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Assessment of Oil Content and Fatty Acid Composition Variability in Two Economically Important *Hibiscus* Species

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Supporting Information

ABSTRACT: The *Hibiscus* genus encompasses more than 300 species, but kenaf (*Hibiscus cannabinus* L.) and roselle (*Hibiscus sabdariffa* L.) are the two most economically important species within the genus. Seeds from these two *Hibiscus* species contain a relatively high amount of oil with two unusual fatty acids: dihydrosterculic and vernolic acids. The fatty acid composition in the oil can directly affect oil quality and its utilization. However, the variability in oil content and fatty acid composition for these two species is unclear. For these two species, 329 available accessions were acquired from the USDA germplasm collection. Their oil content and fatty acid composition were determined by nuclear magnetic resonance (NMR) and gas chromatography (GC), respectively. Using NMR and GC analyses, we found that *Hibiscus* seeds on average contained 18% oil and seed oil was composed of six major fatty acids (each >1%) and seven minor fatty acids (each <1%). *Hibiscus cannabinus* seeds contained significantly higher amounts of oil (18.14%), palmitic (20.75%), oleic (28.91%), vernolic acids (VA, 4.16%), and significantly lower amounts of stearic (3.96%), linoleic (39.49%), and dihydrosterculic acids (DHSA, 1.08%) than *H. sabdariffa* seeds (17.35%, 18.52%, 25.16%, 3.52%, 4.31%, 44.72%, and 1.57%, respectively). For edible oils, a higher oleic/linoleic (O/L) ratio and lower level of DHSA are preferred, and for industrial oils a high level of VA is preferred. Our results indicate that seeds from *H. cannabinus* may be of higher quality than *H. sabdariffa* seeds for these reasons. Significant variability in oil content and major fatty acids was also detected within both species. The variability in oil content and fatty acid composition revealed from this study will be useful for exploring seed utilization and developing new cultivars in these *Hibiscus* species.

KEYWORDS: *Hibiscus germplasm, H. cannabinus, H. sabdariffa, variability, oil content, fatty acid composition, unusual fatty acid, seed oil utilization, nuclear magnetic resonance (NMR), gas chromatography (GC)*

INTRODUCTION

The *Hibiscus* genus contains more than 300 species. Most species produce showy flowers, and the plants are often used in landscaping as shrubs or small trees. *Hibiscus syriacus* L. is the national flower of South Korea. Flowers and calyces from *Hibiscus sabdariffa* L. can be used for making tea or beverages. People in Jamaica and other islands make sorell drink from flowers of *H. sabdariffa* (roselle). Roselle leaves and calyces can also be used as vegetables. Another species, *H. cannabinus* L. (kenaf), is intensively used for making rope and paper products.[1] *Hibiscus* species also produce small seeds which can be potentially used for producing edible oils or feeding livestock.[2,3] However, *Hibiscus* seed oil contains two unusual fatty acids: dihydrosterculic acid (DHSA) and vernolic acid (VA). When *Hibiscus* seed or oil is consumed by animals or human, DHSA in *Hibiscus* (similar to cottonseed oil) can have detrimental health effects such as toxicity and physiological disorders;[2,4] whereas oil rich in VA has potential utility in industrial applications such as in plastics or adhesives in painting materials.[5,6] Kenaf and roselle are probably the two most economically important species in the genus.[7] In the U.S., the *Hibiscus* germplasm collection is maintained in the Plant Genetic Resources Conservation Unit (PGRCU), USDA-ARS, Griffin, Georgia.[1] Morphological traits and anthocyanin and flavonol content have been evaluated from selected species in the collection.[7] However, variability in oil content and fatty acid composition (especially for these two unusual fatty acids) within *Hibiscus* species has not been determined. Since roselle and kenaf are the two most economically important species, assessment of oil content and fatty acid composition was focused on these two species. Therefore, the objectives of this study were to (i) determine the variability of oil content and fatty acid composition in these two species; (ii) compare the level of oil content and fatty acid composition between two species; (iii) determine the correlations among fatty acids and between oil content and fatty acids; and (iv) identify accessions with high oil content and/or higher quality fatty acid composition for utilization or as parents for the development of new *Hibiscus* cultivars.

MATERIALS AND METHODS

Germplasm Accessions and Seed Acquirement. Among the 329 *Hibiscus* accessions evaluated, 222 and 107 were *H. cannabinus* and *H. sabdariffa*, respectively. Seeds for these accessions were acquired from two locations: 162 accessions (with prefix PI) from the USDA-ARS, Plant Genetic Resources Conservation Unit, Griffin, GA, and 167 accessions (with prefix NSSL) from the USDA-ARS, National Center for Genetic Resources Preservation, Fort Collins, CO. The inventory number, species name, and country of origin or collection site for these accessions are listed in Table S1 in the Supporting Information.

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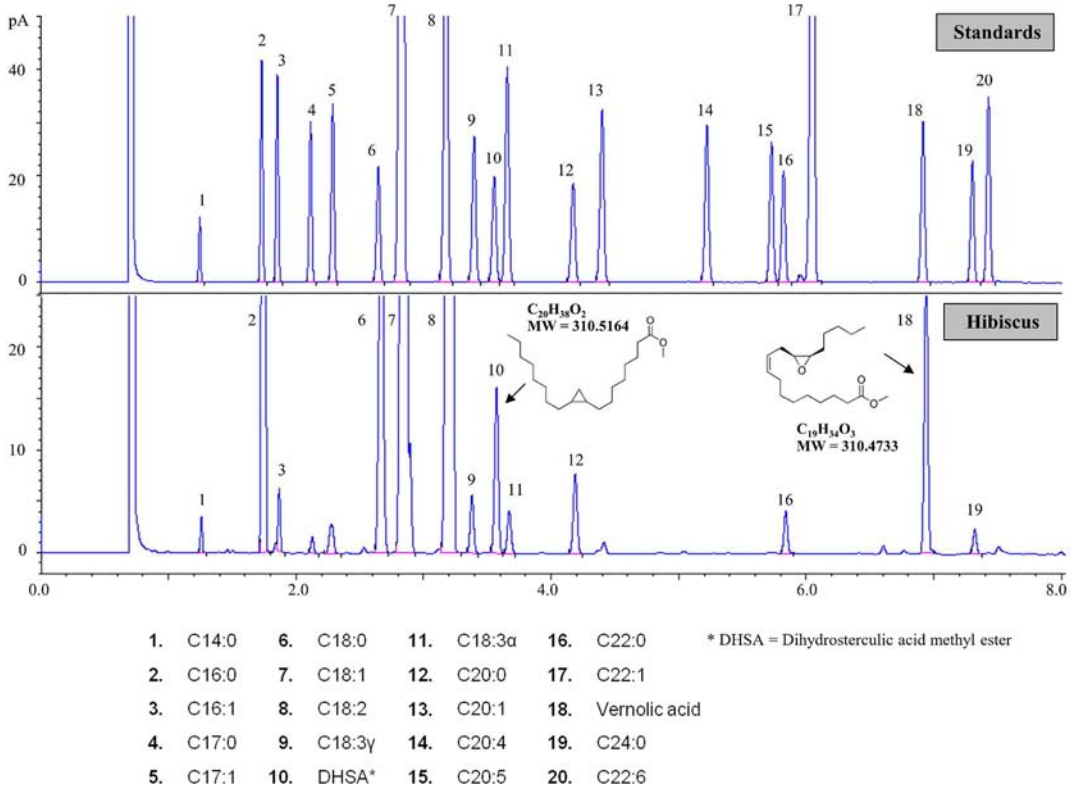

Figure 1. Chromatograms of methyl esters from standards and *Hibiscus* oil generated by GC analysis.

Oil Content by NMR Analysis. Oil content was quantified by nuclear magnetic resonance (NMR) analysis. In a magnetic field, certain atomic nuclei resonate at specific radio frequencies. This resonance can be converted to a signal and measured to determine the amount and nature of the particular nuclei in a sample. Solids and liquids containing hydrogen nuclei can easily be distinguished in this manner; and oils and water can also be differentiated by employing specifically timed radio pulses, provided the moisture content is relatively low (<15% of the total mass).[8] NMR signals calibrated against standards of known amounts were generated for measuring oil and water content in *Hibiscus* seed samples. The oil and water content were measured on a Mini-spec mq10 NMR analyzer (Bruker Optics Inc., Houston, TX, USA). The NMR was maintained at 40 °C and operated at a resonance frequency of 9.95 MHz. For each signal acquisition, spin−echo parameters consisted of a 90° pulse of 10.44 μs and reading at 50 μs followed by a 180° pulse of 21.38 μs (pulse spacing = variable) and reading at 7 ms. A 2 s recycle delay between scans was used, and a total of 16 scans were collected for each sample. No prior preparation of the seeds was necessary. For establishing an oil standard curve, nine standards were prepared by weight. Although pure *Hibiscus* oil is not available commercially, cottonseed oil was used because *Gossypium* (cotton) is closely related to *Hibiscus*. For each standard, shredded paper was added to the sample tube to serve as a matrix, along with a carefully measured mass of oil. Moisture standards were prepared using *Abelmoschus* (another closely related genus) seeds of known moisture content. Seed oil and water content were measured, and the mass of each measurement was converted to a percentage of the total weight of each sample. All samples were measured in triplicate (25 seeds for each replication) and the results averaged.

Fatty Acid Composition by GC Analysis. Fatty acid methyl esters (FAMEs) were prepared from seeds by alkaline trans-methylation.[9] Seeds (20−25) were ground in a coffee grinder and stored at 4 °C until analysis. Approximately 50−100 mg of ground material was transferred to a glass tube, and 3.0 mL of *n*-heptane (Fisher Scientific) was added to extract the oil. For conversion of fatty acids to methyl esters, 500 μL of 0.5 M sodium methoxide ($NaOCH_3$) in methanol solution (Sigma-Aldrich, St. Louis, MO) was added to the tube and mixed with the sample. After 2 h, 7 mL of distilled water was added to separate the organic layer containing FAMEs from the seed residue. An aliquot was transferred to a 2.0 mL autosampler vial for analysis by gas chromatography (GC).

Fatty acid composition was determined using an Agilent 7890A GC equipped with a flame ionization detector (FID) and an autosampler. Peak separation was performed on a DB-23 capillary column (15 m × 0.25 mm i.d. with a 0.25 μm film) from Agilent Technologies. The carrier gas was helium set to a constant inlet pressure of 12.000 psi (~1.1 mL/min at 185 °C initial temperature). A fatty acid methyl ester (FAME) standard mix was used to establish peak retention times for 20 unique fatty acids. This mix included two rare fatty acids found in

Table 1. Variability in Investigated Traits among *Hibiscus* Accessions[a]

trait	*n*	mean	SD	min	max
oil %	984	17.88	2.00	11.53	22.81
seed wt (g)	984	2.23	1.52	0.56	4.84
C14:0%	656	0.18	0.05	0.07	0.37
C16:0%	656	20.02	1.76	15.65	25.35
C16:1%	656	0.52	0.13	0.25	0.94
C18:0%	656	4.08	0.64	2.75	7.32
C18:1%	656	27.69	4.93	14.34	49.78
C18:2%	656	40.52	5.68	21.71	61.18
C18:3γ %	656	0.50	0.20	0	1.68
DHSA %	656	1.24	0.50	0.47	3.69
C18:3α %	656	0.37	0.12	0.15	1.02
C20:0%	656	0.49	0.14	0.22	0.88
C22:0%	656	0.28	0.09	0.09	0.77
VA %	656	3.95	2.13	0.26	9.61
C24:0%	656	0.16	0.03	0.09	0.26

[a]Standard deviation (SD), seed oil and methyl esters (%), seed weight (g/100 seeds), dihydrosterculic acid (DHSA), and vernolic acid (VA).

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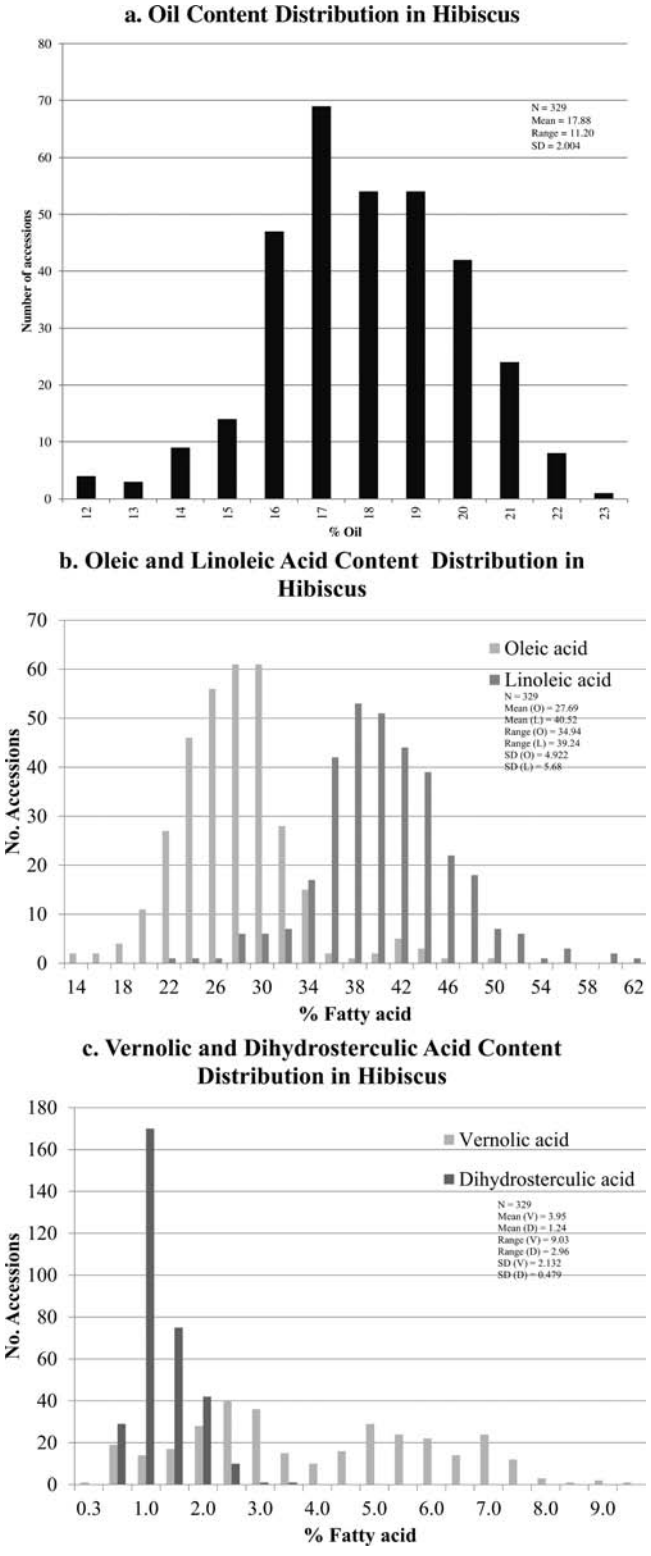

Figure 2. Oil content and some fatty acid distributions in *Hibiscus* accessions.

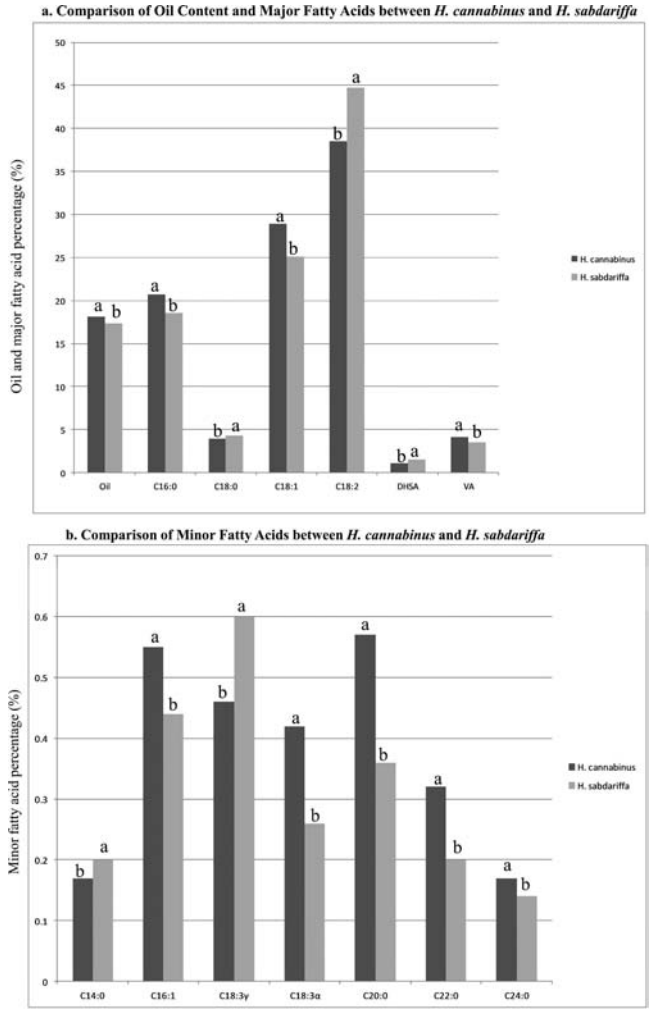

Figure 3. Comparison of seed traits between *H. cannabinus* and *H. sabdariffa*.

oils from seeds in the family *Malvaceae*, vernolic acid (VA) and dihydrosterculic acid (DHSA). All standards were purchased from Sigma-Aldrich except DHSA, which was purchased from Matreya, LLC. One microliter of sample was injected at a 30:1 split ratio into the column with the following thermal profile: 185 °C for 3 min; 185 to 190 at 5.0 °C per min; 190 to 240 at 10 °C per min. The inlet and detector were set to 280 and 300 °C, respectively. Total run time for each sample was 9 min. Fatty acid composition was determined by identifying and calculating relative peak areas. All samples were prepared and injected twice.

Seed Weight. Three samples of available seeds were counted and weighed. The average weight for each accession was expressed as grams per 100 seeds.

Statistical Analysis. Data were recorded by replicates, accessions, and two species. An analysis of variance was performed on the data, and means were separated using Tukey's multiple comparison procedure (SAS, 2008, Online Doc 9.2. Cary, NC: SAS Institute Inc.). Significant correlations between seed traits were determined using Pearson correlation coefficients.

RESULTS AND DISCUSSION

Oil Content, Fatty Acid Composition, and Seed Weight in *Hibiscus*. *Hibiscus* seeds are relatively small, averaging only 2.23 g per 100 seeds and ranging from 0.56 to 4.84 g. There was significant variation in seed weight (almost a 9-fold difference) among accessions. *Hibiscus* seeds contained an average of 17.88% oil, which was composed of thirteen fatty acids. The fatty acid profiles in *Hibiscus* seed oil along with standards identified by GC are shown in Figure 1. The average percentage and range of each fatty acid are listed in Table 1. Among these thirteen fatty acids, six were classified as major fatty acids (each >1%) and seven were classified as minor fatty acids (each <1%). Two unusual fatty acids, dihydrosterculic

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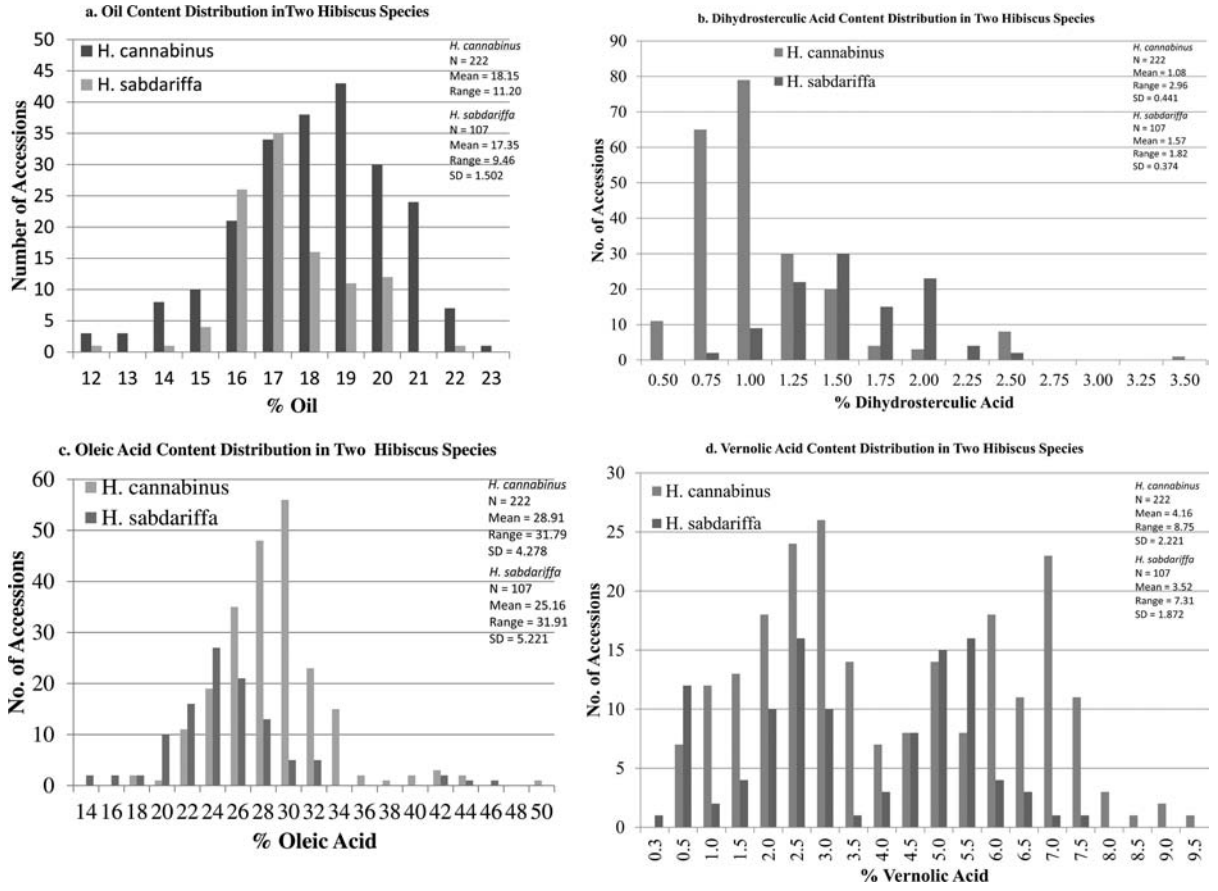

Figure 4. Oil content and some interested fatty acid distribution in two *Hibiscus* species.

acid (DHSA) and vernolic acid (VA), were classified as major fatty acids. Most *Hibiscus* accessions contained about 16−20% oil, but four accessions contained less than 13% oil, and only one accession (PI 343133) contained near 23% oil (Figure 2a). The accession PI 405443 (containing 5% more oil than the average) may be good breeding material to use for developing high oil producing *Hibiscus* cultivars. The six major fatty acids in *Hibiscus* seed oil are linoleic (40.52%), oleic (27.69%), palmitic (20.02%), stearic (4.08%), vernolic (3.95%), and dihydrosterculic acids (1.24%), and each displays a wide range of variation (Table 1).

Oleic acid can be converted to linoleic acid by desaturation (i.e., the addition of a double bond). The levels of these two fatty acids in seed oil are negatively correlated. Since consuming oils with high levels of oleic acid may be beneficial to human health, a high oleic/linoleic acid (O/L) ratio is preferred for developing healthier oilseed cultivars in breeding programs.[10] Oleic and linoleic acids accounted for 67% of the total fatty acids in *Hisbiscus* seed oil with significant variability ranging from 14.34 to 49.78% and from 21.71 to 61.18%, respectively (Figure 2b). Therefore, there is potential to enhance the level of oleic acid and reduce linoleic acid (i.e., to enhance the O/L ratio) via breeding programs. Palmitic (C16:0) and stearic (C18:0) acids were the other two major fatty acids, but these are saturated acids, accounting for 24% of the total fatty acids with significant variability ranging from 15.65 to 25.35% and from 2.75 to 7.32%, respectively. Thus there is potential to reduce the levels of these two saturated fatty acids. The remaining two major fatty acids were less common: dihydrosterculic acid (DHSA) and vernolic acid (VA). Although their levels were low (1.24% and 3.95%), there was significant variability ranging from 0.47 to 3.69% and from 0.26 to 9.61%, respectively (Table 1 and Figure 2c). DHSA is also called 2-octyl cyclopropaneoctanoic acid, identified in oils of lychee (*Litchi sinensis*) seeds, *Euphoria longana* seeds, and cotton (*Gossypium hirsutum*) seeds, and has been confirmed to have detrimental health effects on livestock.[4,11,12] To enhance *Hisbiscus* seed oil utilization, the level of this fatty acid should be reduced to as low as possible. There were 11 accessions which had low levels of DHSA (<0.6%). These accessions may be useful for developing low DHSA cultivars. Vernolic acid is a C_{18} fatty acid also named 12-epoxyoctadec-*cis*-9-enoic acid. It was first found in *Vernonia anthelmintica* (L.) and has also been identified in other species such as *Stokesia* and *Crepis*, but its physiological function is unknown.[5] Oils with a high level of vernolic acid can be used as industrial raw materials to manufacture plastic formulations, adhesives, and protective coatings.[13,14] There were two accessions (PI 405443 and PI 370652) containing 9.30% and 9.14% vernolic acid, respectively which were significantly higher than the other accessions (minimum significant difference = 0.858%). These two accessions may be useful to develop high vernolic cultivars for industrial utilization.

Comparison of Traits between and within Species. There were significant differences in investigated seed traits between *H. cannabinus* and *H. sabdariffa* (Figure 3a and Figure 3b). Oil content in *H. cannabinus* (18.14%) was significantly higher than in *H. sabdariffa* (17.35%). *Hibiscus cannabinus* seeds contained a significantly higher level of oleic acid (28.91%) and significantly lower levels of linoleic (38. 49%), stearic (3.96%), and dihydrosterculic acids (1.08%) than *H. sabdariffa* seeds (25.16%, 44.72%, 4.31%, and 1.57%). Based on these fatty acid profiles,

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Table 2. Pearson Correlation Coefficients, Probability, and Number of Observations for Oil Content, Seed Weight, and Fatty Acid Composition within *Hibiscus* Species[a]

	oil	SdWt	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3γ	DHSA	C18:3α	C20:0	C22:0	VA	C24:0
oil	1	0.20	−0.25	0.12	0.05	−0.02	0.30	−0.13	−0.21	−0.41	−0.24	0.20	0.07	−0.32	0.04
		<0.0001	<0.0001	0.0027	0.2470	0.6331	<0.0001	0.0008	<0.0001	<0.0001	<0.0001	<0.0001	0.0571	<0.0001	0.3243
SdWt		1	−0.18	0.04	0.27	−0.27	0.15	−0.17	0.10	−0.34	−0.24	−0.12	−0.09	0.24	0.01
			<0.0001	0.2882	<0.0001	<0.0001	<0.0001	<0.0001	0.0124	<0.0001	<0.0656	0.0022	0.0446	<0.0001	0.8551
C14_0			1	0.18	−0.26	0.27	−0.08	0.00	0.16	0.28	−0.10	0.15	0.03	−0.15	0.21
				<0.0001	<0.0001	<0.0001	0.0477	0.9428	<0.0001	<0.0001	0.0146	0.0001	0.4974	<0.0001	<0.0001
C16:0				1	0.29	−0.14	0.17	−0.49	−0.13	−0.38	0.24	0.55	0.50	0.13	0.50
					<0.0001	0.0002	<0.0001	<0.0001	0.0008	<0.0001	<0.0001	<0.0001	<0.0001	0.0008	<0.0001
C16:1					1	−0.47	−0.03	−0.18	0.02	−0.26	0.19	−0.11	0.08	0.45	−0.11
						<0.0001	0.4188	<0.0001	0.6373	<0.0001	<0.0001	0.0066	0.0316	<0.0001	0.0051
C18:0						1	0.20	−0.19	0.13	0.34	−0.15	0.31	−0.06	−0.23	0.15
							<0.0001	<0.0001	0.0006	<0.0001	0.0001	<0.0001	0.1531	<0.0001	<0.0001
C18:1							1	−0.85	−0.12	−0.15	−0.05	0.56	0.28	−0.23	0.42
								<0.0001	0.0018	0.0001	0.1957	<0.0001	<0.0001	<0.0001	<0.0001
C18:2								1	0.02	0.15	−0.07	−0.64	−0.44	−0.19	−0.53
									0.6322	0.0001	0.0979	<0.0001	<0.0001	<0.0001	<0.0001
C18:3γ									1	0.44	−0.10	−0.25	−0.07	0.12	0.17
										<0.0001	0.0086	<0.0001	0.0668	0.0023	<0.0001
DHSA										1	0.05	−0.19	−0.04	−0.10	0.05
											0.2226	<0.0001	0.2647	0.0126	0.1885
C18:3α											1	0.38	0.59	0.01	0.24
												<0.0001	<0.0001	0.7211	<0.0001
C20:0												1	0.76	−0.19	0.71
													<0.0001	<0.0001	<0.0001
C22:0													1	−0.01	0.70
														0.8742	<0.0001
VA														1	−0.14
															0.0002

[a]SdWt, seed weight (g/100 seeds); DHSA, dihydrosterculic acid; VA, vernolic acid. Number of data points, 984 for oil and 656 for other traits, was used for calculation of the correlation coefficients.

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H. cannabinus is a better oil source than *H. sabdariffa* for edible consumption. But *H. cannabinus* also contained a significantly higher amount of palmitic acid (20.75%) than *H. sabdariffa* (18.52%). This trait needs to be improved by reducing the level of saturated fatty acids. *Hibiscus cannabinus* also contained a significantly higher level of vernolic acid (4.16%) than *H. sabdariffa* (3.52%), and its oil may also be a better source than *H. sabdariffa* for industrial utilization. Seven minor fatty acids represented only 2−3% of the total fatty acids. Although there were significant differences in each minor fatty acid between the two species (Figure 3b), their contribution for improving oil quality may be small. From the investigated seed traits, overall *H. cannabinus* is a better potential source of oil than *H. sabdariffa* for both edible consumption and industrial utilization.

Significant variability in seed traits was also detected within species, and the results for some interesting traits are shown in Figure 4. The ranges of variability in oil content for *H. sadbariffa* and *H. cannabinus* were 12−22% and 12−23%, respectively. The former range was completely encompassed by the latter range as shown in Figure 4a. Similarly, the range of variation in DHSA for *H. sadbariffa* (0.75−2.50%) was also completely covered by *H. cannabinus* (0.5−3.5%, Figure 4b). The range of variability within *H. cannabinus* was larger than within *H. sadbariffa.* This may be due to more accessions investigated in *H. cannabinus* (222) than in *H. sadbariffa* (107). However, the upper ends of the variability ranges for oleic and vernolic acids (Figure 4c and Figure 4d) in *H. sadbariffa* were covered by *H. cannabinus*, but the lower ends of the ranges were not. This incomplete coverage may be explained by the following reasons. (a) There are no significantly statistical differences between the covered and uncovered regions in the low ranges. (b) Since the median value was much smaller in *H. sabdariffa* than in *H. cannabinus*, the whole distribution for *H. sabdariffa* was shifted from right to left (Figure 4c and Figure 4d). Two extremes in the lower end of the range may reflect the real situation for these two species. (c) The sample number from *H. cannabinus* may still not be large enough to cover the lower extremes. Variability or accessions distributed in the extreme regions can be useful in choosing accessions for specific utilization.

Correlations among Different Seed Traits. There were 15 seed traits investigated in this study, and their correlations are shown in Table 2. Oil content was significantly correlated (at $p < 0.0001$ level) with seed weight, myristic acid, oleic acid, linolenic acids, dihydrosterculic acid, arachidic acid, and vernolic acid, but the highest correlation (r) value was −0.41 with DHSA. This implies that if the oil content increases, the DHSA content may decrease. This may be desirable because DHSA has detrimental effects on animal or human health. Seed weight was significantly correlated (at $p < 0.0001$ level) with most of the traits, but none of the correlation (r) values exceeded 0.5. Seed weight may change, but it has no significant effect on the other traits. Among thirteen fatty acids, six (C14:0, C16:1, C18:0, C18:3γ, DHSA, and VA) had significant correlations (at $p < 0.0001$ level) with other fatty acids, but none of the correlation (r) values were >0.5. However, palmitic acid was significantly correlated (at $p < 0.0001$ level) with arachidic ($r = 0.55$), behenic ($r = 0.50$), and lignoceric acids ($r = 0.50$). Oleic acid was significantly negatively correlated with linoleic acid ($r = -0.85$) but positively correlated with arachidic acid ($r = 0.56$) at $p < 0.0001$ level. Linoleic acid was significantly negatively correlated (at $p < 0.0001$ level) with arachidic ($r = -0.64$) and lignoceric acids ($r = -0.53$). Linolenic acid (C18:3α) was significantly correlated (at $p < 0.0001$ level) with behenic acid ($r = 0.59$). Arachidic acid was significantly correlated (at $p < 0.0001$ level) with behenic ($r = 0.76$) and lignoceric acids ($r = 0.71$). Behenic acid was significantly correlated (at $p < 0.0001$ level) with lignoceric acid ($r = 0.70$). These significant correlation coefficients are useful for potentially altering fatty acid composition in *Hibiscus* via breeding programs.

Evaluation of Accessions by Different Traits. There were 329 *Hibiscus* accessions evaluated for oil content and fatty acid composition in this study. There were two accessions (PI 343133 and PI 343127) with oil content over 22%, significantly higher than many of the other accessions. Both accessions are from *H. cannabinus* and were originally collected from France. Their oleic acid content (32.7% and 30.0%) was above average (27.69%), and their DHSA content (0.84% and 0.83%) was below average (1.24%). If these two accessions are to be cultivated for edible oil, their oleic acid content still needs to be increased and their DHSA content needs to be decreased. There were two accessions (PI 405443 and NSSL 370652) with vernolic acid over 9%, significantly higher than many of the other accessions. These two accessions were also from *H. cannabinus*. The accession NSSL 370652 was collected from China and contained 9.14% vernolic acid with 21.15% oil, much higher than the average (17.88%). The accession PI 405443 was collected from Tanzania and contained 9.3% vernolic acid but only 16.13% total oil, lower than the average. If this accession is to be cultivated for use as an industrial oil (e.g., as a paint additive), its oil content needs to be increased through breeding programs.

In this study, two species were evaluated for oil content and fatty acid composition but not for other traits such as anthocyanin and flavonoid content. There are more *Hibiscus* species available in the USDA germplasm collection. Consuming products from *Hibiscus* has some positive effects on human health. For example, drinking *Hibiscus* tea can lower blood pressure in prehypertensive and mildly hypertensive adults.[15] Therefore, more species and their phytochemical traits should be evaluated to fully explore their potential utilization.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of information for evaluated *Hibiscus* accessions from two species and table of oil content and fatty acid composition for each accession. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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