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Studies on *Shorea robusta*: Analysis of Seed Oil and Defatted Content

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Abstract

Nontraditional seed oil and the defatted matter of *Shorea robusta* have been analysed, in our effort to screen alternative sources of edible oils as well as proteins to the ones already in use.

The oil was extracted in an yield of 19.7%. The saponifiable fraction after working up, was found to contain palmitic stearic, arachidic, oleic and linoleic acids. The unsaponifiable fraction (1.1%) contained sitosterol and stigmasterol.

Protein was extracted from the defatted seeds in an yield of 7.2%. Amino acids (including essential amino acids) were identified from the protein hydrolysate. The defatted sample was sequentially treated with water, sodium chloride, ethanol and sodium hydroxide to separate different fractions. By gel electrop0horesis of these, four protein subunits could be identified.

High temperature heating of the saponifiable fraction resulted in formation of dimer of methyl linoleate whereas heated unsaponifiable content on high temperature heating yielded oxy derivatives of sterols.

Introduction

The study was undertaken to analyse seed oil and the defatted seeds from the non-traditional source. Seeds of *Shorea robusta* (Dipterocarpaceae) were collected from nearby/local forests.

The saponifiable fraction of the seed oil was analysed for its fatty acid composition to ascertain if it could be used as an alternative to conventional ones, after preliminary toxicity and nutritional evaluation.

The non-saponifiable fraction contained sterols.

Defatted seeds were analysed for protein, with an aim to develop new sources of plant proteins, due to their demand for edible purpose.

In the last phase of study, the isolated saponifiable and non-saponifiable fractions were put to high temperature heating (as is done in frying involving edible fats) to see the changes in their chemical compositions.

Experimental

The raw material was extracted with petroleum ether (60-80º) in Soxhlet for oil content. The oil was saponified in presence of alkali. The saponified mixed fatty acids were converted to methyl esters. TLC of the ester was done using ether: hexane: acetic acid (20:80:1) as solvent system and charring at 120º. GC was done using 1µl sample, O.V. 101 column with nitrogen as carrier gas (100ml/min) and F.I. detection. Authentics (Sigma) were used for co-chromatography and spiking. For nutritive evaluation, rats of Wistar strain and groundnut oil as diet standard were taken. Fat intake (1:10 ratio of oil sample to diet was given) and digestibility were recorded.

The non-saponifiable part was taken in ether, washed and dried. It was put to Liebermann-Burchard reaction, tested by TLC using pet ether : ethyl' acetate (90:10) and IR. Sterol standards were used for co-chromatography.

Studies on defatted seeds

For protein content, the defatted seeds were macerated with 10% saline solution and protein precipitated by adding hydrochloric acid. After filtering and drying, the protein extract was hydrolysed by refluxing with 6N HCl. The solution was decolorised, excess of acid evaporated and hydrolysate was analysed for its amino acid composition by taking it in 10% isopropanol for paper chromatography using n-butanol: acetic acid : water (40:10:50) as the solvent system. Ninhydrin was used for detection and spots were visualised at 60ºC. Amino acid standards were used for comparison.

The defatted sample was sequentially treated for two hours with cold water, sodium chloride, ethanol and sodium hydroxide (1:20), centirfuged (30,000 × g, 15') and the supernatant collected in each case to separate the fractions (four). Filtrates containing the respective proteins were dialysed against water for fortyeight hours (4ºC) dried and stored in fridge. Polyacrylamide gel electrophoresis was carried using solutions containing 5 gm protein/mL of buffer (Tris HCl, SDS, glycerol, β mercapto ethanol and urea) and standards (BioRad labs). Defatted protein fractions were taken in phosphate buffer. The electrophoresis was performed on stacking gel (Bio Rad) using tris-glycin buffer (1M) containing SDS (0.4%). Gels were stained (0.2% silver nitrate), fixed (methanol-acetic acid-water-formic acid) and developed (sodium carbonate- sodium thiosulphate-formic acid).

High temperature heating of oil

Saponifiable portion

In view of the good yield and possible suitability for edible purpose,[2] the saponifiable portion was subjected to high temperature (280ºC) heating as is done frying, using vegetable fat/oil.

The saponifiable portion of the oil was heated to 200ºC for 6 hours. The content

was then subjected to column chromatography using Hexane : ether (90:10) for nonpolar triglycerides and ether for polar triglycerides. These were converted to methyl esters. The methyl esters of the polar fraction were subjected to column chromatography using hexane : ether (88:12) and ether. TLC was performed using hexane : diethylether : acetic acid (80:20:1). For GLC, OV-I column was used with initial temperature of 300ºC and final temperature of 330ºC, flame ionisation detector (temperature 360ºC) and nitrogen as carrier gas. GC-MS was done using Shimadzu QP-2000 with EI source at 70 ev.

Nonsaponifiable portion

The portion was heated at 200ºC for 6 hours. A column of silicic acid was prepared. It was prewashed with hexane. Sample after loading on the column was washed with about one column volume of hexane : ether (3:2 v/v). Preparative TLC plates were coated with silica gel. Sample was applied as band on plate, alongwith a band of standard (cholesterol oxide from Sigma). The plate was developed in diethyl ether : cyclohexane (9:1 v/v). After drying, it was sprayed with phosphomolybdic acid (1gm in 5 ml concentrated H_2SO_4). The plate was kept at 120ºC for 10 minutes. The sprayed zones were scrapped off individually and each eluted with 5ml chloroform : methanol (2:1 v/v) and concentrated.

No standard sample of sterol oxides are available. For preparing standard dispersing medium was prepared by mixing 250 mg stearic acid (Sigma) in ethanol with 300 mg trisodium phosphate (Sigma) in ethanol with 300mg trisodium phosphate (Sigma) in ethanol, with stirring. After diluting the medium with distilled water, 1 gm of mixed sitosterol and stigmasterol (Sigma) were dissolved in hot ethanol and added to the medium with stirring at 80ºC (4 hours). After acidification (pH 6), it was extracted with ether, the ether phase was washed with 2% KOH and distilled water. It was dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in ethanol and kept at 4ºC.

For derivatization of sterol oxide rich samples as well as above reference standards to trimethylsilyl ethers, 0.5 ml pyridine, 0.15 ml hexamethyldisilazane and 0.05 ml trimethyl chlorosilane (Sigma) were added. The mixture was staken and kept for 5 minutes before injecting to GC-MS, which was performed on Shimadzu 2000 gas chromatograph coupled to mass spectrometer. The TMS derivatives of the sterol oxides were separated on WCOT capillary column (Chromopack). Hydrogen was used as carrier gas. The oven temperature was kept initially at 60ºC (2 minutes) and finally at 265ºC (30 minutes). The mass spectra were recorded at an electron energy of 70 ev; and the ion source temperature was 200ºC. Each of the sterol oxidation product separated on preparative TLC plates was individually injected to GS-MS followed by that of the standard reference.

Result and Discussion

The seeds contained 19.7% fatty oil. Palmitic, stearic, arachidic oleric and linoleric acids were found to be the constituents as determined by GLC of the methylester content (Fig.1).

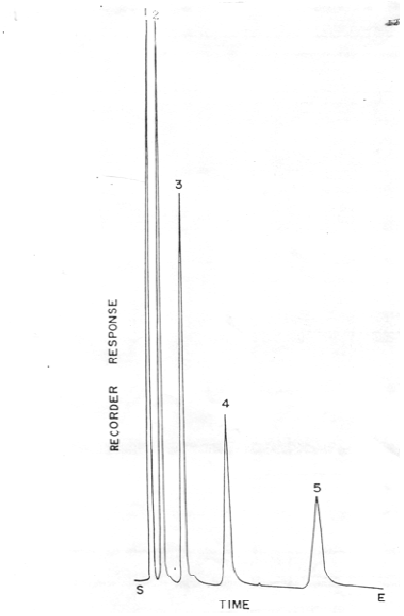

Figure 1

The test oil was found nonrepllent against *Periplaneta americana*. No activity was noted against Gram+ (*Straphylococcus aureus*) Gram- (*Salmonella paratyphi*) and pathogenic fungi e.g. plant pathogenic (*Alternaria alternata*), animal pathogenic (*Aspergillus fumigatis*) and human pathogenic (*Microsporum gypseum*). On finding the above results negative, the oil was fed to Wistar rats and the digestibility was found to be 96%. The test sample did not reveal any abnormality in the animal during the test period of four weeks. The test sample may be used as a feed, in place of groundnut which contains aflatoxin.[3,4]- The nutritionally potential oil seed may be of use for edible purpose.

The yield of the nonsaponifiable fraction was 1.1%. On the basis of IR absorptions 3450 and 1055 cm^{-1} (OH), 1640 cm^{-1} (C=C) 1460 and 1381 cm^{-1} (C-CH_3), the constituents of the saponifiable fraction has been identified to be sitosterol and stigmasterol (Fig.2).

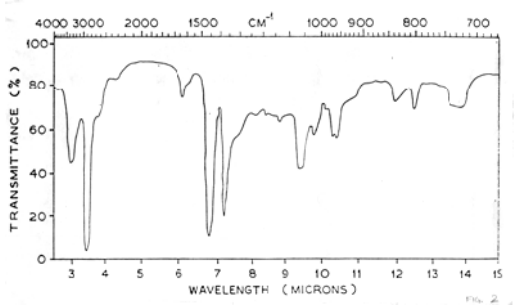

Figure 2

The defatted seeds contained 7.2% crude protein. In the hydrolysed protein content, the essential amino acids present were, methionine, thereonine, histidine, lysine, arginine and valine apart from presence of aspartic acid, glutamic aicd, serine, proline, glycine, tyrosine, cystine and alanine.

The sequentially treated fractions- cold water (albumin) sodium chloride (globulin) ethanol (prolamin) and sodium hydroxide (glutelin) were subjected to electrophoresis alongwith standards and crude protein extract. The PAGE profile (fig.3) shows standard (lane 1) crude protein extractive (lane 2) albumin (lane 3) globulin (lane 4) prolamin (lane 5) and glutelin (lane 6) respectively.

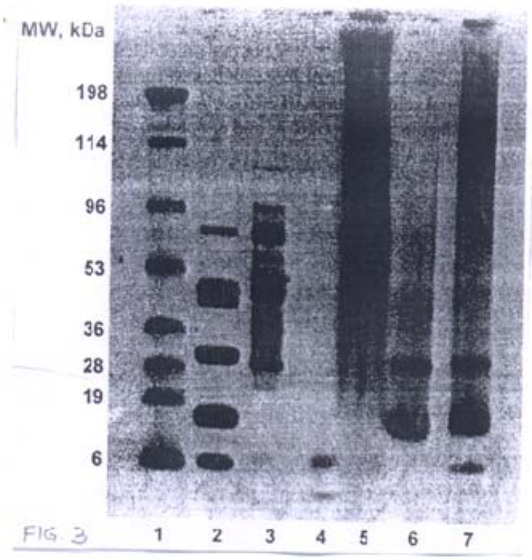

Figure 3

Whereas albumin shows molecular weight subunits between 30 to 96 kDa, globumin shows protein subunits around 6 kDa. There is masking by nonprotein components in the case of prolamin (lane 5). The glutelin content (lane 6) shows two protein subunits - molecular weight subunits of 15 and 30 kDa.

For analysis of heated saponifiable fraction which results in dimerization[5] of oil fraction, separation by GLC was difficult due to high molecular weight components. For identification, GC-mass was done (Fig.4). The mass spectral data gave evidence for presence of dihydroxy dimer of the dehydrodimer of methyl linoleate (m/z 620). An ion at m/z 602 can result from the molecular ion by loss of H_2O. The ions at m/z 503 and 520 are obtained from the molecular ion by substraction of one or two OH groups and the alkyl fragment -CH $(CH_2)_4$ CH_3. An ion (m/z 586) results from loss of both -OH groups from molecular ion. The ion at m/z 293 results from cleavage of the C-C bond joining the two linoleate portions and can represent non-substituted or substituted moiety with loss of two OH groups. The ion at m/z 309 results from cleavage of C-C bond of dimer with loss of a OH group from resulting segment.

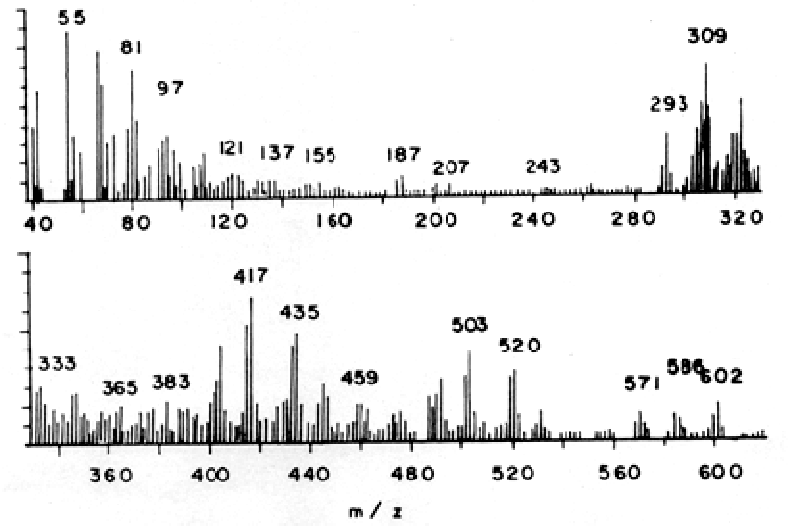

Figure 4

The heated oil (dimerised) showed lesser % digestibility (by 13%) compared to unheated oil when tested against rat.

In the case of heated unsaponifiable content, preparative TLC was done and the bands individually collected and concentrated (Rf values 0.29, 0.34, 0.40, 0.44). GC-MS (Fig.5) of I fraction (Rf 0.29, RT 1.40) shows M+ at m/z 502, peak of m/z 412 (loss of 74 due to Mclafferty rearrangement and of 16 due to additional oxygen, total 90) peak at m/z 397 (loss of 15 due to methyl radical of TMS), fragment ion at 129 (loss of 15 due to methyl radical of TMS and 1 due to H from chain, total 16) from TMSOCO $(CH_2)_2$ having m/z 145. The oxide product has been identified as epoxy sitosterol. GC-MS (Fig.6) of II fraction (Rf 0.34, RT 1.47) shows M+ at m/z 500 (2H less than sitosterol), peak at m/z 410 (loss of 74 due to McLafferty rearrangement and of 16 due to additional oxygen, total 90), peak at m/z 395 (loss of 15 due to methyl radical of TMS) fragment ion at 129 (loss of 15 due to methyl radical of TMS and 1 due to H from chain, total 16) from TMSOCO $(CH_2)_2$ having m/z 145. The oxide product has been identified as epoxy stigmasterol. GC MS (Fig.7) of III fraction (Rf 0.40, RT 1.60) shows M+ at m/z 500, peak of m/z 410 (loss of 74 due to McLafferty rearrangement and of 16 due to additional oxygen, total 90), peak at m/z 395 (loss of 15 due to methyl radical of TMS), fragment ion at m/z 129 (loss of 15 due to methyl radical of TMS and 1 due to H from chain, total 16) from TMSOCO$(CH_2)_2$ having m/z 145. The oxide product has been identified as ketositosterol. Finally GCMS (Fig.8) of IV fraction (Rf 0.44, RT 1.65) shows M+ at m/z 498 (2H less than sitosterol), peak at m/z 408 (loss of 74 due to McLafferty rearrangement and of 16 due to additional oxygen, total 90), peak at m/z 393 (loss of 15 due to methyl radical of TMS), fragment ion at m/z 129 (loss of 15 due to methyl radical of TMS and 1 due to H from chain, total 16) from TMSOCO$(CH_2)_2$ having m/z 145. The oxide product has been identified as ketostigmesterol.

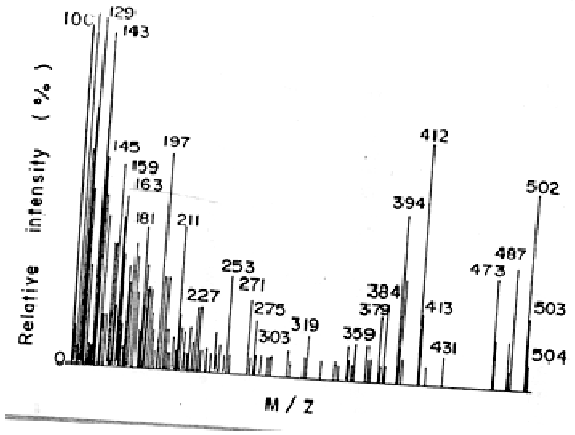

Figure 5

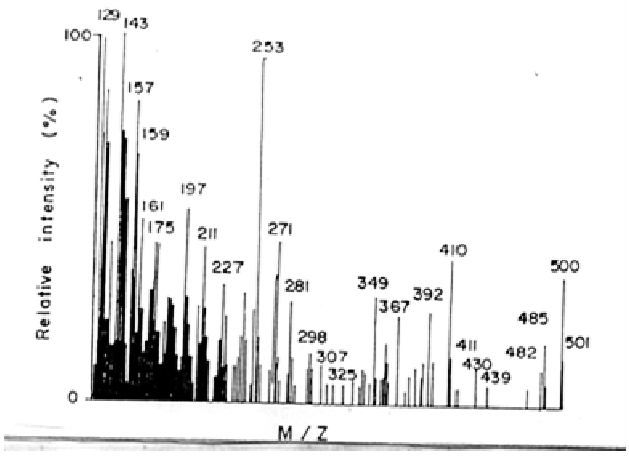

Figure 6

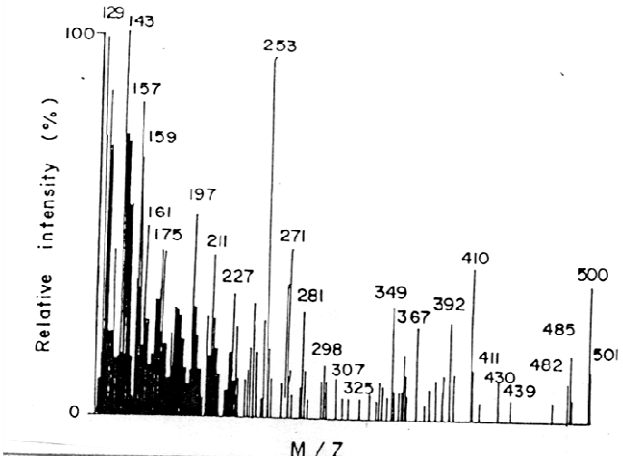

Figure 7

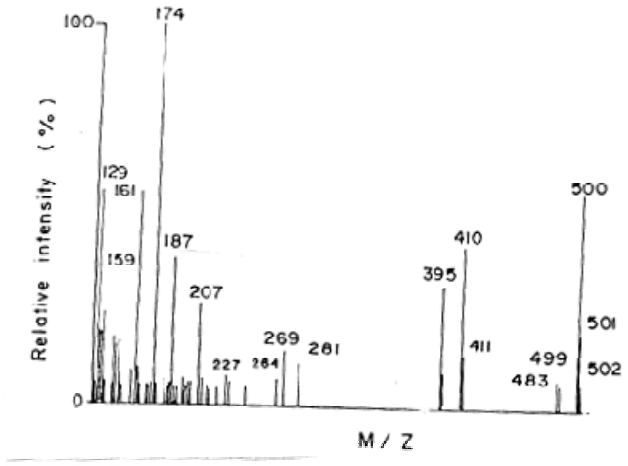

Figure 8

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