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Thladiantha Seed Oils - New Source of Conjugated Fatty Acids: Characterization of Triacylglycerols and Fatty Acids

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Abstract: In this study, seed oils of *Thladiantha nudiflora* and *Thladiantha dubia* were found to contain 55.5 and 44.4% mole of conjugated octadecatrienoic fatty acids, respectively. The presence of moieties of conjugated fatty acids was confirmed by a series from physical methods: UV, IR, ^{1}H and ^{13}C NMR. The triacylglycerols (TAGs) isolated of the seed oils were studied by RP-HPLC with diode array and mass spectrometric detections. It was shown that all 15 TAGs of *Thladiantha dubia* contain moieties of conjugated fatty acids – punicic, (9*Z*,11*E*,13*Z*)-octadeca-9,11,13-trienoic acid (35.6% mole) and 8.9% mole α-eleostearic, (9*Z*,11*E*,13*E*)-octadeca-9,11,13-trienoic acid. Meanwhile, 24 TAGs of *Thladiantha nudiflora* seed oil contain both acids in approximately equal proportions (27.4:28.2 % mole). The enrichment for polyunsaturated fatty acids of the hydrolysis product of the seed oils due to urea inclusion complex formation was discussed.

Key words: *Thladiantha* seed oils, triacylglycerols, α-eleostearic acid, punicic acid

1 Introduction

Triacylglycerols (TAGs) are the most important components of animal and vegetable oils. The qualitative and quantitative determination of TAG species of the seed oils provides information, about the so-called "fatty acid composition" that determines health benefit of the oil. On the other hand, the information about the specificity of the moieties distribution in a series of TAGs is a valuable characteristic that may be used for an indication of seed oil falsification[1].

Particular attention has recently been paid to fatty acids with conjugated double C = C-bonds due to beneficial bioactivities, including anti-cancer effects[2], anti-diabetes[3], anti-obesity[4] with the ability to normalize the fatty acid metabolism in the body, and anti-inflammatory properties[5]. Conjugated linolenic acids (CLnAs) are unusual octadecatrienoic fatty acids containing three conjugated C = C-bonds. The physiological functions of CLnAs reviewed by Hennessy and coauthors[6] and Salsinha *et al.*[7] were cytotoxic to a group of cancer cells, such as HepG2 (hepatoma); A549 (lung); U-937 (leukemic cells); MDA-MB-231, MDA-ERa7v, MCF-7 (breast); MKN-7 (stomach); PC12, SH-SY5Y, NG108-15 (neuronal); DLD-1 (colorectal); T24 (human bladder); PC-3, LNCaP, DU 145 (prostate) and 3T3-L1 (preadipocyte) cells. Moreover, CLnAs have been shown to reduce the risk of obesity. For instance, CLnA mixtures significantly diminished perirenal adipose tissue weight in Sprague-Dawley rats when compared with other polyunsaturated fatty acids[8]. More known conjugated linoleic acids (CLAs) are derived from bovine milk and other ruminant animals' products in small quantities[5]. Meanwhile, the CLnAs have been reported to be synthesized as the main oil components in seeds of some rather rare and specific plants, and seven CLnA isomers with different structures are discovered by now[9].

Thladiantha nudiflora Forbes & Hemsl (1887) and

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Thladiantha dubia Bunge (1831) are two species belonging to the genus *Thladiantha*, Cucurbitaceae family, which are native to Eastern Asia[10]. Some species *Thladiantha* have been applied in Chinese traditional medicine[11, 12]. However, there is no information on the TAG composition of the oils, as well as on fatty acid composition of these species. The purpose of this paper is to determine the qualitative and quantitative composition of triacylglycerols and fatty acid of *Thladiantha nudiflora* Hemsl. and *Thladiantha dubia* Bunge seed oils.

2 Materials and Methods

2.1 General experimental procedures

The FTIR spectra were recorded by Shimadzu IR-Prestige spectrometer in thin-film transmittance technique in KBr cell with the scan resolution of 4.0 cm^{-1} in the range from 450 to 4000 cm^{-1}.

The UV spectra of seed oils in *n*-hexane were measured in quartz cells (1 cm) with a Shimadzu UV 1550 spectrophotometer.

The ^{1}H NMR 600 Hz and ^{13}C NMR 151 Hz spectra were recorded on a JNM-ECA spectrometer (JEOL KOREA LTD.) in $CDCl_3$ using TMS as an internal reference standard.

RP-HPLC Agilent 1260 Infinity coupled with a system with diode array (PDA) and mass spectrometric detectors (MS) (6130 Quadrupole MS, Agilent Technologies, US) and Shimadzu LC20 with refractive index detector (RID 10 A) were used for the analysis of TAGs and FAs, respectively.

2.2 Abbreviation of Fatty acid and TAGs

Triacylglycerols were labelled by letters representing acid moieties without identification of their position in the molecule: Pu represents the radical of C18:3^{9Z,11E,13Z} (punicic acid); αE - C18:3^{9Z,11E,13E} (α-eleostearic acid); L - C18:2^{9Z,12Z} (linoleic acid); O - C18:1^{9Z} (oleic acid); P - C16:0 (palmitic acid); and S - C18:0 (stearic acid). i.e. PuOP means TAG that contains moieties of punicic, oleic and palmitic acids.

2.3 Materials and Reagents

Oils were extracted for plant seed *Thladiantha nudiflora* Forbes & Hemsl collected from Di Linh, Lam Dong, Viet Nam, and identified by Dr Nguyen Quoc Binh (Department of Biology, Vietnam National Museum of Nature, VAST); voucher specimens (VNMN-B 2018.121), *Thladiantha dubia* Bunge and *Punica granatum* were grown in Botanical Garden of National Research University, Belgorod, Russia (2018).

HPLC grade acetonitrile and propan-2-ol (*i*-PrOH) were purchased from Merck (Darmstadt, Germany). Other reagents for isolation, extraction and purification were analytical grade reagents without further purification.

2.4 Oil Extraction and Purification

The process of oils extraction from *Thladiantha* seeds (2 g) by *n*-hexane at a room temperature by 10 mL portions of *n*-hexane was repeated 8 times. All portions were combined, and the extracted oil mass was determined gravimetrically after solvent evaporation on a vacuum rotary evaporator. Oil content as % per DW of *Thladiantha nudiflora* seeds was determined to be 37.1% ± 0.2 (n = 3, p = 0.95), refractive index $n_D^{25} = 1.493$ and for *Thladiantha dubia* – 40.3% ± 0.3 (n = 3, p = 0.95); $n_D^{25} = 1.489$.

The purification of the oils was performed by solid-phase extraction on DIAPAK C cartridges (BioChemMak ST, Moscow) using acetone–CH_2Cl_2 (1:1, v/v.) for oil desorption.

2.5 Chromatographic conditions

The chromatographic columns Kromasil 100-5C18 250 × 4.6 mm and Kromasil 110-3.5C18 2.1 × 150 mm (for MS detection) were used. Temperature of column thermostat was set at 30℃. Mobile phases of the systems "Propan-2-ol–Acetonitrile" and "Water–Acetic acid–Acetonitrile" (4:1:95, v/v/v) were for TAGs and FAs separation. MS detection was carried out in atmospheric pressure chemical ionization, APCI, mode with mass-to-charge range:100–1000 *m/z*; dry-gas (N_2) flow: 4 L/min; vaporizer: 400℃, and a fragmentor voltage of 150 V. To enhance the formation of ions ammonium formate (10 mM) was added to the mobile phase.

Chromatograms were recorded and handled using the Agilent ChemStation software and MagicPlot Student software for the separation of the so-called "problem" TAG – that with a low value of R_S.

2.6 Identification and Quantification Analysis of TAG

The determination of the triacylglycerol species composition was performed by the incremental approach[13, 14], and the compositions of TAG were confirmed by the parameters of both their positive-ion MS and electronic absorption spectra. The capacity factors *k* was calculated using column void time (t_M), determined by the retention times of a series of TAGs, assuming that the retention factors (*k*) in the series of $X_3 \rightarrow X_2L \rightarrow XL_2$ increase by the same value of logarithmic units of retention[15].

Mole fraction of TAGs on the chromatogram, registered at isosbestic wavelengths, 278 nm[16], was expressed by the formula: $\alpha(TAG_i) = \frac{\frac{S_i}{n_i}}{\sum_i \frac{S_i}{n_i}} \times 100$;

where n_i - the number of conjugated octadecatrienoic acid substituents in TAG;

α(TAG) - mole fraction of TAG;

S_i - corresponding peak area.

2.7 Saponification and fractionation fatty acid

The saponification of seed oils was performed by the ad-

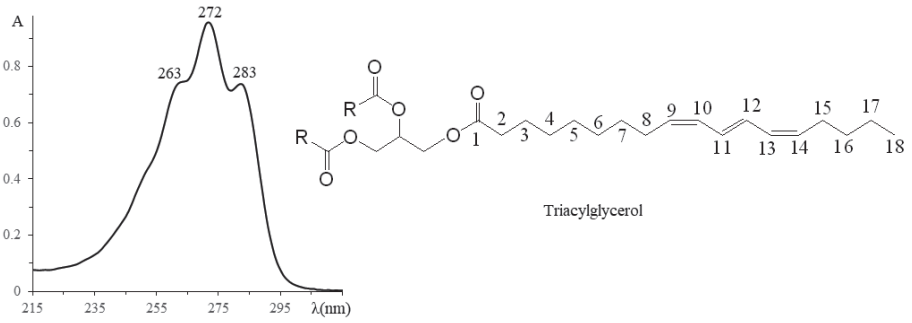

Fig. 1 UV spectrum of *Thladiantha nudiflora* seed oil in *n*-hexane.

dition of the 20% mole excess of 2M NaOH ethanolic solution to the ethanolic solution of the oil at room temperature and reflexing for at least 3 hours. The resulting mixture was acidified with concentrated HCl solution to pH ≈ 3, and fatty acids were extracted by *n*-hexane. *n*-Hexane solution was evaporated under vacuum in a rotary evaporator and FAs were stored at −20℃.

Enrichment of mixture by conjugated fatty acids was performed by urea inclusion complexes fractionation. Typically, 2.0 g of fatty acids were dissolved in 50 mL of ethanol at room temperature and 6.0 g of urea was added. The solution was stirred to homogeneity. The obtained solution was transferred to refrigerator and maintained at −20℃. After 12 hours the mixture was rapidly filtrated under the vacuum. The solid residue was dissolved in 100 mL of slightly acidified water (pH = 5) and extracted by 10 mL of *n*-hexane. The FAs of the filtrate were obtained after ethanol evaporation on the rotary evaporator. The FAs of urea complex were obtained after *n*-hexane evaporation.

2.8 Statistical analyses

All quantitative results of triacylglycerols and fatty acids were calculated from triplicate measurements and are supplemented with ± standard deviations, SD.

3 Results and Discussion

3.1 Confirmation of the presence of conjugated acid substituents in *Thladiantha* seed oils TAG

The presence of conjugated octadecatrienoic acid substituents was confirmed due to the specificity of the oil electronic absorption spectra in *n*-hexane oils solution. The spectra of *Thladiantha* seed oils (Fig. 1) is characterized by the absorption maxima at 263, 272 and 283 nm, being characteristic to that of conjugated trienoic compound[17].

In addition, the FTIR spectrum of the *Thladiantha* seed oils were compared with IR spectra of *Punica granatum* seed oil, containing more than 70% mole of punicic acid[18]. According to the literature data[19], conjugated trienoic fatty acids exhibit selective absorption in the 900 - 1000 cm^{-1} regions. The recorded IR spectra of *Thladiantha* seed oils contains absorption bands at 989.5 cm^{-1}(s), 980 cm^{-1}(w) and at 937.4 cm^{-1}(m) which are similar to the bands of *Punica granatum* seed oil spectrum.

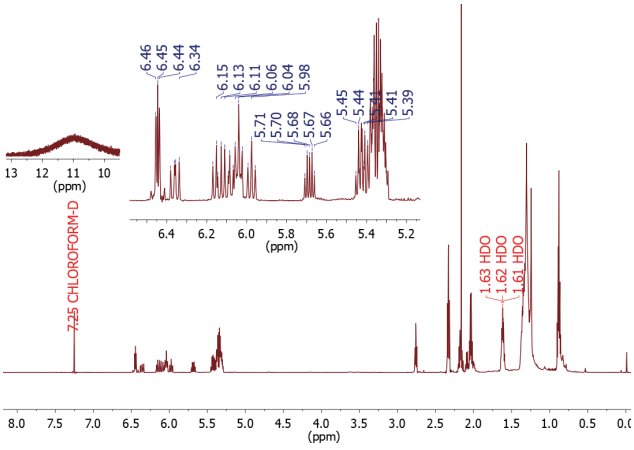

Fig. 2 ^{1}H NMR spectrum of fatty acids in *Thladiantha nudiflora* seed oil.

The presence of CLnAs also was confirmed by ^{1}H NMR and ^{13}C NMR spectra of fatty acid obtained after *Thladiantha* seed oils hydrolysis. The ^{1}H NMR spectrum (Fig. 2) shows the signals above 5.0 ppm (5.35 - 6.80 ppm) that may be attributed to six olefinic hydrogen atoms of the three conjugated C = C bonds[20].

^{13}C NMR spectra of fatty acids were also measured, and the chemical shifts of the carbon atoms of *Thladiantha nudiflora* seed oil fatty acid are compared with that of punicic acid and α-eleostearic acids (Table 1). The results reveal essentially the same chemical shifts for six carbon atoms of three conjugated C = C bonds.

3.2 Determination of compositions TAGs of *Thladiantha* seed oils

Due to the presence of conjugated moieties in seed oils of two species *Thladiantha*, the conditions of RP-HPLC method for the separation of TAG with spectrophotometric detection were developed. The separation of TAGs of Thladiantha seed oil was performed using isocratic elution mode with a mobile phase acetonitrile: *i*-PrOH (60:40, v/v).

Table 1 Comparation of ^{13}C chemical shifts (ppm) of punicic and α-eleostearic acids.

Position (C)	Punicic acid (C18:3^{9Z,11E,13Z})		α-eleostearic acids (C18:3^{9Z,11E,13E})	
	Present work	[20]	Present work	[20]
9	128.92	128.92	128.81	128.84
10	132.57	132.45	132.93	132.92
11	127.88	127.87	126.02	126.00
12	127.98	127.99	135.27	135.15
13	132.79	132.63	131.86	131.72
14	128.81	128.83	130.64	130.66

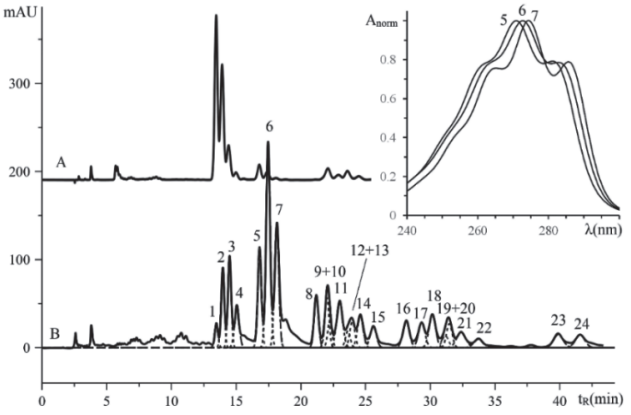

Fig. 3 Chromatograms of *Thladiantha nudiflora* (*B*) and *Punica granatum* (*A*) seed oils. Column 4.6×250 mm Kromasil 100×5 C18, mobile phase compositions: *IPA*: CH_3CN (4:6, v/v), 1 mL/min, for peak numbers, see Table 2.

The chromatograms of *Thladiantha nudiflora* seed oil with subsequent comparison with the chromatogram of the *Punica granatum* seed oil are presented (Fig. 3).

On the chromatogram (Fig. 3B) of *Thladiantha nudiflora* seed oil, five groups of peaks were observed, and 24 TAGs were separated. The TAGs of the six peaks (No. 1, 5, 8, 16, 18, 23) have identical electronic absorption spectra which absolutely coincide with electronic spectra of tripunicin (Pu_3) of *Punica granatum* seed oil with λ max at 274 nm, indicating the presence of punicic acid moiety in these TAGs. The electronic spectra of the peaks No. 4, 7, and 17 are characterized by a small hypochromic shift of the absorption band with the maximum at 271 nm. This indicates the presence of only α-eleostearic acid moiety in these three TAGs. The electronic spectra of other peaks showed intermediate wavelength of absorption maxima as a consequence of the simultaneous existence of the two isomeric conjugated trienoic acid substituents in the molecules of TAGs. From the Fig. 3 it is obvious that the separation of the main part of TAGs was achieved under the current chromatography condition except of two TAG pairs, αEL_2 + Pu_2O as well as αE_2O + Pu_2P. But this "critical" TAGs with a low value of R_s are easily handled by *Magicplot Student* 2.7.2 software with representation of individual components by unmodified Gaussians.

The composition of all peaks was calculated by the increment approach and is shown in Table 2. The presented data showed that the increments for TAG structure variation in corresponding TAG pairs are equivalent: for the substitution of punicic acid moieties with the linoleic (L) one, 0.116 logarithmic units; for linoleic with oleic (O), 0.136; for oleic with palmitic (P), 0.033; and for palmitic with stearic (S), 0.130. Meanwhile the increment for the replacement between two conjugated substituents (punicic acid substituent with the α-eleostearic (αE)) is only 0.019 logarithmic units. The minor value of the increment Δ (Pu→αE) is a reason of problems of the separation of octadecatrienoic isomers in RP-HPLC condition. The increments for the two *Thladiantha* species seed oils are just the same as for *Punica granatum* seed oil. The results of TAGs composition determination were verified by the parameters of both mass spectra of the obtained for adducts of ammonium ion with TAG [$M + NH_4^+$] and electronic absorption spectra, Table 2.

In our preliminary study[17], we reported that the molar fraction of punicic acid of *Thladiantha dubia* seed oil slightly exceeds 35%. In the present work, composition of 15 TAGs that are listed in Fig. 4 was determined.

The quantitative determination of TAG compositions for oils with acid substituents with different chromophores by spectrophotometric detection demands the proper choice of wavelengths for detection. According to our previous findings[16] for TAGs with isomeric conjugated octadecatrienoic acid moieties a wavelength of detection in an isosbestic point (278 nm) can be used for direct quantitative analysis. The results of the calculation of TAGs relative content in seed oils of the two species *Thladiantha* are given in Table 3.

According to data of Table 3 mole fraction of TAGs species of the two plants belonging to the same genus are significantly different. Pu_2L, $Pu\alpha EL$, PuL_2 and PuLO are the mains TAGs (mole fraction more than 5% for each of them) of the two *Thladiantha* seed oils. However, the total number of TAGs of *Thladiantha nudiflora* seed oil is obviously larger than that of *Thladiantha dubia* seed oil.

Table 2 Chromatography parameters and triacylglycerols components of seed oils *Thladiantha nudiflora*.

No	TAGs	t_R (min)	logk	Increments Δ(j→i) (±0.002)					*m/z* $[M+NH_4]^+$
				Pu→αE	Pu→L	L→O	O→P	P→S	
1	Pu_3	13.47	0.642						890.8
2	$Pu_2αE$	13.96	0.661	0.019					
3	$PuαE_2$	14.49	0.681	0.020					
4	$αE_3$	15.06	0.701	0.020					
5	Pu_2L	16.82	0.758		0.116				892.7
6	PuαEL	17.47	0.777	0.019					892.8
7	$αE_2L$	18.13	0.796	0.019					892.7
8	PuL_2	21.20	0.874		0.116				894.7
9 10	$αEL_2$ + Pu_2O	22.06	0.893	0.020		0.135			894.8
11	PuαEO	23.00	0.914	0.020					894.8
12 13	$αE_2O$ +Pu_2P	23.90	0.932	0.019			0.039		894.8 + 868.7
14	PuαEP	24.59	0.946				0.033		868.7
15	$αE_2P$	25.60	0.966	0.019					
16	PuLO	28.13	1.011			0.137			896.7
17	αELO	29.34	1.031	0.020					
18	PuLP	30.16	1.044				0.033		870.8
19	Pu_2S	31.30	1.061					0.129	896.7
20	αELP	31.45	1.064	0.020			0.033		870.7
21	PuαES	32.56	1.080	0.019					896.7
22	$αE_2S$	33.80	1.098	0.018				0.132	
23	PuLS	39.90	1.175					0.131	898.8
24	αELS	41.57	1.194					0.130	898.7
Mean value				0.019	0.116	0.136	0.033	0.130	–
Punica granatum seed oil									
Mean value				0.019	0.116	0.135	0.033	0.131	–

This is a consequence of the fact that biosynthesis of both punicic and α-eleostearic acids in approximately equal proportions occurs in *Thladiantha nudiflora* seed oil, while in the case of *Thladiantha dubia* seed oil only punicic acid is a main conjugated acid.

3.3 Characteristics of FAs compositions of *Thladiantha* seed oils

The calculation of mole fraction of fatty acids based on the results obtained for *Thladiantha* seed oils TAG composition (Table 4) indicated that the seed oils are abundant sources of conjugated octadecatrienoic fatty acid (sum of conjugated acids exceeds 45%).

The obtained results are in a good coincidence with the results of the determination of fatty acids after seed oils saponification. To increase the nutritional value of the products of vegetable oils processing after converting them to fatty acids, it is desirable to enrich the product by fatty acids with conjugated double bonds due to separation of the usual saturated fatty acids that are always present in the saponification product. In this case, fractionation methods using the methods of "green chemistry" are of fundamental importance, including the preparation of inclusion complexes of urea with fatty acids[21]. In the present work, the enrichment of conjugated fatty acid of *Thladiantha* seed oils was achieved by fractional crystallization of inclusion complexes at low temperature.

Mixtures FAs–urea–ethanol (1:3:17, w/w/w) was fractionated at −20℃ for 12 h. The complex was filtered and the composition of fatty acids in these filtrates was analyzed by RP-HPLC.

The results show that the mole fraction of polyunsatu-

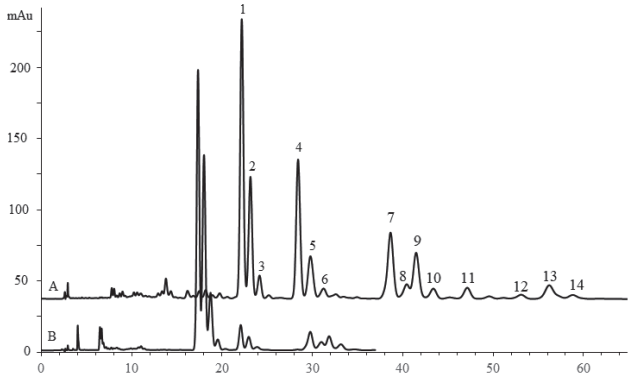

Fig. 4 Chromatograms of *Thladiantha dubia* (*A*) and *Punica granatum* (*B*) seed oils. Column 4.6 × 250 mm Kromasil 100 × 5 C18, mobile phase compositions: *i*-Pr: CH_3CN (3:7, v/v), 1 mL/min. The peaks: 1- Pu_2L; 2- PuαEL; 3- αE_2L; 4- PuL_2; 5- $\alpha EL_2 + Pu_2O$; 6- PuαEO; 7- PuLO; 8- αELO; 9- PuLP; 10- αELP; 11- αE_2S; 12- PuO_2; 13- PuLS and 14- αELS.

Table 3 Triacylglycerides composition of *Thladiantha nudiflora* and *Thladiantha dubia* seed oils.

TAGs	Mole fraction of TAGs, %			
	Thladiantha nudiflora		*Thladiantha dubia*	
	Mean	± SD[a]	Mean	± SD
Pu_3	0.88	0.06	nd[b]	–
$Pu_2\alpha E$	2.82	0.10	nd	–
$Pu\alpha E_2$	3.24	0.11	nd	–
αE_3	2.04	0.09	nd	–
Pu_2L	6.04	0.13	19.18	0.22
PuαEL	13.86	0.20	8.95	0.14
αE_2L	8.38	0.15	1.75	0.09
PuL_2	7.15	0.09	23.22	0.20
$\alpha EL_2 + Pu_2O$	8.42	0.12	4.53	0.10
PuαEO	4.50	0.10	1.30	0.08
αE_2O+Pu_2P	3.40	0.14	nd	–
PuαEP	2.85	0.10	nd	–
αE_2P	2.11	0.08	nd	–
PuLO	5.53	0.12	15.37	0.14
αELO	5.03	0.12	3.79	0.11
PuLP	6.81	0.14	10.70	0.15
Pu_2S	1.88	0.08	nd	–
αELP	4.05	0.12	2.75	0.09
PuαES	2.24	0.11	nd	–
αE_2S	1.25	0.07	1.45	0.08
PuO_2	nd	nd	1.24	0.08
PuLS	3.91	0.09	4.62	0.11
αELS	3.61	0.09	1.34	0.07

[a] Standard deviation (n=3); [b]nd – not detected (< 0.02%)

Table 4 Fatty acid composition of *Thladiantha* seed oils and its composition after the enrichment.

Fatty acid	Mole fraction of fatty acids, %			
	Thladiantha nudiflora		*Thladiantha dubia*	
	Initial	after the enrichment	Initial	after the enrichment
Punic acid (Pu)	27.44 ± 0.23	28.71 ± 0.22	35.57 ± 0.20	39.11 ± 0.25
α-Eleostearic acid (αE)	28.02 ± 0.23	27.44 ± 0.25	8.87 ± 0.12	9.05 ± 0.09
Linoleic acid (L)	28.18 ± 0.17	37.99 ± 0.18	39.74 ± 0.20	44.95 ± 0.22
Oleic acid (O)	6.07 ± 0.09	5.86 ± 0.10	8.18 ± 0.09	6.89 ± 0.15
Saturated (P+S)	10.29 ± 0.07	nd	6.89 ± 0.06	nd
The recovery rate of FAs	82.1 ± 0.4		80.5 ± 0.3	

rated fatty acids of *Thladiantha* seed oils obtained by the method of urea inclusion was significantly increased, and saturated fatty acids were removed. The recovery rate of fatty acids was approximately 80%.

4 Conclusion

The seeds of *Thladiantha dubia* and *Thladiantha nudiflora* contain 37.1 and 40.3 mass.%, of unique oils, respectively. The qualitative composition of 15 TAGs of *Thladiantha dubia* seed oil, 24 TAGs *Thladiantha nudiflora* seed oil were determined using incremental approach and analysis of both MS and electronic absorption spectra. *Thladiantha dubia* seed oil contain 36.17% punicic and 8.97% α-eleostearic acid substituents while for *Thladiantha nudiflora* seed oils content of these conjugated octadecatrienoic acid substituents was of 27.01% and 28.42% of punicic and α-eleostearic acid, respectively. The enrichment for polyunsaturated fatty acid of these oils was archived by the method of urea inclusion complex formation due to the saturated fatty acids complete removal.

Acknowledgments

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Supporting Information

Chromatography parameters of *Thladiantha dubia* seed oil, ^{13}C-NMR, IR spectra of *Thladiantha* seed oils, Chromatograms of fatty acids of *Thladiantha* seed oils.

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