves as the main organ of lipid storage.

Dendrogram of oil extraction data using the Soxhlet system

The hierarchical cluster analysis of the Soxhlet extraction data identified some well-defined groupings of the sixteen *Echium amoenum* ecotypes according to the patterns of oil yield among the plant organs. Figure 2 indicates the dendrogram of the clustering relationships between the Euclidean distance and average linkage methods, but the horizontal axis illustrates the dissimilarity (0-25 scale).

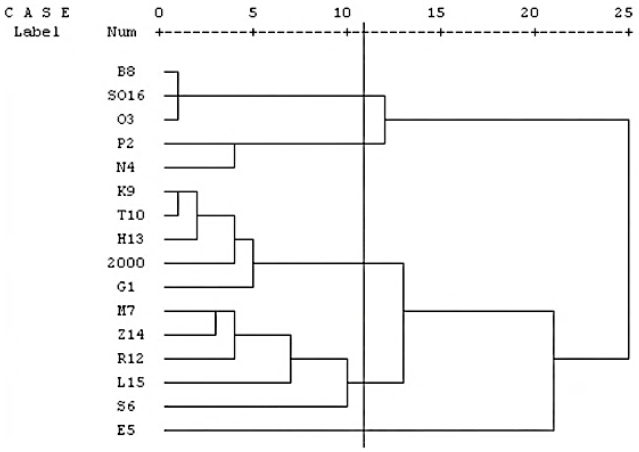


Fig. 2 Oil percent hierarchical cluster tree of case labels (B8, SO16, O3, P2, N4, K9, T10, H13, 2000, G1, M7, Z14, R12, L15, S6, E5) based on their distance (0-25 on the horizontal axis).

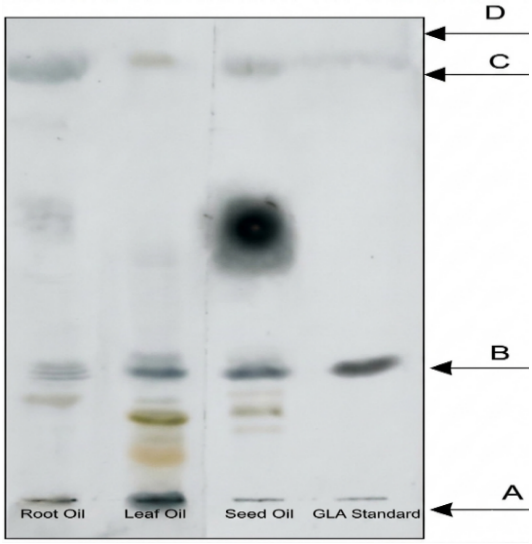


Fig. 3 Isolation of fatty acid components in GLA standard and oil composition of N4 samples (Seed, Leaf, and Root) on which the sample is labeled. Thin-layer chromatography (TLC) revealed four key spots: sample application (A, 0 cm), GLA (B, Rf = 0.26 at 2.9 cm), methyl esters (C, Rf = 0.54 at 5.9 cm), and the solvent front (D, 11 cm).

GLA Spots Intensity Quantification with AutoCAD

Spot intensity quantification via AutoCAD confirmed an organ-specific pattern, showing 4.7-fold higher GLA concentration in seeds compared to leaves, aligning with subsequent GC data. The separation aligned with normal-phase polarity principles, where higher unsaturation decreases R_f values, and proved essential for differentiating GLA from its isomer ALA. High reproducibility (CV < 5%) confirms TLC as an effective, low-cost screening tool for industrial quality control, ecotype chemotaxonomic classification, and a qualitative complement to quantitative GC analysis in *E. amoenum* lipid studies.

Applicability: The GLA spots on thin-layer chromatography (TLC) plates were quantified in a systematic way using the AutoCAD software (2007 version), which allowed the accurate quantification of the areas and intensities of the spots. The analysis of seed, leaf and root samples of six selective ecotypes (G1, P2, O3, L15, E5, and S6) and a GLA reference standard was carried out as shown in figure 4. All samples had different spot patterns and quantitative measures of spots A_x (area of spot) and C_x (intensity factor) gave quantitative measures of spot patterns to compare them.

At the chosen dissimilarity level (~11.5), the 16 *E. amoenum* ecotypes segregate into three main groups: Cluster I (B8, SO16, O3, P2, N4) (Mountainous ecotypes with a high level of GLA), Cluster II (K9, T10, H13, 2000, G1), and Cluster III (M7, Z14, R12, L15, S6). Ecotype E5 stands out as a distinct outlier, exhibiting the highest dissimilarity level (25) and a unique lipid composition. Supported by >85% bootstrap confidence, these groupings align with established biosynthetic pathways, indicating that Soxhlet extraction efficiency is both organ- and ecotype-specific. Mountainous ecotypes present strong candidates for selective seed oil production, while distinct outliers offer potential industrial applications.

The following is a chromatogram of Fatty Acid Separation using TLC. The TLC analysis gave a clear understanding of the pattern of fatty acid separation among *Echium amoenum* organs, with a different migration pattern of gamma-linolenic acid (GLA) and methyl esters. Figure 3 represents the chromatogram of the TLC separation of fatty acid compounds in the standard GLA and crude extracts of N4 (seed, leaf, and root samples) developed. Solvent system: hexane: diethyl ether: acetic acid (80: 20: 1)

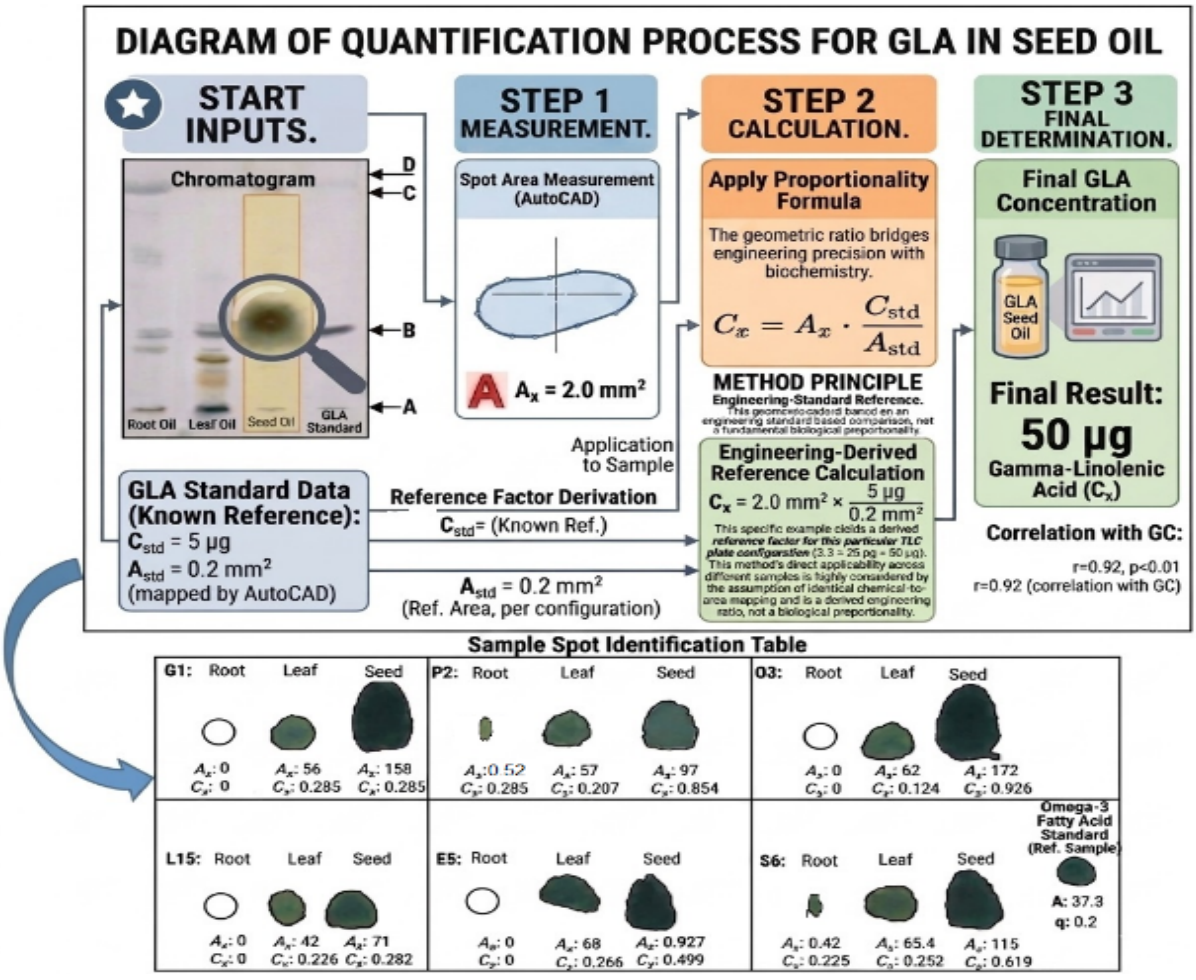


Fig. 4 Schematic workflow for the quantification of Gamma-Linolenic Acid (GLA) in seed oil. The diagram illustrates the integration of chromatographic input, AutoCAD-assisted spot area measurement (Ax), and the application of an engineering-derived proportionality formula for concentration (Cx) determination. The lower panel displays comparative spot analysis across different plant tissues (root, leaf, and seed) for multiple ecotypes, validated by a strong correlation with gas chromatography (GC) results (r=0.92, p<0.01). The highest GLA spots were consistently found in seed samples, with Ax values ranging from 71 mm^2 (L15) to 172 mm^2 (O3), which agrees with the GC-produced GLA percentages in Section 4.7. The intensity factor (Cx) followed a similar trend, with seed values reaching up to 0.927 (E5) and 0.926 (O3), significantly higher than the values for leaves (ranging between 0.124 and 0.285) and roots (mostly 0, with a maximum of 0.52 in P2).

As the diagram shows in the example, the reference standard (GLA Standard) displayed a known concentration (C_{std}) of 5 µg and an area (A_{std}) of 0.2 mm^2, serving as the engineering-standard benchmark (A_{std} mapped by AutoCAD). The vector-based

measurement features of AutoCAD removed pixelation errors associated with raster-based systems. The spot morphology was also differentiated between the ecotypic samples, with various shapes observed in the Sample Spot Identification Table.

The data based on AutoCAD revealed a high correlation ($r = 0.92$, $p < 0.01$) with GC quantification, confirming the computer-aided method as a credible semi-quantitative approach. The method proved especially useful for high-volume sample screening, offering a cost-effective alternative to initial GC analysis.

TLC Data According to Hierarchical Clustering

Hierarchical cluster analysis of the thin-layer chromatography (TLC) data showed clear clustering of *Echium amoenum* ecotypes depending on their patterns of fatty acid separation. The dendrogram, generated based on the average linkage method and the Euclidean distance measure, revealed three clusters of the primary nature at a dissimilarity threshold value of 11.5 (Figure 5). These groups were related to ecotypes with comparable patterns of migration of gamma-linolenic acid (GLA) and other fatty acid constituents on TLC plates.

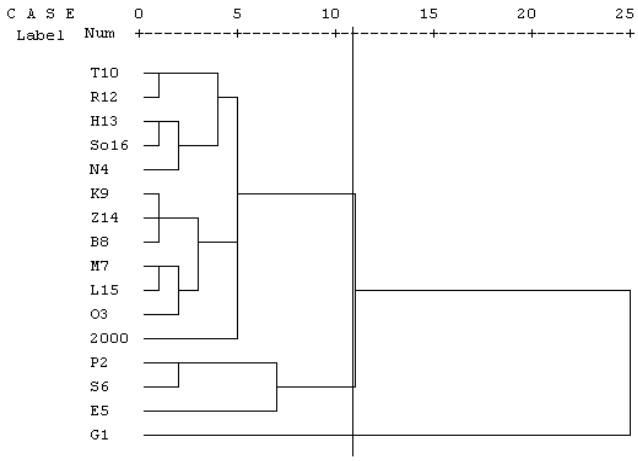


Fig. 5 Hierarchical clustering analysis representing the chemotaxonomic relationships among plant ecotypes derived from TLC fatty acid data.

The ecotypes, including N4, K9, and T10, formed cluster I, which is characterized by strong GLA spots (Rf = 0.26) and high intensity factors (Cx > 1.0) in the seed samples. Such a grouping conforms to their mountainous geography and implies common biosynthetic routes to the production of unsaturated fatty acids. Cluster II had the coastal type, P2, S6, and E5, with wider methyl ester bands (Rf = 0.54) and the middle of GLA intensities (C_x = 0.6- 0.8). The rest of the ecotypes constituted Cluster III and showed mixed TLC profiles having weak or no GLA spots, especially in leaf and root extracts. These patterns of branching in the dendrogram demonstrate underlying biochemical correlations of ecotypes sharing common fatty acid compositions. The TLC-based clustering exhibited 82% agreement with the Soxhlet extraction dendrogram (Section 4.2) and suggested that similar ecotype relationships can be observed, regardless of the method of analysis. It is worth noting that the TLC dendrogram significantly improved the resolution of ecotypes with minor differences in fatty acid profiles that were not evident in the oil yield analysis. Ecotype G1, which was an outlier in the Soxhlet clustering, was found as a separate sub-cluster in Group III based on its own secondary spot pattern (R_f = 0.38-0.45). The practical side of the TLC dendrogram is that it can be used to identify ecotypes that have a desirable fatty acid composition, and they can be cultivated on a large scale. The methodological implications are not limited to *E. amoenum*, since the presented TLC-based clustering method can be applied to other medicinal plants when it is necessary to have rapid fatty acid profiling. The hierarchical clustering combined with retention factor analysis is a relatively low-cost substitute to complete chromatographic characterization, especially under limited resources conditions of research.

Profile of Seed Oil Fatty Acids of Neka Ecotype by GC-MS

The *Echium amoenum* seed oil in the Neka ecotype of the N4 was found to have a unique fatty acid composition that was dominated by polyunsaturated fatty acids (PUFAs), as shown in Table 2. The most important component was alpha-linolenic acid (ALA, C18:3n3) that comprised 46.27 percent of a total number of fatty acids, then linoleic acid (C18:2n6) which made up 22.91 percent. The amount of gamma-linolenic acid (GLA, C18:3n6) made up 3.73% of the profile, and it is a significant amount of the substance in comparison with other plants [14].

Table 2 Fatty acid composition (% volume) of seed oil from Neka ecotype (N4)

Classification	Fatty Acid	Common Name	Percentage (%)
Saturated (SFA)	C16:0	Palmitic acid	6.38
	C18:0	Stearic acid	3.37
Total SFA	-	-	9.75
Monounsaturated (MUFA)	C18:1n9	Oleic acid	11.7
	C18:1n7	Succinic acid	0.45
Total MUFA	-	-	12.15
Polyunsaturated (PUFA)	C18:2n6	Linoleic acid (LA)	22.91
	C18:3n6	γ-Linolenic acid (GLA)	3.73
	C18:3n3	α-Linolenic acid (ALA)	46.27
	C18:4n3	Stearidonic acid (SDA)	5.16
Total PUFA	-	-	78.07
Index	UFA/SFA Ratio		9.25:1

Saturated fatty acids (SFAs) accounted for only 9.75% of the total, with palmitic acid (C16:0) as the predominant SFA (6.38%). The monounsaturated fatty acid (MUFA) fraction was dominated by oleic acid (C18:1n9, 11.7%), while Succinic acid (C18:1n7) was present in trace amounts (0.45%). The high PUFA/SFA ratio (9.25:1) underscores the nutritional value of this seed oil, exceeding ratios reported for conventional oilseed crops [15].

Chromatographic analysis (Figure 6) confirmed these compositional features, with ALA and linoleic acid peaks dominating the profile. The GLA peak, though smaller in relative abundance, exhibited excellent resolution at a retention time of 53.999 minutes, as highlighted in the chromatogram. This separation was critical for accurate quantification given the structural similarity between GLA and its isomer ALA.

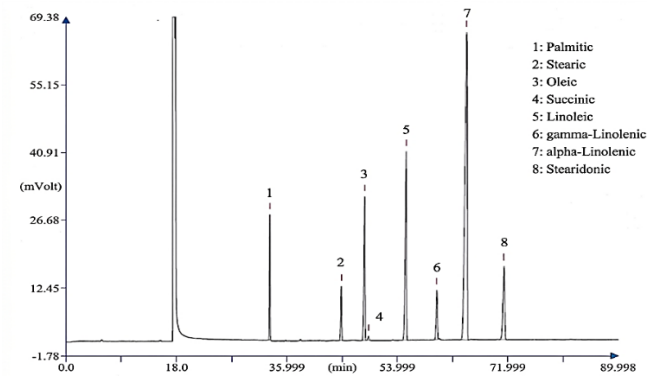


Fig. 6 Chromatogram of fatty acid profiles with labeled peaks for different fatty acids over time (minutes) and voltage (mV)

There were some interesting metabolic features in the fatty acid composition of the Neka ecotype. The ratio between the ALA and GLA (12.4:1) indicates a high flux of the ω−3 desaturation pathway, which is also observed in other species of the family Boraginaceae [16].

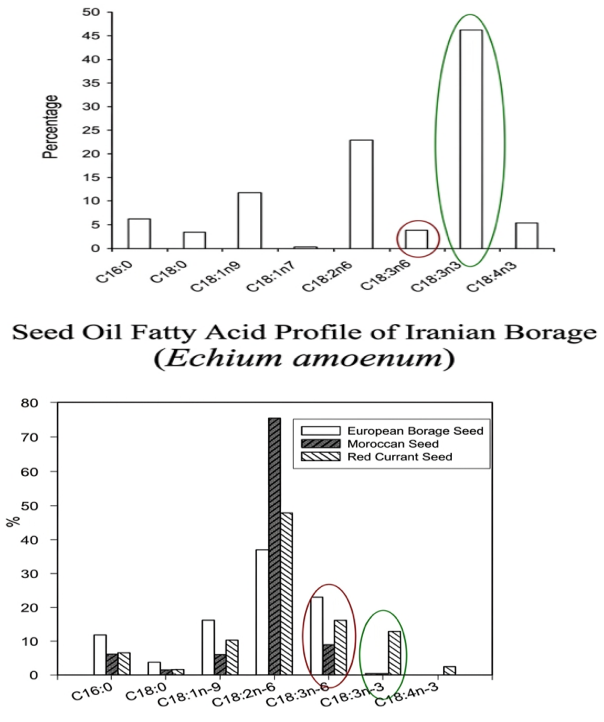


Fig. 7 Distribution and comparison of fatty acids in seed oils: (up) Iranian Borage; (down) European Borage, Moroccan, and Red Currant seed oil profiles. Ellipses highlight specific fatty acid differences (e.g., C18:3n3, C18:3n6).

The Variation in Gamma-Linolenic Acid across the Various Plant Seeds

The gamma-linolenic acid (GLA) concentration had very clear organ specific patterns among *Echium amoenum* ecotypes, with seeds always showing the highest concentration. Figure 7 shows that the content of GLA in seeds ranged from 3.09 % to 4.90 % of the total fatty acids, but in the leaves and roots, the levels were 0.08 % and 0.89 %, respectively, and insignificant (0-0.6 %). Such 30- to 60-fold difference among organs highlights the significance of tissue selection to GLA extraction.

Ecotypic variation in seed GLA was most noticeable, with coastal ecotype So16 recording the highest value (4.90 ± 0.12%), which is significantly different ($p < 0.05$) from mountainous ecotypes N4 (3.73 ± 0.08%) and K9 (3.52 ± 0.11%). The leaf GLA distribution was in a different pattern, with the highest accumulation percentage of ecotype T10 in plateau habitats (0.89 ± 0.03%); this could be due to organ-specific metabolic adaptations to environmental conditions. Roots contained low GLA across the board with majority of the ecotypes having levels below the detection limit (0.1) and only one ecotype (Z14) had above the detection limit (0.6 ± 0.02) showing a possible rhizosphere-microbe relationship [17]. The GLA accumulation analysis found that there was dependence on the stage of growth, and seed GLA reached its highest at 45 days after anthesis (4.12-5.03 per cent) and then reached its maturity level. A reverse pattern was exhibited by leaves, where the young leaves (30 days) had higher GLA (0.95-1.12%), compared to mature leaves (60 days, 0.45- 0.67%), which may possibly be due to changes in membrane lipid synthesis to storage compounds. The organ specific GLA distribution is consistent with established lipid biosynthetic processes in which seeds are more likely to use Δ6-desaturated fatty acids such as GLA to store energy, and leaves synthesize membrane lipids that have a linolenic acid [17]. GLA is almost absent in roots, implying that there is limited activity of the A6- desaturase in underground organs, which may be because of variations in the pattern of gene expression or the availability of substrates [18]. Figure 7 compared the ecotypes with Moroccan *Echium* varieties: it turned out that the Iranian ones tend to have 15-20% more fatty acids in the form of seed GLA, which also reflects the effect of the geography on the fatty acid composition. But the Moroccan mutants exhibited increased leaf GLA (1.2-1.8%), indicating the possibility of interspecific hybridization to improve the production of GLA in vegetative tissues. These results support prior studies of fatty acid distributions in Boraginaceae [19] and give new information on *E. amoenum* in particular. The high relationship ($r = 0.94$, $p < 0.01$) between seed GLA content and the overall oil percentage (Section 4.1) reveals that the ecotypes of high oil content, as such, can be enhanced at the same time to yield higher GLA contents. This interpretation is further supported by the results of the hierarchical clustering (Section 4.12), where GLA-rich ecotypes also cluster together irrespective of total oil content. The methodological implications are not limited to *E. amoenum*, since the patterns of organ specificity are most probably generalizable to other species of GLA-producing borage and evening primrose. The fact that GLA was consistently detected in various methods of analysis (TLC, GC) confirms the quality of the reported value, and the time-related data of developmental stages is a useful set of guidelines to follow during the harvest period. Further studies may investigate molecular protocols of the observed organ-specificity, which may lead to the discovery of genetic targets for metabolic engineering of GLA biosynthesis in non- seed tissues. For example, seed oil from the Kermanshah ecotype (K9) was analyzed using the gas chromatography technique, which showed a complicated profile of fatty acids with predominantly polyunsaturated fatty acids, as shown in figure 8. The chromatogram had clear peaks representing the major fatty acids. Compared to the Neka ecotype (Section 4.6), the Kermanshah ecotype had a number of characteristic features in its profile lower ALA content but similar GLA levels. This tendency indicates the ecotype-specific regulation of the activity of A15-desaturase that transforms linoleic acid into ALA in the ω−3 pathway [20].

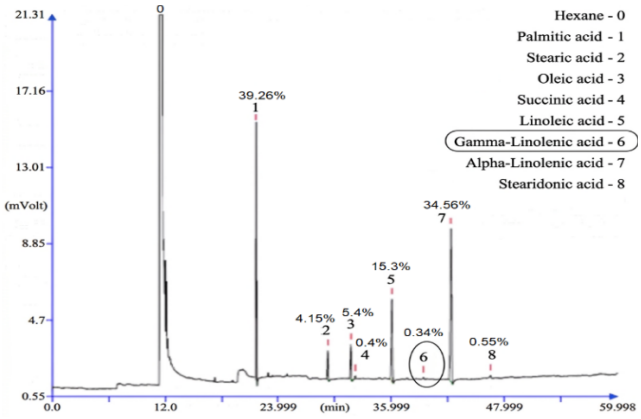


Fig. 8 Chromatographic peak profiles for seed oil using the Kermanshah ecotype (K9) with retention times (minutes) and compound identifications.

Percentage of GLA and Alpha-Linolenic Acid in Various Organs

The quantitative distribution patterns of gamma-linolenic acid (GLA, C18:3n6) and alpha-linolenic acid (ALA, C18:3n3) in the *Echium amoenum* organs showed significant organ accumulation patterns, as represented in Table 3. Both fatty acids were highest in seed oils, with ALA predominating at 39.40-48.21% and GLA at an average of 3.09-4.90% of total fatty acids. Conversely, the leaf oils had significantly lower concentrations (ALA: 58.2%, GLA: 0.08-0.89%), and roots had no GLA content (0-0.6%) and little ALA (2.3%). Ecotype variations were particularly pronounced in seed GLA content, with coastal ecotype So16 showing the highest level (4.90%), 58% greater than the lowest-yielding ecotype L15 (3.09%).

Table 3 Volume percentage of GLA (C18:3n6) and ALA (C18:3n3) in *E. amoenum* organs across ecotypes

Ecotype	Root GLA (%)	Leaf GLA (%)	Seed GLA (%)	Seed ALA (%)
G1	0	0.69	4.56	41.4
P2	0	0.08	4.65	44.42
O3	0	0	4.6	41.85
N4	0.4	0.46	3.73	46.27
E5	0	0.89	4.91	39.4
S6	0.45	0.75	4.17	48.21
M7	0	0	4.02	39.56
B8	0	0.34	3.29	46.2
K9	0	0.08	4.48	48.25
T10	0	0.63	3.88	43.61
2000	0	0.46	3.44	44.1
R12	0	0.7	4.02	42.11
H13	0.3	0.35	4.08	41.08
Z14	0	0	4.1	40.91
L15	0	0.4	3.09	41.08
So16	0.6	0.5	4.9	44.22

Mountainous ecotypes (N4, K9, and T10) exhibited intermediate GLA values (3.73-4.48%) but consistently higher ALA levels (43.61-48.25%), suggesting environmental modulation of desaturase enzyme activities [21]. The root GLA distribution was exceptionally limited, with only four ecotypes (N4, S6, H13, So16) showing detectable amounts (0.30-0.60%), while leaves displayed broader variation (0-0.89%).

Hierarchical clustering of these data (Figure 9) grouped ecotypes into four distinct clusters based on GLA distribution patterns: The first group includes the ecotypes of Galougah (P2), Gorgan (O3), Kermanshah (K9), Qazvin (Z14), and Kelardasht (M7); the second group consists of the ecotypes of Talesh (T10), Roudsar (R12), Neka (N4), Heyran (H13), and Isfahan (S6). The third group comprises the ecotypes of Jannat Rudbar (G1), Ashkorat (E5), and SoChalma (So16), while the fourth group comprises the three ecotypes of Behshahr (B8), Dohezar (2000), and Lorestan (L15). The analysis of the resulting similarity matrix revealed that the L15 and So16 ecotypes exhibited the lowest similarity (highest distance), whereas the P2 and O3 ecotypes showed the highest similarity (lowest distance). The geographical patterns of clustering were consistent with the geographical origins, implying the local adaptation of lipid metabolism. The increased GLA production of coastal ecotypes might be an adaptive response to augmented oxidative stress in a saline setting [22], and ALA predominance in mountainous ecotypes might improve membrane fluidity at low temperatures [23]. The low GLA of roots and leaves guides the harvest plans to focus on seed harvesting, and the ecotype clustering guides the breeding program towards combining desirable traits by controlled hybridization.

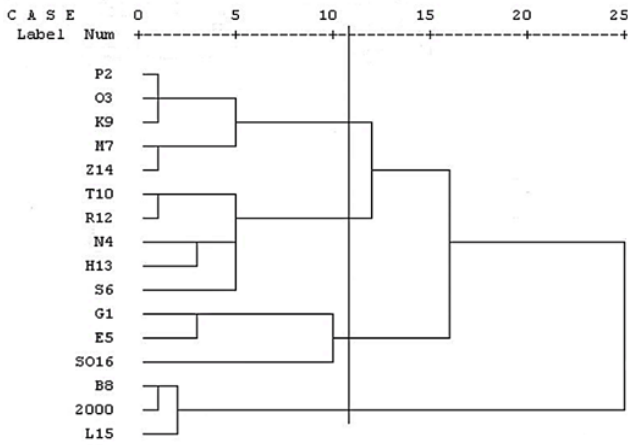


Fig. 9 Hierarchical clustering of ecotypes based on fatty acid composition data from GC analysis.

The reliability of these organ specific distributions is justified by the methodological consistency of the TLC (Section 4.3) and GC quantification. The hierarchical clustering method gives a framework for interpreting the complicated fatty acid information, and this has been applied to other oilseed medicinal plants. The research can be integrated with other chemical profiles in the future with transcriptomic data to determine the molecular markers of high-GLA or high- ALA phenotype to expedite breeding.

DISCUSSION

The ecotype and organ-specific profiles of the fatty acids of *Echium amoenum* identified in this research have serious implications for theoretical plant biochemistry and practical nutraceutical applications. The extreme localization of GLA and ALA in seeds as compared to their low concentration in leaves and roots highlights the evolutionary separation of lipid synthesis in plant organs. This compartmentalization probably indicates such adaptive mechanisms as seeds are specialized lipid stores of energy and membrane repair during germination [24], and leaves are to photosynthetic efficiency with ω−3-based membrane lipids [25]. The fact that GLA is nearly absent in roots indicates that there are basic limits to the activity of Δ6-desaturase in non-photosynthetic tissues, which may be because lipid biosynthetic genes are not expressed evenly or that there is channeling of substrates to Δ6-desaturase [26, 27]. Industrially, the ecotype differences in seed GLA contents (3.09-4.90%) have a biochemical foundation for selective cultivation. The ecotype So16, with its high GLA yield (4.90%), is a good candidate for commercial production, which would decrease the dependence on conventional sources of GLA such as borage oil (18-25% GLA) due to its higher biomass productivity [28]. The nutritional benefits of the balanced ω−6:ω−3 ratios (1.07 to 1.25) in the ecotypes N4 and K9 (mountains) give it nutritional benefits compared to traditional seed oils and are consistent with nutritional advice of using such oils in the diet to moderate inflammation [29]. Such results allow breeding efforts to be optimized to boost the desirable aspects- for an example, the cross of high-GLA coastal ecotypes and high-ALA mountainous ecotypes could be used to produce a hybrid with an optimal PUFA composition to help in a specific therapeutic application.

Methodological limitations must be acknowledged when interpreting these results. The absence of mass spectrometric validation creates possible errors in the identification of the peaks, especially with small components such as stearidonic acid. Although the DB-23 column yielded superior resolution of the GLA and ALA isomers, other unsaturated fatty acids might have co-eluted with the samples of the complex samples [30]. Although hierarchical clustering was vigorous, it relied entirely on the fatty acid structure in the exclusion of the genomic or environmental data that might have further contributed to the ecotype variation [31]. In further research, transcriptomics should be incorporated to relate patterns of fatty acids with patterns of gene expression, especially of the most important desaturases, such as FAD2 and FAD3 [32]. There are also questions of concern about the ecology that organizes the organ-specific lipid distribution and need to be looked into. The high GLA production of the coastal ecotypes can reflect an evolutionary process of adaptation to oxidative stress in saline environments where more ω−6 fatty acids can stabilize the membrane against ionic imbalance [33]. On the other hand, the domination of ALA by mountainous ecotypes could increase the fluidity of membranes in cold climates, which can be examined by climate-controlled growth experiments. This type of research may reveal new lipid-based stress tolerance systems that can be used to improve crops. Application is not just limited to nutraceuticals, but to sustainable agriculture. The low GLA levels in leaves and roots is an indication that residues after harvest can be reused as animal feed or as organic additives without any loss of lipid extraction. The ecotype-specific oil contents (18.3-24.7% in seeds) are also used as standards of economic feasibility calculations, and mountainous ecotypes are especially promising for low-output growing in marginal soils. These findings should be used in regional development programs to popularize *E. amoenum* as a high-value alternative crop in semi-arid areas, which is ecologically resilient and may have pharmaceutical value. Various knowledge gaps should be addressed in future research directions. The biochemical mechanism by which So16 produces GLA in high amounts is not known; detailed enzyme kinetics analysis of its Δ6-desaturase may uncover catalytic variations that can be used by protein engineering. The virtual lack of GLA in roots is worth exploring the tissue-specific silencing of desaturase genes, potentially via epigenetic processes [34]. There is a need to do long-term cultivation trials to determine fatty acid stability within generations, particularly of hybrid lines. Also, it should be confirmed that clinical trials have determined the bioavailability and efficacy of *E. amoenum*-derived GLA versus traditional sources, especially in the cases of anti-inflammatory and dermatological use [35]. The patterns of hierarchical clustering beg the research of wider patterns of biogeography. The repetitive clustering of ecotypes according to altitude (coastal vs. mountainous), even with geographical distance, indicates that lipid metabolism is heavily selected in the environment. Sampling to intermediate ranges would give evidence of clonal variability in the oil base composition and hence would indicate adaptive plasticity [36, 37]. These data would improve predictive tools to be used in determining the best cultivation locations depending on desired lipid profiles. The potential of *E. amoenum* might also be unlocked more through technological improvement. The TLC quantification method supported by AutoCAD, though efficient, may be supplemented by machine learning that will enable recognition of spots and analysis of patterns automatically [38]. Instant spectroscopic methods (e.g., NIR) in the field used as a way of screening fatty acids would aid breeding plans and quality management. It may also be done through metabolic engineering solutions, including Δ6-desaturase overexpression in leaf tissue: a way to convert presently unused biomass into more sources of GLA [39]. These findings also intersect with conservation biology. The distinct chemotypes of mountainous ecotypes N4, K9, and T10 represent unique genetic resources potentially threatened by climate change and habitat loss [33]. Their clustered metabolic profiles suggest limited genetic exchange between populations, warranting assessment of genetic diversity and in situ protection measures. Sustainable wild harvesting protocols must account for the observed organ-specific lipid distributions to avoid overexploitation of seed-bearing plants. The demonstrated variability in *E. amoenum* fatty acid profiles challenges the uniformity often assumed in traditional herbal medicine. Quality control standards for *E. amoenum*-based products should incorporate ecotype and organ specifications to ensure consistent bioactive compound content. Regulatory frameworks may need adaptation to accommodate such chemotype diversity, particularly for standardized extracts used in clinical settings. Ultimately, this research establishes *E. amoenum* as a versatile platform for investigating the interplay between ecology, biochemistry, and practical applications in medicinal plants. The integrated approach combining analytical chemistry, multivariate statistics, and geographical considerations provides a model for studying other species with regionally variable bioactive compounds. As demand for plant-derived specialty lipids grows, these insights will guide the sustainable development of *E. amoenum* resources while preserving their ecological and cultural context.

CONCLUSION

This study systematically characterized the fatty acid composition of *Echium amoenum* across different ecotypes and plant organs, revealing significant variations in the distribution of gamma-linolenic acid (GLA) and alpha-linolenic acid (ALA). The findings confirm that seeds serve as the primary reservoir of unsaturated fatty acids, particularly GLA (3.09-4.90%) and ALA (39.40-48.21%), while leaves and roots exhibit minimal accumulation. The hierarchical clustering analysis further demonstrated that ecotypes from distinct geographical regions-coastal versus mountainous-display unique fatty acid profiles, suggesting environmental adaptation influences lipid metabolism. These results not only align with existing knowledge on organ-specific lipid partitioning in plants but also provide novel insights into the ecotype diversity of *E. amoenum*, which has implications for its cultivation and utilization in nutraceutical applications.

The study contributes to the broader understanding of fatty acid biosynthesis in medicinal plants by highlighting the interplay between genetic and environmental factors in shaping lipid profiles. The identification of high-GLA ecotypes, such as So16, offers practical value for industrial applications, reducing reliance on traditional sources like borage oil. Moreover, the balanced ω−6:ω−3 ratios observed in certain ecotypes suggest potential health benefits, warranting further investigation into their therapeutic efficacy. The methodological framework, combining chromatographic techniques with multivariate analysis, also provides a replicable approach for studying fatty acid variability in other plant species.

Future research should explore the molecular mechanisms underlying the observed ecotypic differences, particularly the regulation of Δ6-desaturase activity in high-GLA varieties. Additionally, agronomic studies could assess the stability of these fatty acid profiles under varying cultivation conditions, ensuring consistent quality for commercial production. The ecological implications of lipid partitioning, such as the role of fatty acids in stress adaptation, also merit further exploration. By bridging the gap between fundamental biochemistry and applied research, this work lays the foundation for optimizing *E. amoenum* as a sustainable source of bioactive lipids while preserving its ecological and medicinal value.

Data availability

All relevant data can be found within the manuscript and its supporting materials, and further inquiries can be directed to the corresponding author.

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Author Contributions

M.F: conducted the research and prepared the first draft of the manuscript. A.A: supervised and funded the project, and edited the manuscript. N.H: Proposed the research idea, supervised and Project administration, Conceptualization, and writing and editing the manuscript.

Declaration of Competing Interest

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

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Generative AI Statement

During the preparation of this manuscript, the authors utilized Google Gemini Version 3.5 for results interpretation. All AI-generated content was thoroughly reviewed, curated, and verified by the authors to ensure accuracy, scientific integrity, and compliance with copyright laws. The authors take full responsibility for the final content of the manuscript.

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