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Dr. Miloslav Rechcigl, Jr., Research Review Director
AID/CDR Program - Office of Research
Room 320, SA-18, R&D/R
Agency for International Development
Washington, DC 20523-1818
U. S. A.

Dear Dr. Rechcigl:

Re: AID-CDR Project No. C12-163
Grant No. TA-MOU-C12-163
Chemical studies on Vernonia oil and genetic improvement of Vernonia galamensis
Dr. David Mills

Enclosed please find four (4) copies of the 6-month performance report, submitted by Dr. David Mills for the above-mentioned project.

With thanks for your cooperation.

Yours sincerely,

Moshe Amir
Director
Department of Research Contracts

cc: Science Attache - US Embassy Tel-Aviv (w/encl 1)
AID Representative, USAID Mission - Kenya (w/encl 1)
CDIE, Washington, DC (w/encl 1)
NAS/BOSTID, Washington, DC (w/encl 2)
Prof. L. Naggan
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THE INSTITUTES FOR APPLIED RESEARCH
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CHEMICAL STUDIES ON VERNONIA OIL AND GENETIC IMPROVEMENT OF *VERNONIA GALAMENSIS*

Semiannual report
August 1993-February 1994

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Submitted to AID-CDR
(File No. 86723101)

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Abstract

- Seeds of *Vernonia galamensis* hybrids between accession V-001 and other accessions produced in the current season will be grown and evaluated in the next season. The performance and phenological characteristics of some hybrids produced two years ago, and grown in the field during 1993, indicate that the crosses were successful, and the hybridization program may thus lead to cultivars suitable for growth under our local conditions.
- During the winter of 1992/3 we carried out many more crosses between accession V-001 and V-029, V-026, V-018, and V-020. We obtained many hybrid seeds that will be evaluated in the field during the spring and summer of 1994, and are currently being used for backcrosses with V-001.
- Observations made in the field revealed that accessions V-004, V-029, and V032, which flower in response to long days, are good candidates for crossing with V-001. These accessions will be used for further hybridization in the next year.
- The methyl ester of vernolic acid was used as a model compound in two types of bromination reaction: direct addition of bromine to the double bond and allylic bromination. The 9,10 dibromo derivative and the 8,11 dibromo derivative of methyl ester of vernolic acid were obtained.
- A hexabromoderivative of vernonia oil was obtained as the main product by direct bromination of the oil.

Please note that the process of approving this program for funding took more than two years. The reporting period thus includes our work from 1992, although funding was approved only in 1993.

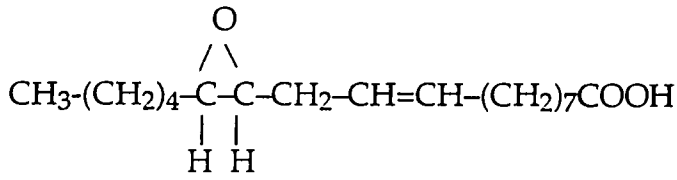
Key words: bisamides, breeding, epoxy oil, *Vernonia galamensis*, methyl ester of vernolic acid, bromination, dibromomethyl ester of vernolic acid

Introduction

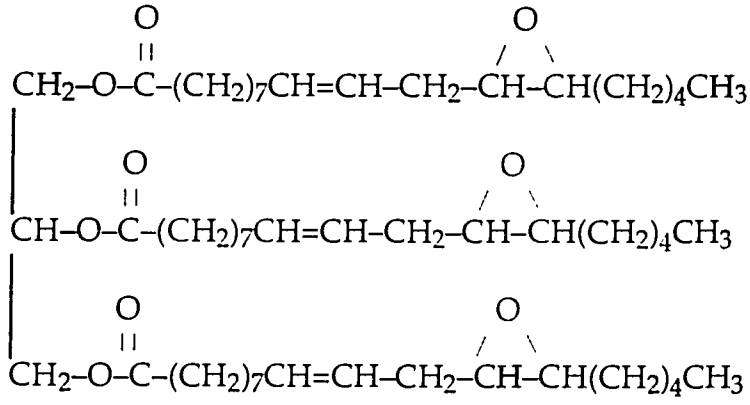
Vernonia galamensis, an annual plant of the Compositae family, is distributed widely throughout Africa. The species grows in a variety of conditions, from areas with an annual rainfall as low as 200 mm (Perdue 1989) to rich forested areas with an annual rainfall of 1,850 mm. The subspecies *afromontana* and *lushotoensis* grow in areas with the most abundant rainfall, which rarely have a prolonged dry period. The two subspecies *nairobensis* and *gibbosa* are distributed in areas of dry evergreen forest, in which rainfall is bimodal and lower than in the habitat with the highest rainfall. The subspecies *mutomoensis* and *galamensis* (varieties: galamensis, petitiana, australis, and ethiopica) are restricted to drier habitats with a well-defined and severe dry season. Populations of the variety petitiana of the subspecies *galamensis* that are native to Sudan and Eritrea provide the most drought-resistant germplasm of *V. galamensis*.

The most important germplasm of *V. galamensis* is accession V-001 (subsp. *galamensis*, var. ethiopica) that was collected by Dr. Perdue near Harer, Ethiopia in 1964 (Perdue et al. 1989). Plants of this accession are high yielding and produce big seeds that are firmly attached in the flower head when ripe. The variety is, however, sensitive to cold, and its flowering occurs in response to short day length, a characteristic that is highly undesirable in a crop grown in the northern hemisphere. Other accessions such as V-004 and V-029 have been reported to flower in conditions of long day length (Phatak 1989).

The potential of the genus *Vernonia* as an industrial crop, which has been recognized since the mid-1950s, lies in the fact that the seeds contain high levels of an oil rich in epoxy acids (Perdue et al. 1989). Since epoxy oils (mainly epoxidized soybean oil) are used in various chemical industries for the production of plastics, protective coatings, and other organic formulations, efforts are being made to develop this plant as a new industrial crop. The seed of *V. galamensis* contains about 40% oil, of which the vernolic acid (cis-12, 13 epoxy, cis-9-octadecenoic acid) content is 72-80%. Vernolic acid is primarily present in the oil as a triglyceride, which is known as trivernolin.



Vernolic acid



Trivernolin

Chemically, the oil is similar to epoxidized soybean and linseed oils. Soybean oil contains the triglycerides of oleic acid, linoleic, linolenic and saturated acids. Linseed oil contains the triglycerides of linoleic, oleic, stearic and palmitic acids. Soybean oil contains just 50% of linoleic acid as its principal fatty acid, and linseed oil contains only 57% of linolenic acid as its principal fatty acid. In contrast, vernolic oil is an especially attractive raw material for industry because it is rich in a single fatty acid—72-80% vernolic acid.

Today, the epoxy oil of *V. galamensis* is being tested as a new raw material primarily for the paint industry (Perdue 1987). Vernonia oil can be used as a reactive diluent, replacing the volatile organic compounds (VOC) used as solvents (such as mineral spirits, toluene and xylene) in the paint and coating industries (Dirlikov et al. 1990). VOC are ecologically hazardous, and their use is being restricted in the U.S.A.

Promoting this potential crop to the stage of commercialization will involve:
i) substantial agronomic research and genetic improvement by breeding and selection efforts, and
ii) the development of novel applications based on epoxy oil.

The overall objective of our program is to develop *V. galamensis* as a new crop. The specific objectives, as specified in the original proposal for a three-year project, are as follows:
1) establishment of an experimental trial plot of a number of promising accessions;
2) genetic improvement, and
3) development of novel applications or products based on vernonia oil.

Materials and Methods

Breeding

A number of accessions, V-001, V-029, V-018, V-025, V-026, and V-020, were established in 10-liter buckets in two different greenhouses—a glass-covered greenhouse (beginning at the end of September 1992) and a plastic-covered greenhouse beginning at the end of December 1992). Rows were arranged in such way that V-001 was always kept inbetween two other accessions. When the plants flowered, crosses were performed between the accessions, always keeping V-001 as the male (the donor of pollen) and all others as females. Pollination was done manually by rubbing flower heads together. Usually, crosses were carried out by bending the stems in order to save the flower heads of V-001 for further assessment. After pollination, flowers were tagged and bagged. Control pollination was carried out within each accession. Pollination was completed by June 1993, and flower heads of each cross were collected. Seeds in each flower (cross) were counted and recorded, and seeds were pooled. Total weight and one-thousand-seed weight were determined. The treatment of crosses in the glass greenhouse has yet to be carried out.

Hybrid evaluation

Vernonia plants, hybrids and parents were either sown directly in the field or transplanted after growing in a greenhouse for 5-8 weeks.

For direct sowing, seeds were planted at a depth of 2-3 cm in beds with 30 cm between rows and 90-100 cm between beds. Seeds were sown either 30 cm apart or continuously at a density of up to 20 seeds per meter. When good germination was obtained, the seedlings were thinned out to a density of up to 7-8 plants per meter.

For greenhouse sowing, seeds were germinated in petri dishes and incubated in closed humidified boxes in a growth room at 25°C. The seedlings were transplanted into speedling trays 1-2 weeks later and grown in a greenhouse for 4-8 weeks prior to transplanting in the field. Vernonia speedlings were planted 0.5 m apart, and each speedling was placed near a dripper. A drip line with 4-liter-h^{-1} drippers every 50 cm was placed in the middle of the rows in a bed. Irrigation was supplied once a week for seven months up to a total quantity of 900 mm, which was 70% of class A pan evaporation. Fertilization was supplied in the form of compressed pellets containing a mixture of chicken and cow manure enriched with phosphorous and potassium. Weeding was performed by hand within the rows, and between the rows weeds were controlled by careful spraying with Dukatalon (perquat and diquat).

Laboratory work-up

Oil extraction and characterization

Vernonia oil was obtained by Soxhlet or room temperature extraction of the ground vernonia seeds of accession V-001 of var ethiopica ssp. *galamensis* grown in the field or greenhouse. The seeds were collected and cleaned, and after coarse grinding the oil was extracted with petroleum ether or hexane. The extraction was performed both with or without lipase deactivation (Ayorinde 1990).

Transesterification of vernonia oil was carried out with sodium methoxide in methanol, and the methyl esters so obtained were analyzed by a GC/MS and compared with fatty acid methyl ester standards. Alternatively, the methyl esters of the free fatty acids were obtained by adding diazomethane solution to the mixture obtained after saponification and acidification. The epoxy and amino groups were determined by potentiometric titration (Jay 1964). IR analysis was carried out on a Perkin Elmer 357 spectrometer. GC/MS was

performed on a Hewlett Packard 5971A instrument equipped with a capillary column 12 m x 0.2 mm, i.d. 0.33, μ film HP101, temperature program from 70 to 250°C, carrier gas helium. ^{1}H- and ^{13}C-NMR spectra were obtained with a Brucker WP-200 SY spectrometer in $CDCl_3$ solution (δ; TMS). TGA analysis was performed on a thermoanalytical system Mettler instrument.

Methylation of free fatty acids

Approximately 5 mg of the oil were transferred to a 10-ml glass vial, to which 1 ml of diazomethane-ether solution was added. The mixture was shaken thoroughly and allowed to stand for 5 min. A sample of 1.0-2.0 μl was then injected into a gas chromatograph TLC (Appendix, Fig. A.1).

Saponification

A mixture of 6.0 g of oil (I, Scheme 2, see below), 1.5 g of potassium hydroxide in 2 ml of water, and 18 ml of 95% ethanol was refluxed for 30 min. The solution was then cooled, diluted with water (100 ml) and extracted with ether (3 x 30 ml) to remove unsaponified matter (solution A). The alkaline liquor was cooled in ice, carefully acidified to pH 5.5-6.0 by dropwise addition of dilute hydrochloric acid, and immediately extracted with ether (3 x 50 ml). The combined ether extracts were dried with magnesium sulfate and the solvent was evaporated off, yielding 3.7 g (61.6%) of mixed fatty acids [see Results and Discussion, Scheme 2 (II); Appendix (TLC, Fig. A.1)]. The solution A was also evaporated to give unsaponified matter; yield 0.220 g (3.6%).

Isolation of methyl ester of vernolic acid (III, Scheme 2)

To 1.2 g of the mixture of fatty acids (II) was added excess (approximately 10 ml) diazomethane-ether solution, and the reaction mixture was allowed to stand overnight. After removal of the ether 1.25 g of a mixture of methyl esters of fatty acids were obtained (TLC, see Appendix, Fig. A.2; GC/MS, see Appendix, Fig. A.3).

The methyl esters were fractionated by column chromatography; the column was eluted with hexane;ether 10:1. The separation process was monitored by TLC and GC. The pure methyl ester of vernolic acid (III) was isolated (0.75 g).

The following analyses were performed:

— TLC (Fig. A.2).

— IR (Nujol, cm^{-1}): 1740 (ester carbonyl), 820, 840 (epoxy group).

— Mass spectrum (Appendix, Fig. A.4), m/z: 310 (M^+); 279 (M^+-CH_3O);267 (M^+-$CH_3CH_2CH_2$); 236/M^+-CH_3 $COOCH_3$);

— ^{1}H-NMR spectrum (Appendix, Fig. A.5), δ, ppm: 0.91 (3H at C-18, t. J = 6.4Hz), 2.34 (2H at C-2, t. J = 6.2 Hz), 2.93 (2H at C-12 and C-13, epoxy protons, m), 3.66 (3H, OCH_3, s.), 5.47 (2H at C-9 and C-10, olefinic protons, m.)

— ^{13}C-NMR spectrum (Appendix, Fig. A.6), δ, ppm: 174.17 (C-1), 132.47 (C-10), 123.89 (C-9) 57.12 (C-12), 56.46 (C-13), 51.35 (OCH_3), 22.53 (C-17), 13.99 (C-18).

Methanolysis of the oil

To 50 ml of a freshly prepared approximately 0.02 N solution of sodium methylate in methanol was added 3.2 g of the oil (I). The reaction mixture was stirred overnight at room temperature. Then, the reaction mixture was neutralized by adding 0.5 N HCl slowly with stirring. The crude reaction product of the methanolysis was immediately extracted with petroleum ether (b.p. 40-60°C). The extract was washed with water and dried with magnesium sulfate. After removal of the solvent, the residue was fractionated by column chromatography, as described above for the methyl ester of vernolic acid. The total yield of pure methyl ester III was 1.12 g.

Bromination of the methyl ester III to the 9,10-dibromo-ester IV (Scheme 2)

To a solution of 350 mg (1.13 mmol) of the methyl ester of vernolic acid (III) in carbon tetrachloride (10 ml) was added dropwise with stirring and cooling (0°C) a solution of 190 mg (1.2 mmol) of bromine in carbon tetrachloride (10 ml). After 3 h the reaction mixture was washed successively with aqueous sodium bisulfite (3%, 10 ml x 3) and water (20 ml x 3) and then dried with magnesium sulfate. Evaporation of the solvent gave a mixture (470 mg, 86.8%) that contains 79% of the main product (GC). The crude product obtained was subjected to column chromatography (140 g of silica gel), the column being eluted with a benzene-methanol 150:1. Fractions of 25 ml were collected. The separation process was monitored by TLC and GC.

The fractions containing the dibromoester IV were collected. These fractions were again subjected to column chromatography (40 g of silica gel), the column being eluted with a hexane:ether 4:5 solution. The pure 9,10-dibromoester IV was obtained (310 mg).

The following analyses were performed:

— IR-spectrum, cm^{-1} : 1730 (ester carbonyl), 840, 810 (epoxy group)

— ^{1}H-NMR-spectrum (Appendix, Fig. A.7), δ, ppm:
0.91 (3H at C-18, t. J = 6.6 Hz), 2.31 (2H at C-2, t. J= 6.7 Hz),
3.02 (H at C-13, epoxy proton m.), 3.16 (H at C-12 epoxy proton m,),
4.18 (H C-9, dt. J = 3.0, 9.4, CHBr), 4.40 (H at C-10, dt. J = 3.1, 9.4, m. CHBr);
2.17 - admixture of acetone.

— ^{13}C-NMR spectrum (Appendix, Fig. A.8), δ, ppm: 174.02 (C-1), 55.04, 56.07, 57.56, 59.30 (C-9), C-10, C-12, C-13 carbons at epoxy group and bromine atoms, 51.35 (OCH_3),22.49 (C-17), 13.90 (C-18). Signals at 54.71, 56.85, 58.50 ppm (intensity 15-20%) obviously correspond to the erythro isomer. Calcd for $C_{19}H_{34}Br_2O_3$, Br, 33.99%, found 33.60%.

<u>N-bromosuccinimide (NBS) bromination of the methyl ester III to the 8,11-dibromoester V (Scheme 2)</u>

A solution of 400 mg (1.3 mmol) of the methyl ester III and 470 mg (2.6 mmol) of NBS in carbon tetrachloride (15 ml) was refluxed under light (300 W) for 3 h. Filtration of the solid succinimide and evaporation of the solvent gave a complex mixture of bromo compounds (550 mg). The mixture was subjected to column chromatography (140 g of SiO_2), the column being eluted with a hexane:ether 10:1 solution: 60% of the main compound was obtained.

The following analyses were performed:

— IR cm^{-1}: 1730 (ester carbonyl), 850, 820 (epoxy group).

— ^{1}H-NMR-spectrum (δ, ppm): 0.91 (3H at C-18, t. J = 6.4Hz), 2.28 (2 H at C-2 t. J = 6.5 Hz), 3.02 (2H at C-12 and C-13, epoxy protons, m), 3.67 (3H, OCH_3, s), 4.49 and 4.67 (2H at C-8 and C-11, CHBr, m), 5.90 (2H at C-9 and C-10, olefinic protons, m). Calcd for $C_{19}H_{32}Br_2O_3$, Br 34.2%, found 36.3%.

Results and Discussion

Characterization of hybrid plants between var petitiana and var. ethiopica
During the period March-May 1993 the following accessions of vernonia were transplanted or sowed in a small plot: V-001 (var. ethiopica), V-004 (var. galamensis), V-009 (var. australis), V-018, V-019, V-025 (ssp. *mutomoensis*), and V-029 (var. petitiana). Emphasis was placed on accessions that had not been tested in previous years. Transplanting was essential for some accessions because of poor germination following direct seeding in the field. For purposes of comparison, accession V-001 was also transplanted. The accessions and numbers of plants established from each are listed in Table 1.

All accessions flowered during summer, with the exception of V-001 (Table 2). V-029 was the earliest accession to flower (mid-May). Accession V-004 flowered two weeks later, followed by V-018, and then all the other accessions. With the exception of V-001, ssp. *galamensis* requires less short days for flowering than other subspecies. V-029 therefore shows promise, since it flowers under conditions of long days. This trait was also exhibited in the two weeks difference in flowering between plants sown at a difference of four weeks.

Table 1. Germination and flowering of parent and hybrids of vernonia grown in the field in 1993

Subspecies	Variety	Accession	Field sowing date	Field transplanting date	Germination date	Flowering date
galamensis	ethiopica	V-001	March 3		March 11	None
				March 30*		None
				May 9**		None
galamensis	petitiana	V-029	March 3		April 12	May 15
				March 30*		June 8
				May 9**		June 26
F1 hybrid		V029 X V001	-	March 30		May 15
mutomoensis	-	V-025	March 3	-	March 23	June 27
F1 hybrid		V025 X V001	-	March 30		June 3

* Sown in a petri dish on January 24 and transplanted to a speedling tray on February 10
** Sown in a petri dish on February 23 and transplanted to a speedling tray on March 11

Table 2. Seed yield and one-thousand-seed weight of parent and hybrids of vernonia grown in the field in 1993

Subspecies	Variety	Accession	Field sowing date	Field transplant-ing date	Yield/ plant	1000 seed wt
galamensis	ethiopica	V-001	March 3		None	None***
				March 30*	None	None
				May 9**	None	None
galamensis	petitiana	V-029	March 3		5.8	2.6±0.1
				March 30*	1.8	
				May 9**	2.5	
F1 hybrid		V029 X V001	-	March 30	2.7****	3.5±0.3
mutomoensis	-	V-025	March 3	-	0.6	3.3
F1 hybrid		V025 X V001	-	March 30	1.6	3.3

* Sown in a petri dish in January 24 and transplanted to a speedling tray on February 10.
** Sown in a petri dish in February 23 and transplanted to speedling tray on March 11.
*** One-thousand-seed weight of other lots of V-001 is 3.9±0.1 g.
**** Partial yield.

In general, all accessions produced seeds in the field—as opposed to the high degree of sterility that was exhibited in the greenhouse as a result of the absence of insects. It should be emphasized that all our accessions, with the exception of V-001 and perhaps also with the exception of V-004 (currently under investigation), are not self-fertile. In the field, plants did not produce high yields with the exception of V-004 (Table 3). One-thousand-seed weight varied from 2 to 4 g, being low in V-032, V-029, and V009 and high in V-020.

Accessions of ssp. *mutomoensis* (VA-018, V-019 and V-025), and *afromontana* (V-020) were more robust than ssp. *galamensis* (V-001, V-0045, V-029, and V-032), although accession V-009 was an exception. In general, the former subspecies had less flower heads and less seeds per plant. V-029 is a short plant and did not grow vigorously under the conditions of the Northern Negev. In contrast to the latter, V-004 is a vigorous plant with many tertiary flowering branches and many flower heads.

Table 3. Phenological characteristics of vernonia accessions and hybrids grown in the field in 1993

Phenological parameter	V-029		V-001		V029 X V001		
	Sown	Trans-planted	1	2	1	2	3
Height (cm)	65	59	72	63	97	90	85
Width (cm)	45	75	49	42	85	60	40
Height of first flower (cm)	22	8	-	-	36	55	23
No. of branches below 1st flower	12	8	-	-	14	30	15
Length of 2ndary branches (cm)	36.1	28.6			56.8	36.8	61.7
SD	3.7	5.9			9.3	8.6	17.7
No. of 2ndary branches	5.4	5.1			7.3	3.5	7.0
SD	1.2	1.6			1.8	1.0	2.8
Main stem diameter (cm)	1.83	1.55	1.55	1.50	1.90	1.45	1.55
2nd branch diameter (cm)	0.61	0.51	0.46	0.50	0.82	0.58	0.62
SD	0.14	0.04	0.06	0.11	0.20	0.28	0.13
Leaf width (cm)	1.2	1.2	1.2	1.2	1.1	0.9	0.9
SD	0.2	0.1	0.0	0.1	0.1	0.2	0.2
Leaf length (cm)	10.4	7.5	15.4	15.0	15.5	14.6	12.9
SD	1.0	1.5	0.5	2.2	1.2	1.8	1.8
Width/length	0.1	0.2	0.1	0.1	0.1	0.1	0.1
SD	0.01	0.03	0.00	0.02	0.01	0.01	0.01
Flower width (cm)	0.78	0.78			1.29	1.23	1.07
SD	0.07	0.04			0.22	0.01	0.08
Flower length (cm)	1.10	1.06			1.52	1.50	1.62
SD	0.07	0.09			0.08	0.00	0.13
Width/length	0.71	0.74			0.85	0.82	0.67
SD	0.06	0.08			0.14	0.01	0.06
Flower color *	2	1			3	3	3
Distance between flower and closest leaf (cm)	0.37	0.42					
SD	0.22	0.16					

* 0 - white; 1 - light blue; 2 - blue; 3 - purple; 4 - dark purple

Table 3. Cont.

Phenological parameter	V-025 1	V-025 2	V-025 3	V-001 1	V-001 2	V-001 3	V-001 4	V-025 x V-001 1	V-025 x V-001 2	V-025 x V-001 3
Height (cm)	162	140	124	120	124	126	131	80	118	93
Width (cm)	64	70	62	20	40	35	25	76	55	60
Height of first flower (cm)	162	138	78					72	98	93
No. of branches below 1st flower	75	100	84					23	52	37
length of 2ndary branches (cm)	63.8	62.0	74.5					62.7	42.3	46.0
SD	3.3	19.8	4.2					9.0	8.2	7.5
No. of 2ndary branches	0.0	0.0	0.0					0.0		0.0
SD	0.0	0.0	0.0					0.0		0.0
Main stem diameter (cm)	2.00	2.20	2.80	1.20	1.70	1.30	1.00	2.30	2.00	1.70
2ndary branch diameter (cm)	0.70	0.55	0.88		0.50	0.36	0.37	0.90	0.55	0.53
SD	0.00	0.13	0.10		0.10	0.09	0.12	0.10	0.13	0.06
Leaf width (cm)	3.0	2.7	2.5	1.5	1.2	1.3	1.2	4.5	4.4	3.1
SD	0.8	0.8	0.5	0.2	0.2	0.1	0.3	0.6	0.6	0.9
Leaf length (cm)	22.0	19.0	19.0	10.4	11.7	13.6	11.3	20.0	21.0	14.0
SD	7.1	5.0	2.7	1.1	1.0	1.4	0.5	1.4	3.9	2.8
Width/length	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2	0.2
SD	0.02	0.01	0.02	0.02	0.01	0.01	0.03	0.02	0.02	0.02
Flower width (cm)	2.08	1.96	2.02					1.48	2.03	1.92
SD	0.30	0.09	0.29					0.20	0.33	0.11
Flower length (cm)	2.60	3.00	3.18					2.40	2.88	2.70
SD	0.14	0.31	0.25					0.10	0.25	0.23
Width/length	0.80	0.66	0.64					0.62	0.71	0.71
SD	0.15	0.07	0.08					0.09	0.13	0.04
Flower color	2	2	2+					2	2.5	2+
Distance between flower and closest leaf (cm)	0.54	0.66	0.58					1.14	0.70	0.94
SD	0.05	0.11	0.18					0.72	0.24	0.63

* 0 - white; 1 - light blue; 2 - blue; 3 - purple; 4 - dark purple

Since V-004 flowers in summer and has a relatively satisfactory yield, it is for the moment considered the best germplasm under our conditions and is the candidate of choice for a field trial that will take place in the spring of 1994 at the Sha'ar Ha'Negev Experimental Station.

Breeding and plant characteristics

In 1993 the following accessions were crossed with accession V-001 in two greenhouses: V-029, V-018, V-025, V-026, and V-020, always keeping V-001 as the male (the donor of pollen) and all others as females. Control pollination was carried out within each accession. The control crosses did not yield any seeds, which leads us to believe that the seeds produced in the cross-pollinated flowers are indeed hybrid seeds. Pollination was completed by June 1993, and flower heads of each cross were collected. Data on the number of crosses and the number of seeds obtained are presented in Table 4. It should be mentioned that not all crosses yielded seeds. This was more pronounced in the cross between V-018 and V-001, of which about only a mean of 3 seeds per flower head were produced in the crosses, whereas 16-24 seeds were obtained with crosses of other accessions with V-001. In this respect, variation was also noted between plants (Table 4).

Samples of F1 hybrid seeds, prepared in 1991 by crossing V-025 and V-029 with V-001, were either sown directly in the field or germinated in petri dishes and then transplanted into speedling trays and finally into the field. Phenological characteristics of the hybrids and their parents are presented in Table 3. It was observed that V-029 x V-001 hybrids grew much more vigorously and were taller than their parents. The other features of the hybrids were intermediate between their parents—height of first flower (in V-001 this flower is quite high), dimensions of the flower (flower size of V-001 is bigger) and seed weight (see also Table 2). The leaf dimensions and the color of the flower resembled those of V-001. Flowering date, and disappointingly also seed retention resembled V-029. In contrast to the latter, as far as we can tell, the hybrid of V-025 x V-001 did not exhibit intermediate features of its parents, although some parameters were different from those of V-025. Backcrosses of the F1 seeds with V-001 are currently being carried out.

Table 4. Hybrid seeds (F1) collected in the plastic-covered greenhouse from different pots (two plants per pot) during the season of 1992/3. Numbers represent number of seeds per capitulum

	Pot										Grand	Grand		
Cross	1	2	3	4	5	6	7	8	9	10	sum	Mean	SE	N
V-026 X	10	35	32	14	3	130	18							
V-001	4	38	36		65	91	4							
	0		34		7	60	0							
			45		6	200	0							
			25		41	2	0							
			75		6	1	0							
			0		2	2	0							
			0		15	118	0							
			0		2	77	0							
			0		10	42	0							
			0		11	27	0							
			0		1	112	0							
			0		37	69	0							
			0		25	20								
			0		15	19								
			0		17	66								
			0		18	60								
			0		34	0								
			0		16									
			0		25									
N	3	2	20	1	20	18	13							
Mean	4.7	36.5	12	14	18	60.9	1.7							
SE	2.9	1.5	4.8		3.6	12.8	1.4							
Sum	14	73	247	14	356	1096	2.2				1822	23.7	4.1	77

Table 4 cont.

Cross	Pot 1	2	3	4	5	6	7	8	9	10	Grand sum	Grand Mean	SE	N
V-029o X	27	19	22	26	51									
V-001	30	17	12	24	34									
	26	2	22	25	24									
	38	9	31	25	31									
	32	27	40	35	19									
	16	1	23	48	9									
	31	31	37	28	13									
	28	12	42	34	13									
	18	18	22	21	30									
	13	16	46	6	3									
	29	24	28	11	47									
	0	18	26	24	2									
	0	4	27	32	19									
	0	10		31	48									
	0			4	0									
	0			19	0									
	0			25	0									
	0			0	0									
	0			0	0									
	0			0	0									
					0									
N	20	13	12	19	20									
Mean	14	14.9	29	21	16									
SE	3.2	2.52	2.8		4									
Sum	288	208	378	418	343						1635	18.6	1.5	88

Table 4 cont.

	Pot										Grand	Grand		
Cross	1	2	3	4	5	6	7	8	9	10	sum	Mean	SE	N
V-029G X	20	53	30	23	29									
V-001	52	41	13	11	45									
	12	16	37	55	26									
	29	49	34	16	3									
	13	55	58	30	43									
	19	50	4	22	18									
	27	23	25	34	25									
	48	29	44	0	5									
	34	50	0		50									
	19	59	0		34									
	70	40	0		32									
	5	43	0		1									
	55	28	0		34									
	30	37	0		17									
	21	9	0		5									
	8	0			21									
	6	0			0									
	34	0			0									
	57	0			0									
	19	0			0									
	31	0			0									
	9	0			0									
	9				0									
	66				0									
	0				0									
	0				0									
	0				0									
	0				0									
	0				0									
	0				0									
	0													
	0													
N	32	22	15	8	30									
Mean	22	26.5	16	24	13									
SE	3.7	4.72	5.1	5.8	3									
Sum	693	582	245	191	388						2099	19.6	1.9	107

Table 4 cont.

	Pot										Grand	Grand		
Cross	1	2	3	4	5	6	7	8	9	10	sum	Mean	SE	N
V-020 X	79	43	45	28	8	3	3	23	1	1				
V-001	1	79	29	29	4	3	24	1	1	0				
	1	48	1	2	1	4	29	20	20	0				
	107	80	0	3	5	37	18	23	4	0				
	15	78	0	1	1	1	19	23	0	0				
	75	9	0	8	66	16	1	1	0	0				
	15	118	0	8	102	28	2	8	0	0				
	17	47	0	35	24	8	2	0	0	0				
	0	19	0	4	1	2	1	0	0	3				
	0	195	0	12	3	25	1	0	0	7				
	0	76		3	1	15	36	0	0					
	0	115		7	25	9	0	0	0					
	0	57		13	92	18	0	0	0					
	0	48			33	14	0	0	0					
	0	29			10	2	0	0	0					
	0	100			1	13	0	0	0					
		60			100	1	0		0					
		0			100	2	0							
		0			0	1	0							
		0			0	0	0							
		0			0	0								
		0				0								
		0				0								
		0				0								
N	16	24	10	13	21	24	20	16	17	10				
Mean	19	50	7.5	12	27	8.42	6.8	6.19	1.5	1.1				
SE	8.7	10.1	5.1	3.2	8.4	2.1	2.6	2.45	1.2	0.7				
Sum	310	1201	75	153	577	202	136	99	26	11	2790	16.3	2.3	171

Table 4. cont

Cross	Pot 1	2	3	4	5	6	7	8	9	10	Grand sum	Grand Mean	SE	N
V018 X	1	3	3	3	2	1	1	52	1	2				
V-001	1	3	15	1	1	0	1	0	5	1				
	0	4	9	5	25	0	3	0	2	5				
	0	1	5	13	1	0	1	0	8	2				
	13	8	1	2	35	0	4	0	1	1				
	83	14	1	5	7	0	2	0	0	1				
	13	46	6	0	5	0	1	0	0	0				
	33	11	4	0	12	0	1	0	0	0				
	26	17	10	0	0	0	2	0	0	0				
	17	21	28	0	0	0	0	0	0	0				
	12	4	3	2	0	0	0	0	0	0				
	40	2	0	20	0	0	0	0	0	0				
	1	0	0	0	0	0	0	0	0	0				
		0	0	2	0	0	0	0	0	0				
		0	0	7	0	0	0	0	0	0				
		0	0	6	0	0	0	0	0	0				
		0	0	4	0	0	0	0	0	0				
		0	0	0		0	0	0	0	0				
			0	4		0	0	0	0	0				
				1		0	0	0	0	0				
						0	0	0	0	0				
						0	0	0	0	0				
						0	0	0	0	0				
						0	0	0		0				
						0	0	0		0				
						0	0	0		0				
						0	0	0		0				
								0						
								0						
								0						
								0						
								0						
								0						
								0						
								0						
N	13	18	19	20	17	27	27	36	23	27				
Mean	18	7.44	4.5	3.8	5.2	0.04	0.6	1.44	0.7	0.4				
SE	6.5	2.73	1.6	1.1	2.4	0.04	0.2	1.44	0.4	0.2				
Sum	240	134	85	75	88	1	16	52	17	12	720	3.17	0.6	227

Chemical work-up

Oil extraction and characterization

It was found that Soxhlet extraction yielded somewhat more oil (38%) than stirring ground vernonia seeds with hexane at room temperature (35%). The oil obtained at room temperature contained less free fatty acids, and the oxirane value was higher (3-6%). Other experiments for the extraction of the oil were performed under conditions for lipase deactivation. A method involving lipase deactivation yielded 43-44% of the oil, and as expected under these conditions the oil contained only a small amount of carboxylic groups (0.13-0.33%), approximately 0.85-2.16% of free fatty acids (FFA).

A lower content of vernolic acid was obtained by the Soxhlet extraction (Table 5) (in this case the oil was obtained without lipase deactivation). By extraction of the oil at room temperature the content of vernolic acid was relatively high (80%).

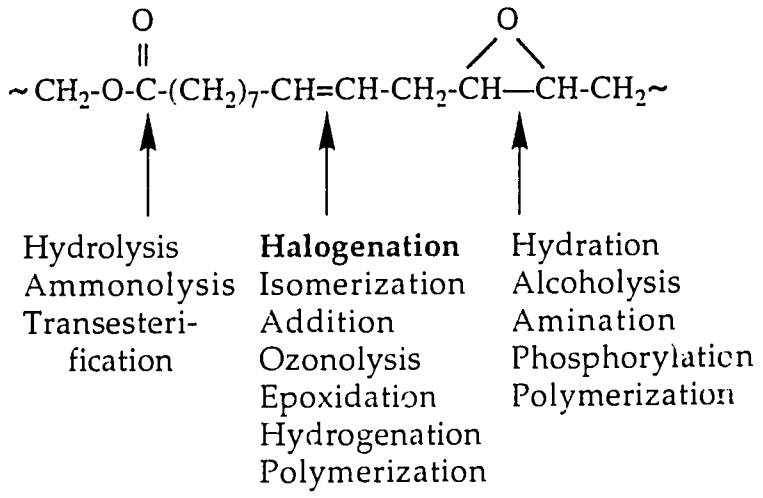
Bromination reactions

The unique structure of trivernolin enables us to perform a wide variety of reactions characteristic of the ester group, of the double bond, and of the epoxy group (Scheme 1).

Table 5. Fatty acid composition* of *Vernonia galamensis* seed oil (accession V-001 of var. ethiopica, ssp. *galamensis*)

	Soxhlet extraction (%)**	R.T. extraction (%)
Vernolic acid	61-62	79-80
18:2	20	10-12
18:1	5-6	3-4
18:0	3-4	2-3
16:0	3-4	3-4

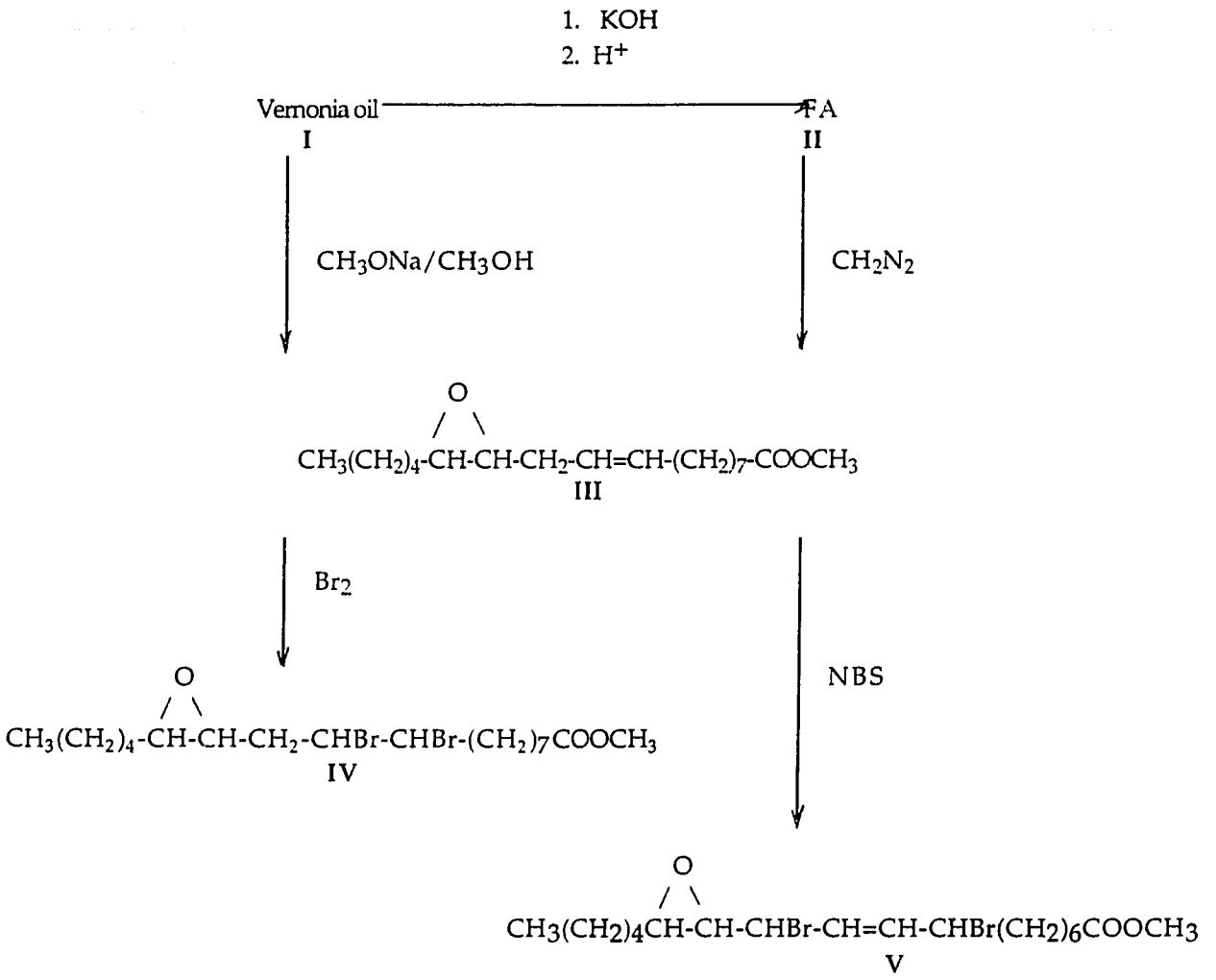
*As methyl esters by GC/MS.
**The mixture also contains 4-5% of an unidentified product.



Scheme 1

We began our studies with the halogenation of the double bond, and in this report we describe the bromination of the methyl ester of vernonia oil. The methyl ester was chosen as a model compound that would subsequently enable us to study the chemistry of the more complicated trivernolin molecule with the ultimate aim of preparing brominated vernonia derivatives. These derivatives will find application as intermediates in organic synthesis and as reactive monomers in the plastic industry.

The methyl ester of vernolic acid (III, Scheme 2) was obtained by two methods. The first comprised saponification of the oil (I) with an ethanolic solution of potassium hydroxide; isolation of the fatty acids (II) after acidification [Smith et al. 1959]; and methylation with diazomethane. The relative weight percent of the fatty acids in the oil (I) was determined by GC/MS (Appendix, Fig. A.3). The methyl ester of vernolic acid (III) was isolated from the mixture of esters by column chromatography. The spectral data (IR, NMR, mass spectra) for this sample fully matched the structure III. In the second method of synthesis, the ester III was prepared by methanolysis of the oil (I), followed by isolation of the product by chromatography (Barford et al. 1963). The yields of ester III prepared by these two methods are approximately the same, but the second method is preferable in terms of ease of obtaining preparative amounts.



Scheme 2

The bromination of the 9,10 double bond of the ester III was carried out at different temperatures in various solvents (carbon tetrachloride, dichloromethane, and acetonitrile). The best results were obtained with carbon tetrachloride at 0°C. A chromatographically pure sample of the dibromine IV was isolated by column chromatography (TLC, GC).

From the ^{1}H-NMR spectrum it is obvious that addition of bromine to the molecule occurred at the 9,10 double bond. Decoupling of signals at 3.02, 3.16 (epoxy protons) and 4.18, 4.40 (CHBr protons) showed that each pair of protons is vicinal and there was no interaction between these couples. Since it is known that bromine adds trans to the double bond, we can expect the ester IV to be a *threo* isomer (a partial structure is shown in Fig. 1a). However, in the ^{13}C-NMR spectrum there are low intensity signals (15-20%), which obviously correspond to the *erythro* isomer (partial structure, Fig. 1b).

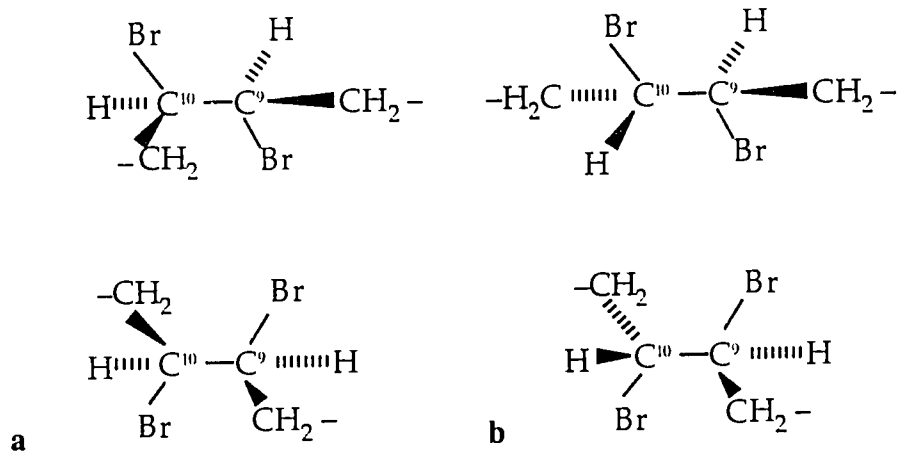


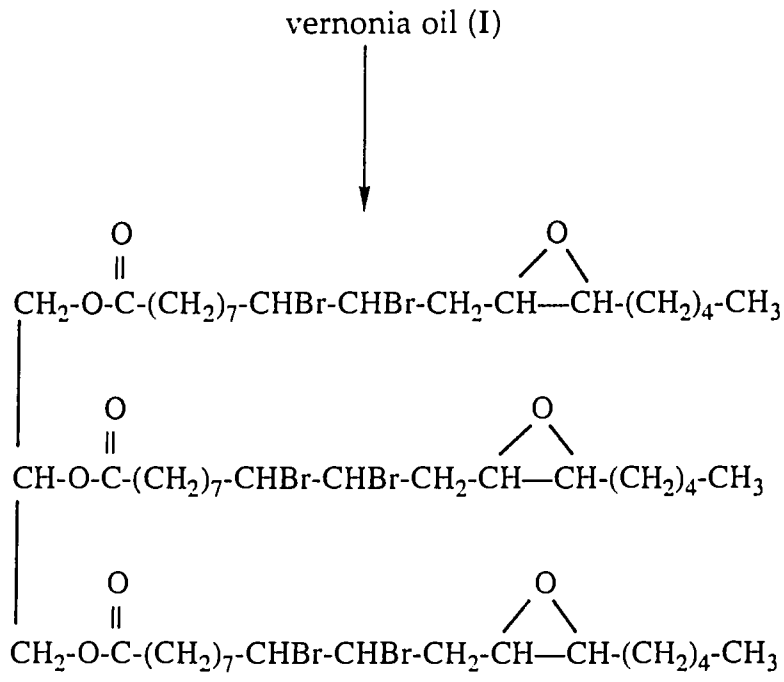
Fig. 1. Partial structures of a) the *threo* and b) the *erythro* isomer of the ester IV

We also carried out bis-allylic bromination of the ester III with NBS. Interaction of the ester III with NBS under different conditions gave a complex mixture of bromo compounds. The product obtained by column chromatography contained only 60% of the main substance. In the ^{1}H-NMR spectrum of this substance there were signals of the epoxy group 2H at 3.02, the CHBr group 2H at 4.49 and 4.67, and olefinic protons 2H at 5.90 ppm. (V, Scheme 2).

Based on the results obtained from the bromination of the methyl ester of vernolic acid, we began a series of bromination reactions of vernonia oil.

From the NMR spectrum of the bromo compound obtained, it is obvious that bromine added to the 9,10 double bond: the signals of olefinic protons are absent (the small signals at 5.26 ppm belong to the secondary proton of the

glycerol moiety). The signals of the protons of the CHBr groups overlap with the signals of the primary protons of the glycerol moiety—the multiple peaks at 4.08-4.57 ppm. We can see that the bromination reaction resulted in considerable changes in the signals of the epoxy protons: they appear as two small multiplets at 2.97 and 3.15 ppm. TLC data and NMR spectra indicated that the bromination of the oil gave rise to a complex mixture of bromo compounds. However, we can assume that the structure of the main compound correspond to the structure VI (Scheme 3).



VI

Scheme 3

To determine the optimal conditions for the bromination reaction different parameters (solvent, molar ratio of reagents, temperature) will be studied, and the reaction will be performed with a series of brominating reagents with the aim of elucidating the structure of the final compound. The results will be presented in the annual report.

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APPENDIX. SPECTRA OF VERNONIA OIL AND ITS DERIVATIVES

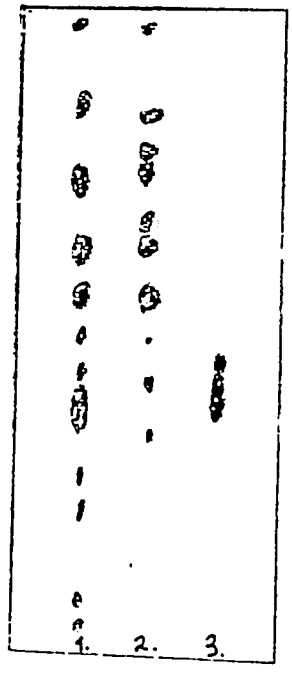

Fig. A.1. TLC (hexane:ether 4:5)
1. Vernonia oil
2. Oil after methylation with CH_2N_2
3. Fatty acids from vernonia oil
(the main compound is vernolic acid)

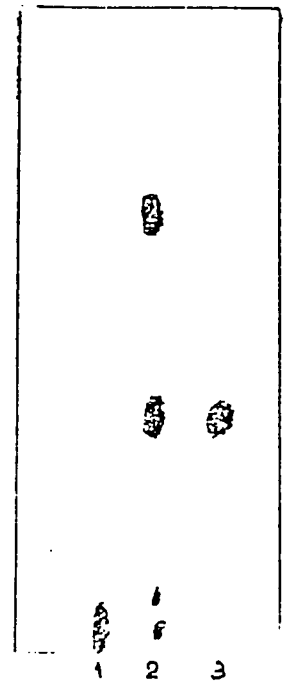

Fig. A.2. TLC (hexane:ether 10:1)
1. Fatty acids from vernonia oil
2. Methyl esters of fatty acids
3. Methyl ester of vernolic acid

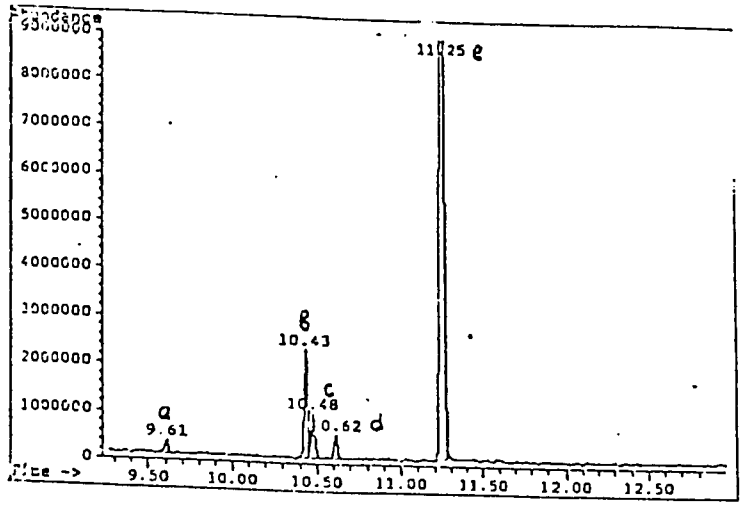

Fig. A. 3. GC/MS of methyl esters of fatty acids (FA) of vernolic oil
Relative weight percent of FA:
a. Palmitic acid - 0.94
b. Linoleic acid - 7.44
c. Oleic acid - 2.86
d. Stearic acid 1.72
e. Vernolic acid 87.02

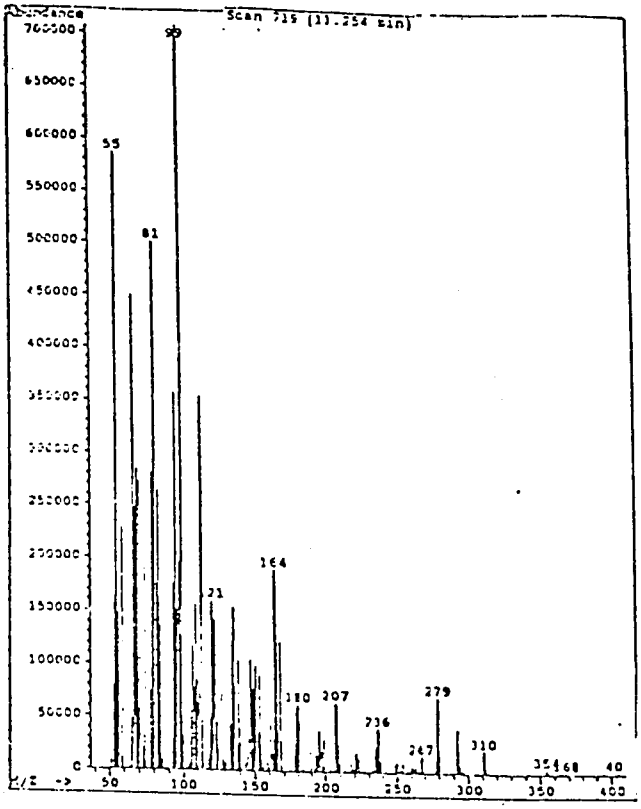

Fig. A.4. Mass spectrum of the methyl ester of vernolic acid

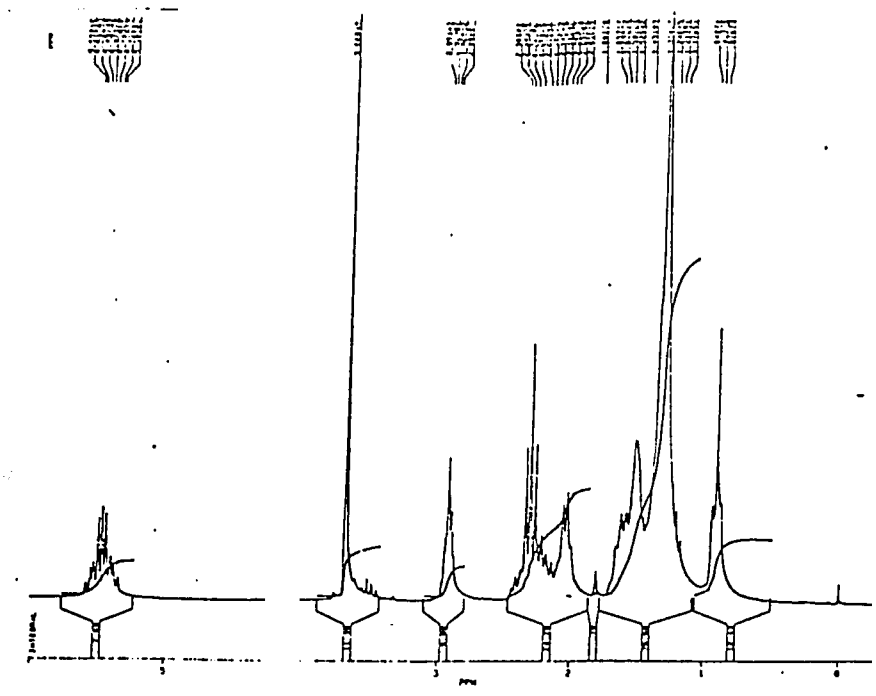

Fig. A.5. ^{1}H-NMR spectrum of the methyl ester of vernolic acid (III)

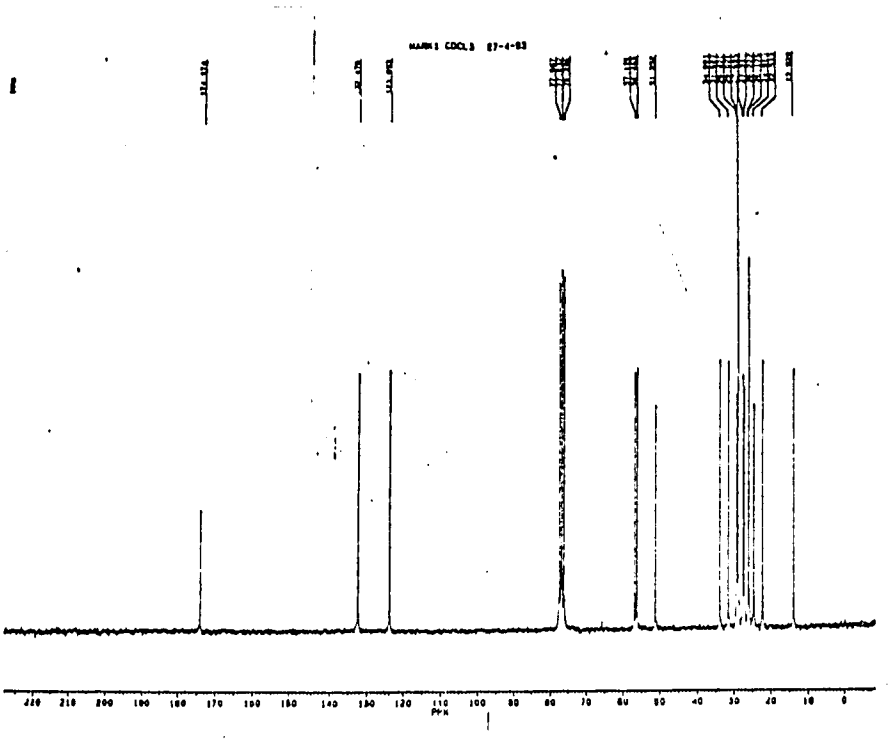

Fig. A.6. ^{13}C-NMR spectrum of the methyl ester of vernolic acid (III)

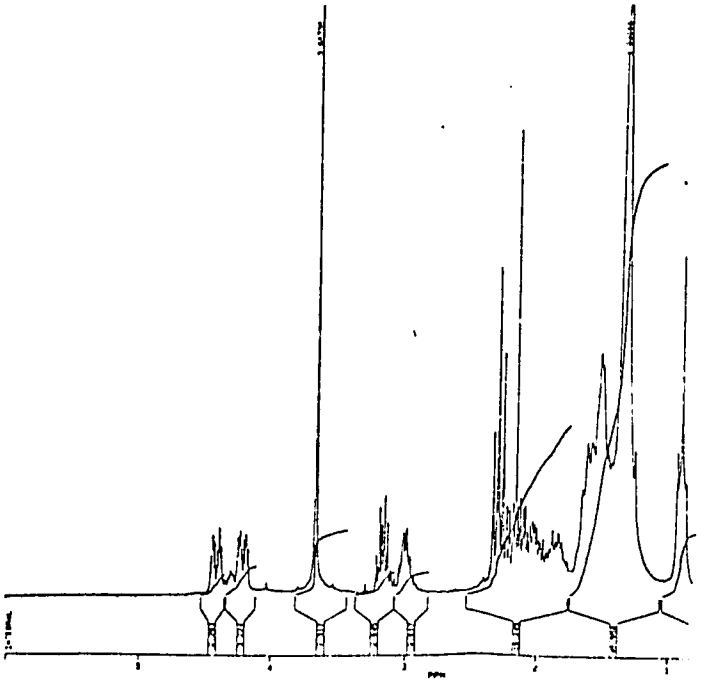

Fig. A.7. ^{1}H-NMR spectrum of the 9, 10-dibromoester (IV)

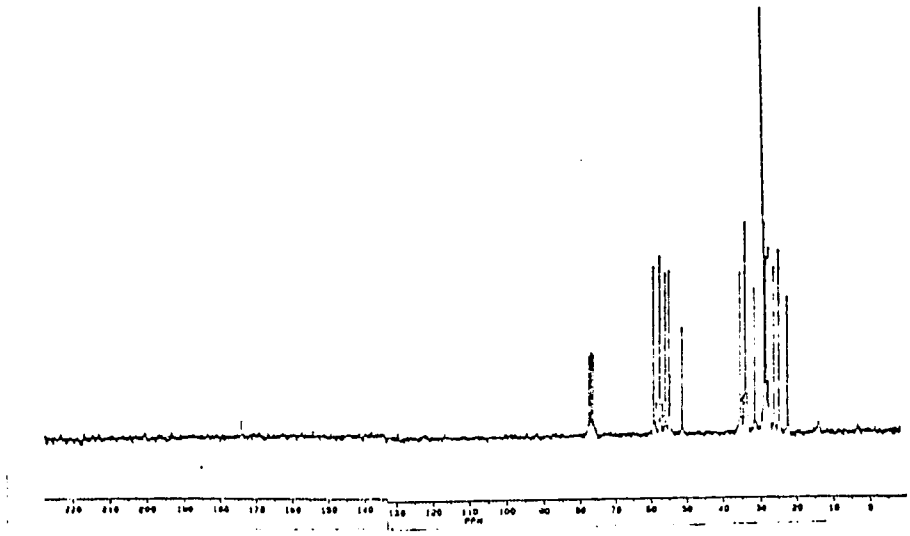
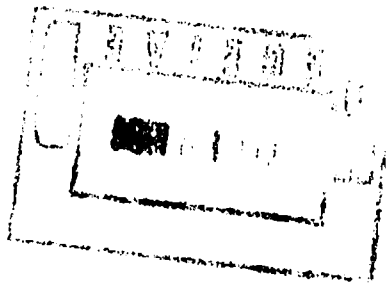
Fig. A.8 ^{13}C-NMR spectrum of the 9, 10-dibromoester (IV)