3. PRESENT WORK

The phytochemical survey and chemical screening of forest origin minor-oilseeds have been carried out for medicinal importance and also for the industrial utilization. Thus, the physico-chemical study of unusual fatty acids of minor-oilseeds is the primary aim of the present investigation. Therefore, the chemical screening has revealed the natural sources of unusual fatty acids in several minor-oilseeds. The new and interesting unusual fatty acids present in high concentration in certain seed oils are being exploited for the industrial utilization. These fatty acids of unusual structures are highly important to the oleochemical industries as raw materials for the production of a variety of oleochemicals.

Considering the extensive applications of unusual fatty acids such as hydroxy and keto fatty acids isolated from natural oil and fats for the medicinal and industrial importance, an attempt has been made to isolate new and interesting unusual fatty acids from the different species of 12 minor-oilseeds which are belonging to the different plant families, viz., Sapotaceae, Euphorbiaceae, Leguminosae, Magnoliaceae, Guttiferae, Sapindaceae, Valerianaceae, Apocynaceae.

The occurrence of ricinoleic acid together with the other normal fatty acids has been reported in the seed oils of Mimosops elangi, Emblica officinalis, Caesalpinia bonducuella, Bahuniavarigete, Sesbaniagrandiflora, Butea monosperma. The occurrence of keto fatty acid together with the
other normal fatty acids has also been reported in the seed oils of *Michaelia champaca, Garcinia indica, Sapindus mukorossi, Sesbinia aegyptica, Valeriana jatamansi, Rauvolfia serpentina.*

The physico-chemical study and structural elucidation of unusual fatty acids has been studied by the chemical and spectroscopic methods (FTIR, $^1$H NMR, $^{13}$C NMR and MS). The component of fatty acids has been identified by GLC analyses.

4. GENERAL EXPERIMENTAL PROCEDURE

Materials and Methods

Sources of oilseeds

Minor-oilseeds samples belonging to different plant families were collected from various parts of Karnataka State of forest origin.

Solvents

Petroleum ether (b.p. 40-60 °C), ethanol, methanol, benzene, acetone and diethyl ether were of reagent grade. All the solvents were distilled. The anhydrous diethyl ether was prepared by distilling the commercial samples over anhydrous calcium chloride and the distillate was stored in contact with sodium wire. The super dry ethanol or methanols were prepared using magnesium turnings and iodine.
Extraction of oils

The air-dried seeds were ground, powdered and extracted thoroughly with light petroleum ether (b.p. 40-60 °C) in a Soxhlet extractor for 24 hours. The petroleum ether extracts were dried over anhydrous sodium sulphate and solvent was removed in vacuo at 40° C to get the oil. The oil contents were determined according the AOCS methods.

Chromatographic methods

Thin layer chromatography¹ (TLC)

Analytical

The clean glass plates (20 x 10 cm) were coated with slurry of silica gel ‘G’ in distilled water (1:2 w/v) to get the film of 0.2mm thickness using a “Camag” applicator. The coated plates were air-dried and activated at 100 °C for 1 hour and cooled in a desiccator.

Preparative thin layer chromatography

Glass plates were coated to get a silica gel ‘G’ layer of 1mm thickness, using “Camag” applicator. The coated plates were air-dried and activated at 100° C for 1 hour and cooled in a desiccator.

Development and visualization

The plates after applying samples as spots or bands were developed with suitable solvent systems in a closed glass-developing chamber. The
separated spots or bands were located by exposing to iodine vapour or spraying with 0.2% ethanolic solution of 2,7-dichloro-flourescein followed by viewing under UV light or spraying with 50% sulphuric acid.

*Reversed phase thin layer chromatography*

The reversed phase thin layer chromatography of the esters was carried out on siliconised silica gel plates using acetonitrile:water:acetic acid (70:20:10 v/v/v) as developing solvent. The spraying was done with chromic acid and subsequent charring made the components visible.

*Argentation thin layer chromatography*

The silica gel coated glass plates impregnated with 20% silver nitrate were used for argentation TLC. The solvent system, petroleum ether:diethyl ether (92:8 v/v) was used for developing the plates. The spots were detected by spraying with sulphuric acid and subsequent charring.

*Picric - acid thin layer chromatography*

A thickness of 0.2mm silica gel ‘G’ coated, activated glass plate was developed in a solvent system, petroleum ether : diethyl ether : acetic acid (75:25:1 v/v/v). It was sprayed thoroughly with 0.5M picric acid in 95% ethanol and placed in a jar saturated with vapours of diethyl ether : ethanol : acetic acid (80:20:1 v/v/v). After 30 minutes, the plates were removed and exposed to ammonia fumes for few minutes. The orange spot on a yellow background of the plate indicated the presence of epoxy fatty acids.
Column chromatography

The oxygenated ester or acid was purified by using activated neutral alumina in a column. About 500mg of ester or acid was transferred to a column (4'x0.25") containing 40gm of neutral alumina and petroleum ether. Then, it was further eluted with petroleum ether and diethyl ether (7: 3 v/v). The pure material thus obtained was analyzed for IR, $^1$H NMR, $^{13}$C NMR and MS.

Gas liquid chromatography

The quantitative examination of the methyl esters was carried out on a “Shimadzu GC-17A Unit” using a stainless steel column coated with 15% DEGS on chromosorb, W, 45-60 mesh. The temperature at injection port, detector port and oven were 240°C, 240°C and 190°C respectively. The machine recorded the weight percent of individual peaks. The peaks were identified by comparing their retention times with those of standard reference sample under the similar conditions.

Spectroscopic methods

Ultraviolet (UV)

The UV spectra of the methyl esters of oils were taken on Hitachi 150-20 Model Instrument in methanol using cell of 1 cm path length was used. The concentration of solutions was 0.001%.
**Infrared (IR)**

The IR spectra of oils and their methyl esters were recorded on a *Nicolet 5700 FTIR* instrument. IR spectra were determined as liquid films and KBr pallets for liquid and solid samples, respectively.

**Nuclear Magnetic Resonance $^1H$ NMR and $^{13}C$ NMR**

The $^1H$ NMR was recorded from deuterio chloroform and d$^6$ DMSO solutions on *Bruker Avanace-300 (300 MHz)* Model spectrophotometer. The chemical shifts (δ 0-20) were measured in ppm downfield from internal TMSi at δ=0.

**Mass Spectrophotometry (MS)**

The mass spectra of TMSi derivative, diacetyl derivative, keto fatty esters and novel substituted 16-hydroxy-amido-palmitic acids were run on *Finningan Mat with PDP Micro Computer 810* at 70 eV with a source temperature 150° C.

**Chemical methods**

**Halphen test $^3$**

This chemical test was specific for the Cyclopropenoid functional group in fatty oils. A solution of sulphur (1% in CS$_2$) was mixed with equal volume of amyl alcohol and known as Halphen reagent. The fatty oil and Halphen reagent were mixed and the reaction mixture was heated on a water
bath (70-80° C) for few minutes until all the carbon disulphide boiled off. Then the test tube was loosely plugged with cotton and heated for 1-2 hours in an oil bath at 110-115°C. A development of red coloration indicated the presence of Cyclopropenoid fatty acids.

**Saponification**

A known weight of the oil was saponified at room temperature by stirring overnight with 0.8N alcoholic potassium hydroxide solution. The excess of alcohol was removed by distillation under reduced pressure. It was then diluted with water. The non-saponifiable matter was removed by extraction with diethyl ether. After careful acidification of pH 5 with 0.5N sulphuric acid, the mixed fatty acids were extracted with diethyl ether. The ether solution was washed with water several times and the solvent was removed.

**Acetolysis**

A portion of oil (20gm) was stirred for 24 hours at room temperature with 200 ml of glacial acetic acid in 80 ml of 10% of sulphuric acid according to Wilson’s method. It was then diluted with distilled water and extracted with solvent ether repeatedly. The combined ether extracts were washed thoroughly with distilled water and dried over anhydrous sodium sulphate. The solvent was removed in a stream of nitrogen. The acetolysed product was saponified as described above.
Isolation of hydroxy fatty acids

The mixed fatty acids thus obtained were partitioned according to Gunstone's method\(^5\) between equal volume of petroleum ether (b.p 40-60\(^\circ\)C) and 80\% methanol. The yields of oxygenated and non-oxygenated fatty acids were recorded.

Esterification

Methyl esters of fatty acids were prepared by the following methods.

Transesterification method

The seed oil was transesterified with 1\% sodium methoxide in methanol (50ml) under reflux for 1 hour. Then, the reaction mixture was diluted with distilled water (25ml) and extracted with diethyl ether (30ml). The combined ether extracts were washed with distilled water, dried over anhydrous sodium sulphate and solvent was removed in a stream of nitrogen.

Fischer esterification (Methanol-Sulphuric acid)

The fatty acid samples were refluxed in a large excess of absolute methanol containing 1\% sulphuric acid (v/v). In each case, the resulting mixture was diluted with water and then extracted repeatedly with diethyl ether. The combined ether extracts were dried over anhydrous sodium sulphate and solvent was removed in a stream of nitrogen.
**Trimethylsilylation**

The hydroxy fatty acid methyl esters (10mg) were converted into TMSi derivative by dissolving the fatty esters in dry pyridine (1ml), 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane in an anhydrous condition. The mixture was shaken vigorously for 30 seconds and was allowed to stand for 5 minutes. Then the pyridine was removed in a stream of nitrogen and the resulting derivatives were used for MS and GLC analysis.

**Acetylation**

About 200 mg of dihydroxy fatty acid methyl esters were taken in a 250 ml round bottomed flask and a mixture of acetic anhydride and pyridine (15ml) was added. The reaction mixture was refluxed on a water-bath for about 2 hours in an anhydrous condition. The product was cooled and diluted with distilled water (50ml). It was extracted with diethyl ether and dried over anhydrous sodium sulphate. The excess of pyridine was removed in a stream of nitrogen.

**Hydrogenation**

About 50 mg of fatty acid methyl ester(s) with 75 ml of methanol were subjected to catalytic hydrogenation with equal amount of palladium-charcoal (20%) in a Parr-low pressure hydrogenator for about 12 hours at 50-60 lbs/inch. The catalyst was filtered off and the solvent was removed to get the hydrogenated product.
Oxidation Methods

**KMnO₄/Acetic acid method**

The hydroxy acid (2 gm) dissolved in acetic acid was oxidised by gradual addition of powdered potassium permanganate (10 gm) at such a rate that temperature did not exceed 50°C. After 3 hours, at this temperature the solvent was removed under reduced pressure and the residue distilled with water and acidified with dilute sulphuric acid. This was decolourised with sulphur dioxide and then steam distilled. Both the residue and the distillate were extracted with diethyl ether to give crude dibasic acid (1.4 gms) and crude monobasic acid (0.5 gm) respectively. The former was extracted with boiling water and after the concentration of solution to 10 ml and cooling to 0°C gave azelaic acid (0.5 gm) m.p. 106-107°C (from ethyl acetate) undepressed with authentic sample. The volatile acid (0.25 gm), was distilled under pressure which readily gave p-bromophenacyl heptanoate m.p. 66-67°C.

**von Rudloff method**

The oxidation of the unsaturated keto acid was carried out in t-butanol (20 ml). A solution of keto acid in t-butanol (0.25%) was treated with a solution of sodium-metaperiodate (200 mg) in 20 ml of water and potassium permanganate (1 ml) in the presence of potassium carbonate 60 mg. The mixture was stirred at room temperature for 24 hours and the solution then decolourised with NaHSO₃ followed by acidification with
HC1. The mixed acids were extracted with diethyl ether. The ether was removed and the extracts were treated with 10% H₂SO₄ in absolute methanol. The mixture was refluxed for 1 hour and then extracted with diethyl ether. The ether extracts were dried over anhydrous Na₂SO₄. The solvent was removed in a stream of nitrogen.

The GLC analysis of the products as their methyl esters showed that the cleavage fragments were monobasic and dibasic acids respectively.

Analytical methods

Analytical data of oils were determined according to AOCS methods¹⁰.

Iodine value (I.V.)

The fatty material was weighed accurately into an iodine flask (250 ml) and was dissolved in carbon tetrachloride (20 ml). Then Wijs solution (25 ml) was added. (Wijs solution was prepared by dissolving iodine monochloride (4 ml) in one litre of glacial acetic acid). The weight of the sample was maintained in such a way that there would be an excess of Wijs solution of 100 to 150% over the amount required. After swirling, the iodine flask was kept in dark place for 30 minutes at room temperature. The flask was removed from dark storage.

Potassium iodide 15% solution (20 ml) and 100 ml of distilled water were added to the iodine flask. The contents of iodine flask were then
titrated against 0.1N sodium thiosulphate solution using starch as an indicator. A blank titration was also carried out simultaneously.

\[
\text{Iodine value (I.V.)} = \frac{(A-B) \times N \times 12.69}{M}
\]

Where A and B are titre values of blank and samples respectively. Similarly, M and N are weight of sample and normality of sodium thiosulphate solution respectively.

**Saponification value (S.V.)**

1-2 gm of fatty material was weighed accurately and added to 0.5N alcoholic potassium hydroxide (25 ml) in a round bottom flask (250 ml) to which an air condensor was attached. The mixture was refluxed on a water bath for 1 hour and titrated against a standard 0.5N hydrochloric acid using phenolphthalein as an indicator. Simultaneously a blank reading was also carried out.

\[
\text{Saponification Value (S.V.)} = \frac{56.1 \times N \times (A-B)}{M}
\]

Where A and B are titre values of blank and sample, respectively. Similarly M and N are weight of sample and normality of HCl respectively.

**Durbetaki titration**

**Preparation of Durbetaki reagent (0.1 N HBr in HAc)**

20 gms of red phosphorus and 40ml of water were taken in a flask. To this 40ml of liquid bromine was added drop by drop from tap funnel. On
addition of first few drops of bromine lambent green flame appeared but not when the air was displaced. At the end of the reaction, the flask was gently heated and the liberated HBr was passed through a U tube loosely fitted with broken glass smeared with moist red phosphorus (to remove bromine vapours) and was bubbled at a slow rate through 1 litre of glacial acetic acid until desired normality was attained.

**Standardization of the reagent**

About 0.4gms of potassium phthalate was weighed and was dissolved in 10 ml glacial acetic acid. Then it is titrated with HBr solution (Durbetaki reagent) using 5drops of 1% crystal violet indicator (0.1 gm of crystal violet indicator in 100ml acetic acid) to a bluish green end point. Normality of HBr was calculated as follows.

\[
\text{Normality of HBr} = \frac{\text{Weight of pot. phthalate}}{0.2042 \times \text{Titration in ml}}
\]

**Stepwise titration**

About 300-500 mg of the oil sample was weighed in a 50 ml conical flask and was dissolved in 5 ml of distilled benzene. Four to five drops of indicator (crystal violet 1% solution) were added. Durbetaki reagent (HBr in acetic acid 0.1N) was taken in a semi-microburette and was added to the conical flask slowly with constant stirring at 3°C and 55°C, separately. The end point was observed by bluish green colouration.
The amount of HBr reactive fatty acid(s) present in the oil sample were calculated as follows –

\[
\begin{align*}
\text{\% of Cyclopropenoid fatty (CPFAs) at 55\textdegree C} &= \frac{A \times N \times 28.8}{M} \\
\text{\% of epoxy fatty acid(s) (EFAs) at 3\textdegree C} &= \frac{A \times N \times 31.4}{M}
\end{align*}
\]

Where A is titre value in ml, M and N are weight of sample(s) and normality of HBr, respectively.
References