Seeds of *Trichosanthes kirilowii*, an Energy-Rich Diet

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Trichosanthes kirilowii Seeds, Triglycerides, Glycolipids, Phospholipids

The kernels of *Trichosanthes kirilowii* seeds contain a green oil which makes up for 62% of their dry matter. This oil consists up to 95% of triglycerides, 2% of glycolipids, 1.3% of phospholipids and 1.8% of chlorophylls. As fatty acid components the triglycerides, glycolipids and phospholipids contain the unsaturated fatty acids linoleic and oleic acid and the saturated palmitic acid. In the triglycerides 19% of the $C_{18:3}$ acid occur with the configuration Δ^9 *cis*, Δ^{11} *trans*, Δ^{13} *cis*. This acid is called trichosanic acid and is absent in glycolipids and phospholipids which contain instead another $C_{18:3}$ fatty acid, which has conjugated double bounds and occurs with an amount of 21% and 3%, respectively. Typically, these oil seeds contain in addition up to 30% of their dry matter proteins and up to 2.5% mono- and oligosaccharides. The monosaccharides consist of rhamnose, galactose and glucose and the oligosaccharides represent a mixture of tri- and tetrasaccharides.

Introduction

Trichosanthes kirilowii Maximowicz is one of the traditional Chinese medicinal plant species recorded in the „Compendium of Materia Medica", an ancient medicine book published in 1578 during the Ming Dynasty. It is a perennial climbing plant and grows all over China. A great number of studies and investigations have been carried out in order to elucidate the function of the plant with respect to its medical properties (Joh *et al.*, 1995; Kimura *et al.*, 1995; Takeda *et al.*, 1994). It is known that the seeds of the plant have expectorant and febrifugal properties and help digestion and excretion. Therefore, the seeds are used in the traditional Chinese medicine as an anti-inflammatory agent, namely as a cough medicine and expectorant (Li, 1875; Committee for Pharmacopoeia of the Minister of Public Health of China, 1963; Kimura *et al.*, 1997). Since a few years people collect the seeds of *Trichosanthes kirilowii* in the Yiyang District of the Hunan province mainly because of their good taste and smell. The seeds are eaten raw or roasted and the activity of collecting seeds becomes increasingly popular. The seeds are known to contain great amounts of fat (Li, 1875). According to Joh *et al.* (1995) the fatty acid composition of seeds of *Trichosanthes kirilowii* from Corea is known. These analyses were not only carried out by means of HPLC on reversed-phase and silver-impregnated columns and by gas chromatography, but also by mass spectrometry. Also analyses on hydrated sterols of the seeds are available (Kimura *et al.*, 1995, 1997).

Abbreviations: HPAEC, High-performance anion exchange chromatography; TFA, Trifluoro acetic acid; SDS, sodium dodecylsulfate.

Materials and Methods

Seed material

Seeds of *Trichosanthes kirilowii* (Maximowicz) were obtained from the Central South Forestry University of Zhuzhou/Hunan, People's Republic of China. Mature seeds had been harvested in the Yiyang District of the Hunan Province of China, and used for lipid, protein and sugar analyses after a 3 months storage at −20 °C.

Lipid isolation

Seed kernels were isolated from 50 seeds. The kernels were crushed in a mortar together with

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10 ml of a mixture of methanol and chloroform (1:1; v/v). The crushed material was supplemented with additional 140 ml of the methanol/chloroform mixture and extracted for 2 h at boiling temperature and backflow conditions. Further extraction was achieved on a glass sinter funnel with 50 ml acetone and subsequently with 20 ml diethylether. After evaporation of the solvent, lipids were taken up in diethyl ether and separated from insoluble components by centrifugation at 3000×*g*. After distillation of the diethyl ether a lipid mixture is obtained as a slightly greenish oil.

Column chromatography and TLC of lipids

The lipids were fractionated over a silica gel 40 column (mesh 0.063–0.2 mm, Merck No. 1.10180) into unpolar lipid-, glycolipid- and phospholipid-fractions. Dimensions of the silica gel column: height 10 cm, diameter 2.4 cm, volume 75 cm^3. 3.2 g lipids were dissolved in chloroform and given on the silica gel column. Elution of the triglycerides was achieved with 5 times the column volume of chloroform. Glycolipids were eluted with 6 times the column volume of acetone and phospholipids with 4 times the column volume of methanol. Subsequently, after the separation by thin layer chromatography on silica gel G-layers in the solvent chloroform: methanol: acetic acid: water (85:15:10:3.5, v/v/v/v) the glycolipids were quantitatively determined by photometry with anthrone/sulfuric acid and the phospholipids with the Fiske Subbarow reagent (Radunz 1969, 1972, Radunz *et al.* 1998, Bednarz *et al.*, 1988).

Gas chromatography of fatty acids

For the preparation of methyl esters the lipid fractions were subjected to a mild alkaline hydrolysis with simultaneous transesterification according to earlier described methods (Radunz *et al.*, 1998). Gas chromatographic analyses were performed with a 10 m long ethylene glycol–succinate capillary column (Hewlett Packard, Type 5890, Series II plus). Column temperature was 190 °C, detector and injection block temperature 300 °C, respectively. Carrier gas was nitrogen.

Sugar analyses

For the determination of sugar 20 seed kernels were crushed in a mortar with 10 ml water and extracted for 12 h. Insoluble components were removed by centrifugation at 5000×*g* for 20 minutes. The quantitative determination of sugars in the supernatant was carried out with the anthrone reagent according to Roughan *et al.* (1968). For qualitative sugar analysis the lipids present in the supernatant were eluted by chromatography on a RP-18 column and the samples centrifuged at 20000×*g*. Analysis of the monosaccharides was achieved on a Carbo Pac PA1 anion-exchange column (column volume: 4cm × h 250 mm) by using a HPAE-chromatograph from Dionex according to the methods of Köplin *et al.* (1993). The device was equipped with a Dionex metal-free GP-40 gradient pump and a pulsed amperometric detector with a gold electrode. Elution of the sugars was done with 16 mM NaOH. The flow rate was 1 mm per min at room temperature. Data were taken with a Merck/Hitachi D2000 Integrator. Oligosaccharides were also separated from the supernatant fraction by HPAE chromatography on the above described column. For splitting into the monosaccharides, oligosaccharides reacted with 2 molar triflouro-acetic acid (TFA) during 2 h at 120 °C in a sealed ampoule. Trifluoroacetic acid was distilled away in the vacuum evaporator at 40 °C, the remainder washed twice with 500 µl isopropanol and then twice with 200 µl water. The sugar thus freed from trifluoroacetic acid and isopropanol were taken up in 100 µl water and were centrifuged at 13.000×*g* for 10 minutes to remove insoluble components. The monosaccharides were determined from the supernatant by HPAE-chromatography. In parallel to these HPAE-analyses, oligosaccharides were determined by TLC on cellulose layers with n-butanol/pyridine/water, 6/4/3 (v/v/v) as solvent according to van Wyk (1966). Reference substances were glucose, galactose, maltose and raffinose. The spraying reagent used was aniline phthalate and for reducing sugars aniline diphenylamine phosphoric acid.

Protein determination

For the qualitative protein determination 10 seed kernels were crushed with 10 ml of 1.7% sodium chloride in a mortar and extracted for 12 h. Particles were centrifuged at 3000×*g* for 20 minutes. The soluble proteins of the supernatant as well as the insoluble proteins of the sediment were

quantitatively determined with Neßler's reagent (Table I). The qualitative composition of the seed proteins was determined by electrophoresis under native conditions. The composition of the polypeptide subunits of both fractions was determined by means of SDS-polyacrylamide gel electrophoresis according to earlier described methods of He *et al.* (1993).

Chlorophyll determination

Chlorophyll was determined photometrically according to Schmid (1971) in methanol/water (90:10, v/v).

Results and Discussion

Seeds of *Trichosanthes kirilowii* Maximowicz consist of 62% of a greenish oil, of 2.5% of soluble sugars and 3.8% of soluble proteins and of 26% of proteins insoluble in aqueous media (Table I). The seeds belong to the lipid storing fruits having a high nutritional value. Like the seed lipids of most oil fruits, the oil consists of 95% triglycerides (Table II). The etioplast membrane-forming glycolipids such as monogalactolipid, digalactolipid, sulfolipid and sterylglycoside as well as the mitochondrial membrane-forming diphosphatidylglycerol and the other membrane-forming phospholipids as phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine and phosphatidylinositol occur only in low concentrations. Thus, the portion of glycolipids amounts to 1.9% and that of phospholipids to 1.3%. with monogalactolipids being the main component of glycolipid and phosphatidylinositol being the main component of phospholipids. Interestingly, the chlorophyll content is 1.8% which is unusually high for seed kernels with a chlorophyll *a/b* ratio of 1 : 6.4.

Table II. Composition of seed lipids of *Trichosanthes kirilowii.*

Lipids	% of total lipids
Chlorophyll a+b	1.82
Chlorophyll b/a	6.38
Triglycerides	95.0
Monogalactosyldiglyceride	0.82
Digalactosyldiglyeride	0.45
Sulfoquinovosyldiglyceride	0.37
Sterylglycoside	0.27
Cardiolipin	0.11
Phosphatidylethanolamine	0.21
Phosphatidylglycerol	0.15
Phosphatidylcholine	0.36
Phosphatidylinositol	0.47

Values are given in% of total lipids and represent averages of 3 to 5 individual experiments. The maximal deviation is 5 percent.

Soluble sugars are composed, as shown by HPAE-chromatographic analysis on a CarboPAC-Anion exchange column, of monosaccharides and oligosaccharides. The monosaccharides are composed of rhamnose, glucose and galactose, which occur in the ratio of 0.5:1:1, The oligosaccharides are composed of the trisaccharide raffinose and of a tetrasaccharide. By means of thin layer chromatographic analysis of the oligosaccharide fraction on cellulose layers with the solvent *n*-butanol, pyridine, water (6:4:3) the results obtained by HPAE-chromatography were confirmed. Hydrolytic cleavage of the oligosaccharides showed that they were composed of galactose and glucose, occurring in a molar ratio of 1:1. A qualitative peptide analysis by SDS-polyacrylamide gel electrophoresis showed, that the peptides insoluble in aqueous medium were composed of subunits with apparent molar masses of 56, 35, 25 and 23 kDa and of traces of the masses 67, 60 and 10 kDa (Fig. 1). The soluble peptides mainly had a molar mass of 10 kDa and consisted to a minor

Table I. Lipid, sugar and protein content of seeds of *Trichosanthes kirilowii.*

Seeds		Kernels	Lipids		Sugar		Soluble proteins*		Insoluble proteins*	
Number	Weight g	g	% of kernels	% of seeds	% of kernels	% of seeds	% of kernels	% of seeds	% of kernels	% of seeds
50	13.25	7.06	61.6	32.4	2.45	1.85	3.79	2.86	26.2	13.8

* Proteins soluble or insoluble in buffers. Values represent averages of 5 experiments and deviate by maximally 5 percent.

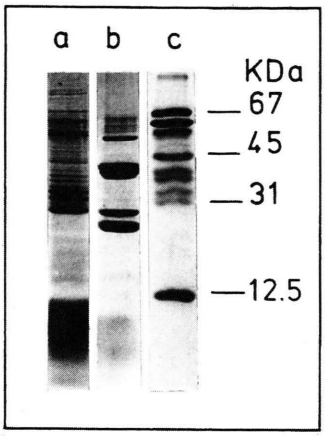

Fig. 1. Fig. 1. SDS-polyacrylamide gel electrophoresis of *Trichosanthes kirilowii.*
a. Soluble proteins;
b. insoluble proteins;
c. marker proteins.

extent of subunits of 25, 26, 28, 30, 33, 34, 37, 60, 67 and 76 kDa. The qualitative peptide composition corresponds to that of other seeds with different quantitative compositions (Yu Ma and Bliss, 1978).

Table III. Composition of the fatty acids of seed lipids of *Trichosanthes kirilowii.* The lipids were separated on a silica gel column into triglyceride, glycolipid- and phospholipid fractions with fatty acids being analysed by gas chromatography.

Fatty acids	Triglycerides	Glycolipids	Phospholipids
$C_{12:0}$	0.2	0.1	0.2
C_{12} unsaturated	0.2	0.4	–
$C_{14:0}$	0.1	0.8	0.2
$C_{16:0}$	8.3	20.7	24.4
$C_{16:1}$	0.1	1.6	0.9
$C_{18:0}$	3.2	7.0	5.1
$C_{18:1}$	22.7	14.3	5.8
$C_{18:2}$	44.9	33.1	58.8
$C_{18:3}$ (Δ^9, Δ^{12}, (Δ^{15})	0.3	1.0	0.8
$C_{18:3}$*	Traces	20.8	2.8
$C_{18:3}\Delta^9$ *cis*, Δ^{11} *trans*, Δ^{13} *cis*	18.7	–	–
C_0	13.0	29.0	30.1
C_{1-3}	87.0	71.0	69.9
C_{16}	8.4	22.3	25.5
C_{18}	86.6	76.2	73.3

C_0, saturated fatty acids; C_{1-3}, unsaturated fatty acids; C_{16}, fatty acids with 16 carbon atoms; C_{18}, fatty acids with 18 carbon atoms. * Isomers of trichosanic acid. Values represent averages of 5 experiments and deviate by maximally 5 percent.

The fatty acids of the triglycerides, glycolipids and phospholipids are compared in Table III. The fatty acid pattern of these 3 fractions is typical for the fatty acid composition of the respective lipid classes (Butt and Beevers, 1966; Gurr, 1980; Werdelmann and Schmid, 1985). In all three lipid classes, linoleic acid, being known as an essential fatty acid, is the main component. In the triglycerides oleic acid, known to occur above all in storage lipids, makes up for more than 1/5 of all acids. In addition in the triglycerides trichosanic acid, also called punicic acid, occurs up to 19%. The acid has conjugated double bounds and the configuration $C_{18:3}$, Δ^9 *cis*, Δ^{11} *trans*, Δ^{13} cis (Ahlers *et al.*, 1954; Crombie and Jacklin, 1957; Crombie and Williams, 1962; Kaufmann and Mankel, 1964). The acid is only detected in storage lipids (triglycerides) and does not occur in the membrane forming glycolipids nor in phospholipids. Trichosanic acid with its conjugated double bounds has an extremely sensitive structure and can be obtained from the triglycerides only by mild alkaline hydrolysis. In the gas chromatographic analysis on an ethylene glycol succinate column the acid has a shorter retention time than the eleostearic acid with the configuration $C_{18:3}$, Δ^9 *cis*, Δ^{11} *trans*, Δ^{13} trans from *Aleurites* seeds (Radunz *et al.*, 1998). Trichosanic acid is widely distributed in seeds from *Cucurbitaceae* and was also detected in *Punica granatum* and *Jacaranda* (Table IV).

The fatty acids of the glycolipid and of phospholipid fractions are characterized by an increase of the saturation degree in particular by an increase in palmitic acid by 8% in triglycerides and by 21% in glycolipids. In phospholipids this acid increases by 24% whereas stearic acid increases by 3% in triglycerides, by 7% in glycolipids and by 5% in phospholipids. In the glycolipid and phospholipid fraction another $C_{18:3}$ fatty acid with conjugated double bounds occurs, which appears in the gas chromatographic analysis with a shorter retention time than trichosanic acid. Also this conjugated fatty acid is only obtained from lipids after mild alkaline hydrolysis. Whereas this acid occurs in the phospholipid fraction by 3% it is present in the glycolipids up to 21%. Hydrogenation of these two C_{18}-unsaturated fatty acids with platinum oxide as catalyst leads to stearic acid. The isomer of trichosanic acid occurs as an ester compound of glycolipids and phospholipids and does not occur in stor-

Table IV. Occurrence of trichosanic acid ($C_{18:3}\Delta^9$ *cis*, Δ^{11} *trans*, Δ^{13} *cis*), (syn. punicic acid) in seed oils of different higher plants of different systematic position.

Plant	Family	% of lipids	Literature
*Trichosanthes kirilowii**	Cucurbitaceae	18	this paper
*Trichosanthes anguina**	dto.	42.8	Chisholm and Hopkins (1964)
*Trichosanthes cucumeroides**	dto.	29	Gunstone *et al.* (1994) Hilditch and Williams (1964)
Trichosanthes dioica	dto.	27.8	Hilditch and Williams (1964)
Fevillea trilobata	dto.		Tulloch and Bergter (1979)
Cucurbita digitata	dto.	17	Chisholm and Hopkins (1965)
Cucurbita palmata	dto.	11	Chisholm and Hopkins (1965)
Cucurbita foetidissima	dto.		Chisholm and Hopkins (1965)
Momordica balsamina	dto.	58	Hopkins and Chisholm (1962)
Momordica charantia	dto.		Khan *et al.* (1963)
Ecballium elaterium	dto.	22	Chisholm and Hopkins (1964)
Punica granatum	Punicaceae	72	Ahlers *et al.* (1954) Hilditch and Williams (1964)
Jacaranda	Bignoniaceae		Hopkins and Chisholm (1962)

* Systematic classification according to Encke (1960).

age lipids. The composition of total fatty acids corresponds to that obtained by HPLC- chromatography on reverse-phase and silver-impregnated columns as well as to that obtained by gas chromatography carried out by Joh *et al.* (1995). Differences exist only with respect to the concentration of individual fatty acid components, which can be certainly due to different maturity stages of the seeds and to the growth habitat.

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Ahlers N. A. E., Dennison A. C. and O'Neill G. A. (1954), Spectroscopic examination of punicic acid. Nature **173**, 1045–1046.

Butt V. S. and Beevers H. (1966), The plant lipids. In: Plant Physiology, Metabolism: Intermediary Metabolism and Pathology (F. C. Stewart, ed.), pp. 265–414, Academic Press, New York, London Vol. **IVB**.

Chisholm M. J. and Hopkins C. Y. (1964), Fatty acid composition of some cucurbitaceae seed oils. Can. J. Chem. **42**, 560–564.

Chisholm M. J. and Hopkins C. Y. (1965), Kamlolenic acid and other conjugated fatty acids in certain seed oils. Am. Oil Chem. Soc. **43**, 390–392.

Committee for Pharmacopoeia of the Minister of Public Health of China (1963), Pharmacopoeia of People's Republic of China. The People's Health Press, pp. 231–232.

Crombie L. and Jacklin A. G. (1957), Lipids. Part VI. Total synthesis of α- and β-eloeostearic and punicic (trichosanic) acid. J. Chem. Soc. 1632–1646.

Crombie L. and Williams J. C. (1962), Lipids. Part VIII. Syntheses of *cis*-13- and *trans*-octadec-13-ene-9,11-diynoic acids, and octadeca-*cis*-13, *trans*-15- and -*trans*-13,*trans*-15-diene-9,11-diynoic acids: their status in nature. J. Chem. Soc. 2449–2454.

Encke F. (1960), Pareys Blumengärtnerei. Paul Parey Publ., Berlin, Hamburg.

Gardiner S. E. and Forde M. B. (1992), Identification of cultivars of grasses and forage legumes by SDS-PAGE of seed protein. In: Modern Methods of Plant Analyses. Seed Analyses (Linskens A. F. and Jackson J. F., eds.). Springer Publ., Hamburg, Vol. **14**, pp. 43–61.

Gunstone F. D., Harwood J. L.-C. and Padley F. B. (1994), The Lipid Handbook, 2nd Ed. Chapman & Hall. Chemical Database, London, Weinheim, New York.

Gurr M. J. (1980), The biosynthesis of triacylglycerols. In: The Biochemistry of Plants. A Comprehensive Treatise (P. K. Stumpf, E. E. Conn, eds.). Academic Press, Vol. **4**, pp. 205–248.

He P., Radunz A., Bader K. P. and Schmid G. H. (1996), Influence of CO_2 and SO_2 on growth and structure of photosystem II of the Chinese tung-oil tree *Aleurites montana*. Z. Naturforsch. **51c**, 441–453.

Hilditch T. P. and Williams P. N. (1964), The Chemical Constitution of Natural Fats. Chapman & Hall, London.

Hopkins C. Y. and Chisholm M. J. (1962), Identification of conjugated triene fatty acids in certain seed oils. Can. J. Chem. **40**, 2078–2082.

Joh Y., Kim S. and Christie W. W. (1995), The structure of the triacylglycerols, containing punicic acid, in the seed oil of *Trichosanthes kirilowii*. J. Am. Oil Chem. Soc. 72, 1037–1042.

Kaufmann H. P. and Mankel G. (1964), Über das Vorkommen von Transfettsäuren. Fette, Seifen, Anstrichmittel, Die Ernährungsindustrie **66**, 6–13.

Khan S. A., Qureshi M. J., Bhatty M. K., Karimulloh (1961), A cristal study of composition of *Momordica cherantia* seed oil. Pakistan J. Sci. Res. **13**, 111–112.

Kimura Y., Akihisha T., Yasukawa K., Takido M. and Tamura T. (1995), Structure of five hydroxylated sterols from the seeds of *Trichosanthes kirilowii* Maxim. Chem. Pharm. Bull. **43**(10), 1813–1817.

Kimura Y., Akihisha T., Yasukawa K., Takase S., Tamura T. and Ida Y. (1997), Cyclokirilodiol and isocyclokirilodiol: two novel cycloartanes from the seeds of *Trichosanthes kirilowii* Maxim. Chem. Pharm. Bull. **45**, 415–417.

Köplin R., Wang G. E., Hötte B., Priefer U. B. und Pühler A. (1993), A 3.9-kb DNA region of *Xanthomonas campestris* pv. *campestris* that is necessary for lipopolysaccharide production encodes a set of enzymes involved in the synthesis of dTDP-rhamnose. J. Bact. **175**, 7786–7792.

Li S. Z. (1875), Compendium of Materia Medica. Republished in 1982, People's Health Press of China.

Radunz A. (1969), Über das Sulfoquinovosyldiacylglycerin aus Höheren Pflanzen, Algen und Purpurbakterien. Hoppe-Seyler's Z. Physiol. Chem. **350**, 411–417.

Radunz A. (1972), Lokalisierung des Monogalaktosyldiglycerids in Thylakoidmembranen mit serologischen Methoden. Z. Naturforsch. **27b**, 1113–1114.

Radunz A. (1976), Localization of the tri- and digalactosyldiglyceride in the thylakoid membrane with serological methods. Z. Naturforsch. **31c**, 589–593.

Radunz A., He P. and Schmid G. H. (1998), Analysis of the seed lipids of *Aleurites montana*. Z. Naturforsch. **53c**, 305–310.

Roughan P. G. and Batt R. D. (1968), Quantitative analysis of sulfolipid (sulfoquinovosyl diglyceride) and galactolipids (monogalactosyl and digalactosyl diglycerides) in plant tissues. Anal. Biochem. **22**, 74–88.

Schmid G. H. (1971), Origin and properties of mutant plants: yellow tobacco. In *Methods in Enzymology* (A. San Pietro, ed.). Academic Press, New York, London, Vol. **23**, pp. 171–194.

Takeda T., Kondo T., Mizukami H. and Ogohara Y. (1994), Bryonolic acid production in hairy roots of *Trichosanthes kirilowii* Max. var. Japonica Kitam, *trans*formed with *Agrobacterium rhizogenes* and its cytotoxic activity. Chem. Pharm. Bull. **42**(3), 730–732.

Tulloch A. P. and L. Bergter (1979), Analysis of the conjugated trienoic acid containing oil from *Fevillea trilobata*. ^{13}C Nuclear magnetic resonance spectroscopy[1]. Lipids **14**, 996–1002.

Van Wyk D. (1966), Mitteilungen für lamellare Strukturproteide VI. Über die Kohlenhydrate im löslichen Strukturproteid von *Antirrhinum majus* und *Spinacia oleracea*. Z. Naturforsch. **21b**, 700–703.

Werdelmann B. and Schmid R. (1985), Biotechnologie der Fette, Umschau **1**, 40–45.

Yu Ma and F. A. Bliss (1978), Seed proteins of common bean. Crop Sci. **18**, 431–437.