EXPERIMENTAL

Melting points were finally checked by Kofler-Block. Optical rotations were generally measured in chloroform (and \( \lambda-D \)) unless otherwise stated. Infrared spectra were mostly taken with potassium bromide and the nuclear magnetic resonance spectra were recorded (in CDCl\(_3\)) with tetramethyl-silane as internal standard (60 MHz). The ultraviolet spectra were taken in methanol. The alumina and silica gel used for different chromatographic purposes were obtained from National Chemical Laboratory, Poona; S. Merck (India); and E. Merck (West Germany).

Different types of compounds were isolated and identified separately from two plants by various physico-chemical methods. The experimental work has been recorded accordingly in two sections 1 and 2.

1. Seseli indicum, W. & A.

2. Zataria multiflora, Boiss.
SESELI INDICUM

Extraction

The dried and powdered seeds of Seseli indicum, W. & A.; (1 kg) were extracted successively with petroleum ether (60-80°, 6.5L), benzene (6L) and ethanol (5L) at their boiling points and the different extracts were obtained as noted below after complete removal of the respective solvents.

1. Petroleum-ether (60-80°) - 30 g.
2. Benzene - 45 g.
3. Ethanol - 50 g.

Petroleum-ether extract

The extract (30 g), taken in ether, was treated with aqueous solution of potassium hydroxide (20%) and then divided into alkali-soluble and alkali-insoluble (neutral) parts in the usual manner. The alkali-soluble portion was acidified with dilute hydrochloric acid and extracted with ether. The ethereal solution was then dried over anhy. sod. sulphate. The residue (10 g) on removal of ether formed the alkali-soluble part.
I. Neutral part

The neutral part (18 g) was saponified in alcohol (150 ml) by refluxing with alcoholic potassium hydroxide (6 g) for two hours. After saponification the alcohol was distilled off. The unsaponifiable matter was then separated from the soap solution by means of ether. The volatile matter from the unsaponifiable portion was then removed by steam-distillation, and a gummy residue (11 g) was obtained. This was taken in benzene and after chromatographic treatment on alumina the following products were obtained.

Product A

Elution with petroleum-ether (40-60°) and crystallisation from alcohol yielded a colourless product (150 mg), m.p. 68-70°; \( \nu \text{max.} 2900, 2830, 1470, 1375, 720 \) and \( 710 \text{ cm}^{-1} \). It appeared to be a saturated hydrocarbon (probably hentriacontane and/or dotriacontane). Finally, this product was subjected to GLC analysis for the verification and final confirmation. It was found to be a mixture of n-alkanes of the series \( C_{23} - C_{35} \) as given below (Table 2). The product was compared with the mixture of authentic specimens of the paraffins.
The above figures indicated that the n-alkanes containing odd numbered carbon atoms predominated in this plant (seeds) also according to the general findings so far collected by Streibl and others (vide: theoretical portion).

**Product B**

Further elution of the column with petroleum-ether: benzene (1:1, v/v), and purification by repeated crystallisation from methanol and chloroform yielded a crystalline product (B-
100 mg), m.p. 137-38°, $\gamma$ = 33.3°. It gave positive Liebermann-Burchard test and yellow colour with tetranitromethane. It was identified as $\beta$-sitosterol by mixed melting point determination and co-TLC with an authentic specimen of the compound, and further confirmed through its acetate.

**Acetate**

The product (B, 50 mg) was mixed with acetic anhydride (1 ml) and pyridine (6 drops) and allowed to stand at room temperature for 24 hours. The mixture was then poured onto ice and the solid filtered off. It was crystallised from methanol as colourless flakes (40 mg), m.p. 126-27°. This derivative did not show any depression in melting point on admixture with an authentic specimen of $\beta$-sitosterylacetate

**Product C**

On further elution with benzene and crystallisation from methanol and chloroform another crystalline product (C, 300 mg; TLC - homogeneous, silica gel/AgNO$_3$-20%) was obtained, m.p. 153-55°; $\gamma$ = 33.3° (yield, 0.02%). It gave positive Liebermann-Burchard test, red colour with Noller's test (cf: cholesterol) and yellow colour with tetranitromethane. The IR spectrum showed the presence of $\sim$OH (3325 and 1050 cm$^{-1}$), C=C (1650 cm$^{-1}$), and $\text{C}=$CHR (830 cm$^{-1}$) groups. Its molecular weight was found to be 412 (mass spectrum - molecular ion and base peak).
According to the fragmentation pattern (m/e 369, 351, 300, 273, 271, 255, 213, 159, 139) the compound appeared to possess steroidal nucleus\[^13\]: 412-139 = 273, and 412-(139+2) = 271.

**Found:** C, 84.00%; H, 11.99%,

**Calculated for C\(_{29}H_{48}O\):** C, 84.40%; H, 11.72%.

The NMR spectrum indicated signals\[^{12a}\] at 0.7 (H-13), 0.75 (H-29), 0.8 (H-26 & 27), 0.85 (H-29), 0.90 (H-26, 27 & 29), 0.967 (H-21), 1.00 (H-19), 1.07 (H-21), 5.12 (2H-vinyl protons), and 5.35 (1H-vinyl proton). Following derivatives were prepared towards its identification.

(a) **Acetate** — The compound (100 mg) was mixed with acetic anhydride (1 ml) and pyridine (4 drops), heated for two hours on a steam-bath, and was then worked up in the usual manner. The crude acetate was chromatographed over alumina and eluted with petroleum-ether (40-60\(^\circ\)). Shining crystals were obtained on crystallisation from methanol and acetone, m.p. 135-38\(^\circ\); \(\int^{22}_{D} \cdot 20^\circ\) (homogeneous, TLC:silica gel/AgNO\(_3\)); \(\gamma_{\text{max.}}\) 1730 and 1240 cm\(^{-1}\); M\(^+\) 454, important peaks at m/e 394, 340, 315, 313, 273, 255, 213, 159 and 139 etc. The NMR spectrum indicated signals at 0.70, 0.75, 0.80, 0.83, 0.90, 0.95, 1.00 and 1.03 (CH\(_3\) protons); 2.0 (s, acetate); 4.60 (3\(^\circ\)H); 5.11 (dt, vinyl protons); and 5.33 (dt, vinyl proton).
(b) **Benzoate** — It was prepared by treating the sterol (100 mg) with benzoyl chloride (1 ml) and pyridine (6 drops) at room temperature (24 hrs.). The crude ester was then chromatographed over alumina, and eluted with light petrol, and crystallised from acetone and petrol, m.p. 160-63° (TLC—single spot, 80 mg).

(e) **3:5-Dinitrobenzoate** — The sterol (100 mg) was treated with freshly prepared 3:5-dinitrobenzoyl chloride (100 mg) and pyridine (1 ml) and heated on a steam-bath for 45 minutes. Aqueous solution of sodium bicarbonate (5%) was added to the cooled mixture, and the solid ester was then filtered. The crude derivative (90 mg) was crystallised from acetone and petroleum-ether, m.p. 231-33°.

Round : N, 4.09%
Calculated for C$_{36}$H$_{50}$O$_{6}$N$_{2}$: N, 4.62%.

(d) **Tetrahydroproduct** — The sterol (150 mg) was hydrogenated in glacial acetic acid (20 ml) using platinum catalyst (PtO$_{2}$, 40 mg). The hydrogenated product$^{14}$ (110 mg) was crystallised from methanol and chloroform (TLC — homogeneous, silica gel/AgNO$_{3}$$^{20%}$), m.p. 136-38°; $\int_{-\infty}^{\infty}J_{D}$ + 20°. It gave negative test with tetranitromethane and did not indicate any vinyl proton
signals in the NMR spectrum but methyl protons signals at 0.65, 0.683, 0.783, 0.85, 0.893 and 1.00; $\nu_{max}$ 3400 and 1040 cm$^{-1}$.

**Acetate** - The above product (d) was treated with acetic anhydride and pyridine for acetylation. After working up as usual the acetate was crystallised from methanol, m.p. 129-30°; $\gamma_D$ +18°.

The tetrahydroproduct and its acetate did not show any depression in melting point on admixture respectively with authentic specimens of stigmastanol$^{14}$ (obtained from $\beta$-sitosterol by hydrogenation with Pd/C-10% in ethanol), and its acetate. Co-IR, co-TLC, and m.m.p. of the tetrahydroproduct and stigmastanol and their acetates respectively suggested that indosterol has stigmastane type carbon skeleton.

(e) *Oxidation of the sterol* - The compound (C, 500 mg) was taken in acetone (distilled over $\text{KMnO}_4$) and cooled into a freezing mixture (5-10°). The Jones' reagent$^{38}$ (chromium trioxide in conc. $\text{H}_2\text{SO}_4$, cooled) was added to the cooled solution dropwise with constant stirring till a reddish brown colour persisted and the reaction mixture was allowed to stand at room temperature for about 30 minutes and then diluted with cold water. The product (443 mg), after extracting with ether, was chromatographed over
silica gel. Elution with benzene and crystallisation from methanol and acetone yielded a compound (TLC-single spot), m.p. 156-57°; $\gamma_{\text{max}} \sim 1690$ and 1660 cm$^{-1}$. The IR spectrum showed bands at $\gamma_{\text{max}}$. 1690 and 1660 cm$^{-1}$. The NMR spectrum$^{16}$ indicated methyl signals at $\delta$ 0.75, 0.8, 0.85, 0.9, 0.98, 1.10 and 1.17, and olefinic signals at $\delta$ 4.9 (t, 1 H) and $\delta$ 5.80 (s, 1 H); $\lambda_{\text{max}}$. EtOH 252 nm, $\varepsilon$ 11,000$^{21b}$. The mass spectrum showed the parent ion peak at m/e 424 and the other important fragments at m/e 381, 312, 285, 137, 81 and 43 etc. The latter fractions of the eluent (benzene) gave a different product which on crystallisation from methanol and chloroform had m.p. 157-58°; $\gamma_{\text{max}} \sim 30^\circ$ (TLC - homogeneous); $\gamma_{\text{max}}$. 1722 cm$^{-1}$ (C=O). It did not show any absorption in the UV region. The former product was found to be an $\alpha,\beta$-unsaturated ketone, while the second a saturated one. Both the products gave positive Zimmermann test.

(f) Epoxidation - The number of double bonds present in the sterol was determined by per-acid titration. The compound (C, 200 mg) in chloroform (10 ml) was treated with perbenzoic acid (freshly prepared - 12 ml) in chloroform (12 ml) and kept for 24 hrs. in a refrigerator and then the contents were titrated against standard solution of sodium thiosulphate (N/30.6) in the usual manner (iodometric) using potassium iodide. Calculations
indicated the presence of two double bonds. A crystalline product, m.p. 123-25°, was also obtained after chromatography and crystallisation (ethyl acetate).

On the basis of the careful survey of the literature and the study of the physical constants and properties of the sterol, its different derivatives and reaction products, it appeared to be a new sterol carrying an important feature (△²^9(11) double bond) in the steroidal nucleus along with △²²(23) double bond in the side chain as has been discussed earlier, and the structure derived.

II. Alkali-soluble part

The brownish gummy alkali-soluble part (10 g) was taken in benzene (30 ml) and subjected to column chromatography over silica gel (300 g). The following coumarins were obtained in pure form (TLC) after elution with different solvents. They emitted fluorescence when exposed to ultraviolet light. No acidic constituent was found in this part.

Product D

Elution with petroleum-ether (60-80°) and crystallisation from methanol and chloroform afforded a product (D), m.p. 83-85°; \( \lambda_{\text{max}} \) 330, 297sh, 254, 245sh, and 224 nm (TLC-homogeneous).
This product has been identified as suberosin\textsuperscript{39} by m.p., m.m.p. and co-TLC with an authentic specimen of the compound.

**Product E**

Further elution of the column with benzene gave another compound which has been identified as bergaptene, m.p. 187-88°, \( \lambda_{\text{max.}} \) 310, 268, 259, 250, 243 and 222 nm. It did not show any depression in melting point on admixture with an authentic specimen of the coumarin.

**Product F**

Chloroform yielded the third crystalline product (F), m.p. 119-20°; \( \lambda_{\text{max.}} \) 330, 292, 283, and 218 nm. It was characterised as seselin by co-TLC and direct comparison with an authentic specimen (m.m.p).

**Product G**

The latter fractions obtained from chromatography with chloroform gave a yellow crystalline product (G), m.p. 151-53°, \( \lambda_{\text{max.}} \) 312, 269, 249, 241 and 223 nm. It was identified as isopimpinellin by m.p., m.m.p. and co-TLC with an authentic specimen of the coumarin.

N.B. The last three coumarins are also obtained in quantity
in the form of a crystalline mixture in the very beginning directly by petrol extract of the seeds on concentration of the extract.

**Alcoholic extract**

**Sugars**

The entire alcoholic extract of the seeds was used as such for detection of free sugars. Glucose and rhamnose have been detected in the extract by descending co-paper chromatography (solvent: n-butanol:acetic acid:water - 4:1:5, upper layer), using Whatman filter paper No. 1 and aniline-phthalate in n-butanol.

**Non-volatile carboxylic acids**

The seeds (250 g) were extracted (under reflux) with alcohol for 12 hours, and then the extract concentrated in vacuo. Ascending paper chromatographic method (Filter paper - Whatman No. 1) was used for characterisation of the water-soluble non-volatile organic acids using n-butanol:formic acid:water (4:1:5, v/v organic layer) solvent system. After developing the chromatogram for 12 hours, it was air-dried overnight, and then heated for 8 hours at 80\(^\circ\) and finally sprayed with bromophenol-blue (0.04% in alcohol). These acids were detected as lemon-yellow spots. The extract was employed in water along with the authentic
specimens of the various known carboxylic acids for comparison. Oxalic, malic and citric acids were finally detected in this extract by repeating the experiment several times for confirmation.

**Amino acids**

The dry and powdered seeds (250 g) were exhausted with petroleum-ether then treated with aqueous sodium chloride solution (2% - 400 ml) for about 8 hours. The sodium chloride extract (100 ml) was then heated with hydrochloric acid (6N, 25 ml) for 20 hours on a steam-bath. Thereafter the mineral acid was completely removed from the hydrolysate and the residue was then extracted with absolute alcohol. This extract was used for examining amino-acids.

Descending paper chromatographic technique\(^\text{11}\) (Whatman Filter Paper No.1) was employed using n-butanol:acetic acid: water (4:1:5, v/v - organic layer - 20 hrs.) as the developing solvent system. The dried chromatogram was then sprayed with ninhydrin in acetone (0.1%). Finally, it was kept in an electric oven at 55\(^\circ\) for 10 mts. and the amino-acids were located as coloured spots using various authentic samples simultaneously. Serine, alanine, leucine, valine, asparagine and, threonine were thus identified in the above extract.
ZATARIA MULTIFLORA

Extraction

The dried and powdered drug (1 kg.- consisting of leaves, stems and seeds) was extracted successively with the following solvents (4.5 litres) at their respective boiling points for twenty hours, and the different extracts were obtained as noted below after complete removal of the respective solvents.

1. Petroleum-ether (60-90°) - 45 g.
2. Benzene - 35 g.
3. Ethanol - 50 g.
4. Methanol - 22 g.

Petroleum-ether extract

The viscous mass (45 g) obtained after removal of the solvent was taken in ether, treated with aqueous solution of potassium hydroxide (5%) and was then divided into neutral and acidic parts in the usual manner. The alkali-soluble portion was acidified with dilute hydrochloric acid, extracted with ether and then the ethereal solution was dried over anhydrous sodium sulphate. This formed the acidic part.
Neutral part

The neutral part (33 g) was saponified in alcohol (200 ml) by refluxing with alcoholic solution of potassium hydroxide (10 g) for six hours. After saponification, the alcohol was distilled off and the residue diluted with water (300 ml). The unsaponifiable matter was then separated from the soap-solution by ether. The volatile matter from the unsaponifiable portion was then removed by steam-distillation and the residue was obtained as a brownish gummy mass (15 g). This was taken in benzene and subjected to chromatographic purification over alumina, and the following products were obtained.

Product A

Elution with petroleum-ether (60-80°) and crystallisation from alcohol yielded a colourless product (A), m.p. 65-66°, which showed single spot on thin layer chromatography (silica gel). It was found to be a saturated hydrocarbon (IR) and the elemental analysis compared with that of triacontane, which showed absorption bands at 2960, 2920, and 2850 cm⁻¹ (C-H saturated); 1470, 1460 and 1375 cm⁻¹ (C-CH₃); 730 & 720 cm⁻¹ (CH₂). The product did not show any depression in m.p. on admixture with an authentic specimen of the n-alkane C₃₀H₆₂ (from Czechoslovakia).

Found: C, 85.56%; H, 14.22%

Calculated for C₃₀H₆₂: C, 85.22%; H, 14.78%
But, on the basis of mass spectrum the molecular weight was indicated as 464.53 corresponding to n-paraffin C\textsubscript{33}H\textsubscript{68} (m.p. 71.8°, Lit.)\textsuperscript{8} (Calcd. for C\textsubscript{33}H\textsubscript{68}: C, 85.26%; H, 14.74%). This anomaly was clarified by GLC analysis which indicated the product to be a mixture of n-paraffins as given below (Table 3), accompanied by traces of some olefins and branched-chain hydrocarbons. Odd numbered homologues predominated\textsuperscript{9}.

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>TABLE - 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. n-Tritriacontane (C\textsubscript{33}H\textsubscript{68})</td>
<td>62.0</td>
</tr>
<tr>
<td>2. n-Hentriacontane (C\textsubscript{31}H\textsubscript{64})</td>
<td>17.0</td>
</tr>
<tr>
<td>3. n-Nonacosane (C\textsubscript{29}H\textsubscript{60})</td>
<td>5.0</td>
</tr>
<tr>
<td>4. n-Dotriacontane (C\textsubscript{32}H\textsubscript{66})</td>
<td>4.0</td>
</tr>
<tr>
<td>5. n-Pentatriacontane (C\textsubscript{35}H\textsubscript{72})</td>
<td>3.5</td>
</tr>
<tr>
<td>6. n-Tetratriacontane (C\textsubscript{34}H\textsubscript{70})</td>
<td>Very small quantity</td>
</tr>
<tr>
<td>7. n-Triacontane (C\textsubscript{30}H\textsubscript{62})</td>
<td>, ,</td>
</tr>
<tr>
<td>8. n-Octacosane (C\textsubscript{28}H\textsubscript{58})</td>
<td>, ,</td>
</tr>
<tr>
<td>9. n-Heptacosane (C\textsubscript{27}H\textsubscript{56})</td>
<td>, ,</td>
</tr>
</tbody>
</table>

Product B

Further elution of the neutral part with benzene and purification by repeated crystallisation from methanol and chloroform gave a crystalline solid (B, shining flakes), m.p. 136-37°, $\gamma$-25°D = 32.5°. It gave positive Liebermann-Burchard
test and yellow colour with tetranitromethane, \( \gamma \) max. 3571 and 3450 cm\(^{-1}\) (OH); 1653 and 840 cm\(^{-1}\) (C=C). It was identified as \( \beta \)-sitosterol by the above facts and by mixed melting point determination with an authentic specimen of the compound and the preparation of its various derivatives.

(a) \textbf{Acetate} - The above product (30 mg) was treated with acetic anhydride (0.5 ml) and pyridine (2 drops) and allowed to stand overnight at room temperature and then heated on a steam-bath for six hours. The solid product obtained was crystallised from methanol and acetone as colourless flakes (18 mg), m.p. 123-24°. This derivative did not show any depression in melting point on admixture with an authentic specimen of \( \beta \)-sitosteryl acetate.

(b) \textbf{Benzoate} - The sterol (50 mg) was treated with benzoyl chloride (1 ml) and pyridine (5 drops). The mixture was allowed to stand overnight at room temperature and then heated for about six hours on a steam-bath. The solid derivative obtained was filtered off, washed with aqueous solution of potassium hydroxide (2%) and water, and finally crystallised from methanol and acetone, m.p. 144-48° (30 mg) (cf: \( \beta \)-sitosteryl benzoate\(^{14} \), m.p. 145-50°).

\textbf{Found: C, 83.41%; H, 9.91%}

\textbf{Calculated for} \( \text{C}_{36} \text{H}_{54} \text{O}_2 \): C, 83.34%; H, 10.49%
(c) 3:5-Dinitrobenzoate - The sterol (30 mg) was treated with freshly prepared 3:5-dinitrobenzoyl chloride (40 mg) and pyridine (0.5 ml) and heated on a steam-bath (45 mts.). The crude derivative was crystallised from acetone and petroleum-ether, m.p. 203-06° (15 mg) (cf: 3:5-DNB of \( \beta \)-sitosterol\(^{14} \), m.p. 202-04°).

Found: C, 71.47%; H, 8.89%; N, 5.09%.

Calculated for \( \text{C}_{36} \text{H}_{52} \text{O}_6 \text{N}_2 \): C, 71.02%; H, 8.61%; N, 4.60%.

**Product C**

On further elution with benzene and chloroform (3:1, v/v) and crystallisation from ethyl acetate another crystalline compound (C, TLC-homogeneous) was obtained, m.p. 254-56°. It gave positive Liebermann-Burchard & Noller's tests, and yellow colour with tetranitromethane, \( \wedge \) max. 880 cm\(^{-1} \) (=CH\(_2\)), 3380 and 1025 cm\(^{-1} \) (OH), 1638 cm\(^{-1} \) (C=C) and 1372 & 1385 cm\(^{-1} \)-CH groups. Its molecular weight was found to be 442 (mass spectrum). By the fragmentation pattern \(^{30} \) (m/e 427, 424, 411, 399, 207, 191 & 189) and the co-TLC with an authentic specimen of the triterpene, the product (C) was identified as betulin and was further confirmed by preparation of its diacetate.
Diaacetate — It (50 mg) was prepared with acetic anhydride (0.5 ml) and pyridine (4 drops) in the usual manner. The crude acetyl derivative (45 mg) was chromatographed over silica gel. Elution with petroleum–ether and benzene (1:3, v/v) and crystallisation from MeOH and acetone gave a crystalline product, m.p. 212–15°. It also gave yellow colour with tetranitromethane; 
\[ \text{\textsuperscript{\textdegree}C=O}, 1638 \text{ cm}^{-1} (\text{C=C}), 1245 \text{ cm}^{-1} (\text{Acetate}), \]
\[ 890 \text{ cm}^{-1} (=\text{CH}_2) \text{ and, } 1360 \text{ & } 1380 \text{ cm}^{-1} \text{ C(CH}_3)_2. \]

Acidic part

The gummy acidic part (8 g) was taken in benzene and ether (9:1, v/v) and chromatographed over silica gel (80 times; Barton)\textsuperscript{40} yielding the following acids:

Product D

On elution with benzene, a crystalline product (D), m.p. 74–75°, was obtained. This appeared to be a saturated (negative TNM test) aliphatic acidic compound, \[ \text{\textsuperscript{\textdegree}C=O}, 1705 \text{ and } 725 \text{ cm}^{-1}, \text{ and showed single spot on TLC. It yielded a methyl ester on treatment with diazomethane (ether), m.p. 56–57° (alcohol), which did not show any depression in melting point on admixture with the authentic specimen of methyl behenate. The} \]
acid was thus identified as behenic acid, $\nu_{\text{max}}$ 3340 cm$^{-1}$ (OH),
1705 cm$^{-1}$ (C=O), 725 & 715 cm$^{-1}$ (CH$_2$)$_n$.

Found: C, 78.00%; H, 12.90%.
Calculated for C$_{22}$H$_{44}$O$_2$: C, 77.58%; H, 13.02%.

But, later by gas-liquid-chromatographic analysis the above product (D) was found to be a mixture of four fatty acids as given below:

**TABLE - 4**

<table>
<thead>
<tr>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lignoceric acid (C$<em>{24}$H$</em>{48}$O$_2$)</td>
</tr>
<tr>
<td>2. Cerotic acid (C$<em>{26}$H$</em>{52}$O$_2$)</td>
</tr>
<tr>
<td>3. Behenic acid (C$<em>{22}$H$</em>{44}$O$_2$)</td>
</tr>
<tr>
<td>4. Montanic acid (C$<em>{28}$H$</em>{56}$O$_2$)</td>
</tr>
</tbody>
</table>

Further elution of the acidic part with benzene and ether (9:1, v/v) and crystallisation from methanol, another acidic product (E) was obtained, m.p. 235-90$^\circ$. The third crystalline product (F) obtained in the latter fractions on elution with benzene and ether (4:1, v/v) showed m.p. 280-85$^\circ$. It gave infrared spectrum identical with the former compound (E), but indicated significant depression in melting point on admixture with it, and thus the two were considered to be different compounds.
Product E

The acid, m.p. 285-90°, \( \leftarrow \alpha_\text{D}^{20} + 85° \), gave positive Liebermann-Burchard & Noller's tests and yellow colour with TNM, \( \gamma \) max. 3450 (OH-Broad); 1690 (C=O), 1620 & 820 (C=C); 1372 & 1385 cm\(^{-1}\) (gem-dimethyl). Mass spectrum showed the parent ion peak at m/e 456, and the fragmentation pattern \(^{35} \) - m/e 248, 233, 207, 203, 191 and 189. It appeared to be a triterpenic acid belonging to the oleanolic acid series (Tschesche et al. \(^{31} \)).

Following derivatives were prepared from this acid towards its identification.

(a) Acetate - The compound (0.5 g) was mixed with acetic anhydride (10 ml) and pyridine (0.5 ml) and the mixture was allowed to stand overnight at room temperature, and then heated for two hours on a steam-bath. The solid, obtained in the usual manner, was chromatographed over silica gel. On elution with benzene and crystallisation from acetone, shining crystals, m.p. 279-81°, \( \leftarrow \alpha_\text{D} + 68.5° \) (450 mg) were obtained (TLC - single spot, silica gel-AgNO\(_3\), 20%); \( \gamma \) max. 1720 (C=O); 1245 (acetate); and 1385 & 1369 cm\(^{-1}\) C(CH\(_3\))\(_2\). Its nuclear magnetic resonance spectrum showed signals \(^{34} \) at 5 3.39 (1H; t, vinyl proton); 4.61 (1H, t, 3\(^{3}\)H); 2.08 (3H, s, acetate protons); 1.26, 1.17, 0.97, 0.90 and 0.76 (CH\(_3\) protons).
(b) **Methyl ester** — It was obtained by treatment with diazomethane (ether), chromatography (alumina) and crystallisation (acetone), m.p. 197-98°; $\alpha_D^{25} = 47.8°$ (single spot - TLC), $\gamma_{\max}$ 3540 (OH); 1736 (C=O); and 1140 cm$^{-1}$ (methyl ester). The NMR spectrum showed signals at 5.33 (1H, t, vinyl proton), 3.68 (s, methyl ester protons); 1.19, 1.03, 0.97, 0.93, 0.75 (CH$_3$-protons).

(c) **Methyl ester acetate** — Prepared from (b)(above) in the usual manner by acetylation. Chromatography (alumina) and crystallisation (acetone) yielded shining crystals (TLC-homogeneous), m.p. 213-15°; $\gamma_{\max}$ 1740 (C=O); 1240 (Acetate); 1160 (Me-ester); and 1365 & 1385 cm$^{-1}$ C(CH$_3$)$_2$ group. It gave positive TNM test for unsaturation.

(d) **Lactone** — Purified acid (E, 200 mg) in CHCl$_3$ (20 ml) was treated with a stream of dry hydrogen-chloride (gas) at room temperature (10 mts.) and then the contents were poured into water (100 ml). The product was divided into acidic and neutral parts in the usual manner. The neutral portion was crystallised from chloroform and methanol, m.p. 244° (148 mg). This product did not give yellow colour with tetranitromethane; $\gamma_{\max}$ 3395 (OH), 1765 (lactone); and 1365 & 1385 cm$^{-1}$ (gem-dimethyl).
(e) Ketone (Jones' Oxidation) - The acid (E, 100 mg) was taken in acetone (distilled over KMnO₄) and cooled in a freezing mixture. The Jones' reagent was added to the cooled solution dropwise with constant stirring till a pale brown colour persisted, and then the reaction mixture was allowed to stand at 0-5° for about 15 minutes and diluted with cold water (25 ml). The product was then extracted with ether and dried over anhydrous sodium sulphate. The product (90 mg), m.p. 162-64°, gave positive Zimmermann test and formed 2,4-dinitrophenylhydrazone, m.p. 256-59°; max. 3390 (COOH), 1690 (C=O), 1365 and 1385 cm⁻¹ (gem-dimethyl), 1708 cm⁻¹ (C=O).

Oleanonic Acid—Found: C, 79.45; H, 10.60%
Calculated for C₃₀H₄₆O₃: C, 79.29%; H, 10.14%.

Although the compound (E) contained an ethylenic bond but did not absorb hydrogen (Pd/c-10% in ethanol) at all, the number of double bonds were determined by peracid—titration in the usual manner. Further, one carboxylic acid group was found to be present in the compound on titrating it with standard alkali solution (n/26.1).

On the basis of the careful survey of the literature and the study of different spectra of the compound and those of its different derivatives it appeared to be oleanolic acid, but the melting points of the compound and those of some of its...
derivatives were not very closely comparable with literature\textsuperscript{14,41} (vide-Table 5). Hence, conclusive identification of the compound E was finally confirmed by its superimposable infrared spectrum with that of the authentic specimen of oleanolic acid (from Prof. Dr. R. Tschesche, Bonn) in addition to the above physico-chemical data.

\textbf{TABLE - 5}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Oleanolic acid</th>
<th>Product E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m.p. °C</td>
<td>( \alpha )</td>
</tr>
<tr>
<td>1. Parent acid</td>
<td>310</td>
<td>+ 80</td>
</tr>
<tr>
<td>2. Acetate</td>
<td>268</td>
<td>+ 75</td>
</tr>
<tr>
<td>3. Methyl ester</td>
<td>201</td>
<td>+ 75</td>
</tr>
<tr>
<td>4. Methyl ester acetate</td>
<td>222-3</td>
<td>+ 70.4</td>
</tr>
<tr>
<td>5. Ketone (Oleanonic acid)</td>
<td>160-200</td>
<td>-</td>
</tr>
<tr>
<td>6. Lactone</td>
<td>278</td>
<td>-</td>
</tr>
</tbody>
</table>

\textbf{Product F}

The second acid, m.p. 280-85\(^\circ\); \( \int \alpha \int_D^{20} + 94\(^\circ\), also gave positive Liebermann-Burchard & Noller's tests and yellow colour with tetranitromethane. The infrared spectrum showed bands at 3459 (OH-Broad); 1690 (C=O); 1640, 835 (C=C), 1375 and 1385 cm\(^{-1}\).
Mass spectrum showed the parent ion peak at m/e 456 and other important fragments at m/e 395, 248, 207, 203 and 189.

The above acid (F, 100 mg) was acetylated as usual with acetic anhydride (<2 ml) and pyridine (5 drops) and the crude acetate was chromatographed over silica gel. Elution with petroleum-ether and crystallization from methanol and acetone gave a colourless crystalline product, m.p. 262-63°, $\lambda_{\text{max.}}$ 3300, 1745, 1705, 1260, 1385 and 825 cm$^{-1}$.

Methyl ester of the product F was also prepared by treatment with diazomethane (ether), chromatography (alumina) and crystallisation (methanol and acetone) having m.p. 214-16°, $\lambda_{\text{max.}}$ 3400, 1740, 1605, 1385, 1375 and 1160 cm$^{-1}$.

Methyl ester acetate was obtained in the usual manner by acetylating the above methyl ester, chromatography (alumina) and crystallisation from methanol and acetone, m.p. 203-06°, $\lambda_{\text{max.}}$ 1740, 1385, 1365, 1240, 1160 and 822 cm$^{-1}$. Its NMR signals at $\delta$ 4.83 (1H, t, vinyl proton) 4.73 (1H, t, 3αH); 3.72 (3H, s, Me-ester protons); 2.09 (3H, s, acetate protons); 1.30, 1.17, 1.02, 0.87 and 0.78 (CH$_3$-protons).

But later, the above acid (product F) on further purification (silica gel: AgNO$_3$-15%), more careful examination, and comparison with authentic specimen (co-TLC) was not found to be a pure compound but a mixture of most probably oleanolic, ursolic and epi-oleanolic acids.
Benzene extract

The viscous mass obtained after removal of the solvent was divided into neutral and alkali-soluble parts. The alkali-soluble part on chromatography yielded mainly oleanolic acid (compared with the product E). The neutral part did not yield any specific additional solid product.

Alcoholic extract

The ethanolic extract (50 g) was triturated successively with petroleum-ether and benzene and the residue left was dissolved in water and then divided into ether-soluble and ether-insoluble parts.

Ether-soluble part

The ether-soluble part (20 g) gave positive Shinoda (Mg/HCl) and ferric chloride tests. It was taken in chloroform (20 ml) and then chromatographed over silica gel (90 times). On elution with benzene, a greenish mass was obtained which gave six spots (closely situated) on a TLC plate (silica-gel) G; solvent system - benzene:pyridine:formic acid - 36:9:5).
Individual components of this complex mixture could not be obtained in pure form either by column or even by preparative TLC. Hence, it was not worked up further. The spots on TLC were examined in the UV light as well.

On further elution with benzene:ethyl acetate (4:1, v/v) a gummy mass was obtained which also gave positive tests for flavonoidal compounds. On repeated chromatography this gummy material afforded a mixture which was then resolved into two components (pure form) by repeated preparative TLC: designated as G (0.70 g) and H (0.13 g). U.V. light was employed for separation of these compounds.

**Product G**

It gave positive Mg/HCl and FeCl₃ tests, m.p. 323-30°. The UV spectra showed absorption at λₘₐₓ. 345, 266sh, 245sh nm; AlCl₃: 420, 301, 270 nm; AlCl₃/HCl: 345, 294, 270; NaOAc: 410, 310, 279, 264 nm; NaOAc/H₃BO₃: 370, 303, 290, 255 nm; and NaOMe: 400, 315, 275 nm. It was identified as luteolin by m.p., m.m.p. and co-TLC and the preparation of its various derivatives.
(a) Methyl ether — The above compound (G, 0.17 g) was dissolved in dry acetone (50 ml) and to the solution were added roasted potassium carbonate (10 g) and dimethyl sulphate (neutral -0.5 ml). The reaction mixture was refluxed on the steam-bath for about 4 hrs. (negative FeCl₃ test). The crude methyl ether was worked up as usual and then chromatographed (silica gel). Elution with chloroform and crystallisation from methanol and chloroform afforded a colourless product (80 mg), m.p. 191-93°. The NMR spectrum showed peaks at 3.85 (d, methoxyl protons); 6.35 (d, 6-H); 6.55 (d, 8-H); 6.9 (d, 5'-H, J= 8.0 cps); 7.3 (2'-H) and 7.4 (6'-H).

(b) Acetate — The compound G (60 mg) was treated with acetic anhydride (1 ml) and pyridine (8 drops), and the mixture was heated on steam-bath and worked up as usual. The product was crystallised from ethyl acetate, m.p. 234-35°; NMR peaks at 2.35 (d, acetate protons); 6.65 (d, 6-H); 6.9 (d, 8-H); 7.4 (d, 5'-H); 7.72 (s, 2'-H) and 7.84 (s, 6'-H).

Product H

This compound (m.p. 289-90°) did not show any depression in melting point on admixture with the authentic specimen of 6-methyl quercetin. It was identified as 6-methyl-quercetin by
co-TLC, m.p., m.m.p. and its methyl ether.

**Methyl ether** — It (product H, 90 mg) was treated with neutral dimethyl sulphate in dry acetone and worked up as usual. The crude methyl ether was purified by passing through a small column of silica gel. It was crystallised from dichloromethane and methanol (36 mg), m.p. 165-66°. Its NMR spectrum showed the peaks at 1.8 (s, 3H); 4.00 (s, OCH\textsubscript{3} protons); 6.6 (s, 8-H); 7.00 (d, 5'-H); 7.4 (s, 2'-H); and 7.5 (s, 6'-H).

**Ether-insoluble part**

The water-soluble part (21 g), obtained after removal of water under reduced pressure, was tested by paper chromatography and found to be a mixture of a number of components. It was then taken in methanol and subjected to preparative paper chromatographic technique (Whatman Filter paper No. 3; solvent system — n-butanol: acetic acid:water — 4:1:5, v/v) for resolution into pure constituents. Three major components were separated in pure form; labelled as I, J & K, the middle product (J) could not be obtained in well crystalline form.
Product I

It gave a positive Mg/HCl test and blue colour with ferric chloride, m.p. 250-55°. The UV spectra\(^{36}\) showed

\[\lambda_{\text{max.}} \quad 343, 268, 250 \text{ nm}; \quad \text{AlCl}_3: 410, 350, 330, 297, 272 \text{ nm}; \]
\[\text{AlCl}_3/\text{HCl}: 390, 245, 296, 278, 262 \text{ nm}; \quad \text{NaOAc}: 405, 266, 252 \text{ nm}; \]
\[\text{NaOAc}/\text{H}_3\text{BO}_3: 370, 364, 252 \text{ nm and NaOMe: 395, 268, 257 nm}; \]
\[\gamma_{\text{Nujol max.}} \quad 3500, 3120, 1675, 1570, 1555, 840 \text{ cm}^{-1}. \]

It was considered to be a flavonoidal glycoside.

(a) Methyl ether - The above product was converted into methyl ether as usual by the treatment with dimethylsulphate in acetone. The crude methyl ether thus prepared was then hydrolysed by methanolic sulphuric acid (10%). The partially methylated product obtained through crystallisation from alcohol had m.p. 285°; \[\lambda_{\text{max.}} \quad 326, 282, 262 \text{ nm}; \quad \text{AlCl}_3: 326, 280, 264 \text{ nm}; \]
\[\text{AlCl}_3/\text{HCl}: 325, 282, 263 \text{ nm}; \quad \text{NaOAc}: 320, 276 \text{ nm}; \quad \text{NaOAc}/\text{H}_3\text{BO}_3: 329, 266, 281 \text{ nm}; \]and \[\text{NaOMe: 328, 276 nm}. \]

(b) Aglycone - It was obtained after hydrolysis of the glycoside (Product I) with methanolic sulphuric acid (10%). After working up as usual, the ether-soluble part (aglycone), crystallised from ethyl acetate and methanol, had m.p. 320-5°. This aglycone did not show any depression in melting point with
an authentic specimen of luteolin. The aqueous part contained sugar moiety and the mineral acid. The latter was removed by ion-exchange technique (Amberlite IR-120). Glucose was identified as sugar moiety by direct comparison with authentic sample of glucose by paper chromatography (solvent:n-butanol:acetic acid:water 4:1:5, v/v). The product I was thus identified as luteolin-7-glucoside.

**Product J**

It also gave positive Shinoda and ferric chloride tests. The UV spectra showed the absorption at $\lambda_{max}$ 340, 268, 256 nm; $\text{AlCl}_3$: 415, 323sh, 297sh, 278, 257 nm; $\text{AlCl}_3/\text{HCl}$: 399, 345, 296sh, 277, 260 nm; $\text{NaOAc}$: 405, 350; 267sh, 257sh nm; $\text{NaOAc}/\text{H}_3\text{BO}_3$: 375, 256 nm and $\text{NaOMe}$: 408, 298, and 274 nm.

Following derivatives have been prepared towards its identification.

(a) **Methyl ether** — It was prepared as usual by dimethyl-sulphate (neutral) and acetone (dry). After working up as usual, the product (which could not be crystallised) was hydrolysed in methanolic sulphuric acid to a partially methylated aglycone and sugars. The sugars are identified as glucose and rhamnose by co-paper chromatography with their authentic specimen. The UV
spectra of the aglycone (partially methylated) were also recorded
\( \lambda_{\text{max}} \): 330, 282, 265 nm; AlCl\(_3\): 330, 282, 265 nm; AlCl\(_3\)/HCl: 330, 282, 265 nm; NaOAc: 340, 310, 274 nm; NaOAc/H\(_3\)BO\(_3\): 330, 281, 266 nm; and NaOMe: 380, 345, 308, 274 nm).

(b) Acetate - The product J was acetylated with pyridine and acetic anhydride, and the gummy crude acetyl derivative was chromatographed over silica gel. Elution with chloroform afforded a semi-solid product. The NMR spectrum\(^{36a,b}\) showed peaks at 1.2 (d, CH\(_3\) rhamnose); 2.0 (d, acetate); 2.4 (d, acetate); 4.7 (1H, rhamnosyl); and 5.3 (m, 1H, glucosyl).

(c) Aglycone - Sulphuric acid (10%) was used for hydrolysis of the product J. The reaction mixture was worked up as above and the aglycone, m.p. 312-16\(^\circ\), thus obtained showed no depression in m.p. on admixture with an authentic sample of quercetin, and the \( R_f \) values of both (the authentic and the natural sample) also were found to be identical by co-TLC.
(d) Methyl ether of the aglycone — It was prepared as above. Methanol afforded crystalline material, m.p. 153-55° (TLC - single spot; solvent: toluene:pyridine: acetic acid — 10:1:1). Quercetin methyl ether\(^{36c}\), m.p. 154-56°.

(e) Acetate of the aglycone — It was as usual prepared by acetic anhydride and pyridine. The crude acetyl product was chromatographed over silica gel. Elution with CHCl\(_3\) and crystallisation from EtOAc afforded a colourless crystalline product, m.p. 190-95°. On the basis of the above experiments and the results the product J was identified as quercetin-7-O-glucoside-3-O-rhamnoside.

**Product K**

It was a pale yellow compound (crystallised from ethyl acetate and water), m.p. 172-73°, and also gave positive Mg/HCl and FeCl\(_3\) tests. The UV was also recorded\(^{36} (\lambda_{\text{max}} 330, 270 \text{ nm}; \text{AlCl}_3: 364, 335, 301, 270 \text{ nm}; \text{AlCl}_3/\text{HCl}: 387, 335, 301, 272 \text{ nm}; \text{NaOAc}: 398, 311, 270 \text{ nm}; \text{NaOAc}/\text{H}_3\text{BO}_3: 328, 270 \text{ nm}; and \text{NaOMe}: 400, 305\text{sh}, 279 \text{ nm}).
(a) Acetate - It was prepared as above and chromatographed over silica gel. Elution with benzene and acetone (3:2), and crystallisation (alcohol) yielded a pure compound (TLC), m.p. 207-08\(^{\circ}\).

(b) Aglycone - The product K was refluxed with H\(_2\)SO\(_4\) (10\%) for 3 hrs. After working up the aglycone, m.p. 347-48\(^{\circ}\), was identified as apigenin by co-TLC (solvent: toluene:pyridine:acetic acid — 10:1:1). The acidic (aqueous) part was neutralised by BaCO\(_3\), filtered, concentrated and then spotted on paper for the identification of sugar moiety. Glucose was found to be present as sugar moiety by direct comparison with an authentic specimen.

Product L

It was also isolated (0.5 g) from the alcoholic extract of the plant material through column chromatography (silica gel). It gave positive Liebermann-Burchard test and yellow colour with tetranitromethane and was a water soluble powder, m.p. 297-99\(^{\circ}\).

\(\gamma\) max. 3500, 1055 (Broad) cm\(^{-1}\). This compound was also isolated along with the compounds I, J & K through chromatography of the water soluble part of the alcohol-extract. Following derivatives were prepared towards its identification.
(a) **Acetate**  - The product (L, 100-mg) was treated with acetic anhydride (1.00 ml) and pyridine (0.5 ml), and allowed to stand over-night at room temperature. The reaction mixture was worked up as usual and the product (85 mg) was crystallised from methanol and chloroform, m.p. 175-77°; $\nu_{\text{max}}$ 1750 cm$^{-1}$, 1245 cm$^{-1}$ (acetate); NMR: $\delta$ 0.70, 0.80, 0.88, 1.00 (CH$_3$ protons); 2.03 (q, acetate proton); 5.25 (vinyl protons). The nuclear magnetic resonance spectrum of this acetate was also superimposable with that of the authentic specimen of $\beta$-sitosteryl-D-glucose acetate (Lit.$^{42}$ m.p. 171°).

(b) **Hydrolysis**  - The product L (200 mg) was hydrolysed in methanolic sulphuric acid (10%). The aglycone was extracted with ether and crystallised from methanol m.p. 131-34°. It also gave positive Liebermann-Burchard test and yellow colour with TNM. This aglycone was identified as $\beta$-sitosterol by m.p., m.m.p. and co-TLC with an authentic specimen of the sterol. Glucose was identified as sugar moiety in the aqueous solution by paper chromatography (solvent:butanol:acetic acid:water—4:1:5, v/v) in the usual manner. Thus, the product L was characterised as $\beta$-sitosterol-D-Glycoside (Lit.$^{42}$, m.p. 290-92°). This was further confirmed by Co-IR with an authentic specimen of sitosterol-$\beta$,D-glycoside (kindly supplied by Dr. Rastogi, C.D.R.I., Lucknow); vide: Spectra L.
Free sugars

The entire alcoholic extract as such was also used for detection of free sugars. Glucose and rhamnose have been detected to be present in the extract by co-paper chromatography in the manner as described earlier.

Non-Volatile carboxylic acids

The dried drug (600 g) was extracted (under reflux and at room temperature separately) with alcohol and aqueous alcohol (50%) for 12 hours, and then the different extracts were concentrated in vacuo. Ascending paper chromatographic technique (Whatman No. 1) was employed for characterisation of water-soluble non-volatile organic carboxylic acids in these four different extracts using n-butanol:formic acid: water (4:1:5, v/v, organic layer)\textsuperscript{10}. After developing the chromatograms for 12 hours they were air-dried overnight, and then heated for 8 hours at 80\textdegree and finally sprayed with bromophenol-blue\textsuperscript{10} (0.04% in rectified spirit). These acids were detected as lemon yellow spots.

Succinic and gluconic acids were detected in the alcoholic (95%) extract, whereas succinic, malic and oxalic acids were, somehow, found to be present in the aqueous alcoholic extract.
The experiments were repeated four times in each case for confirmation.

**Amino-acids**

The air-dried powdered plant-material (containing leaves, stems and flowers) (250 g) was extracted with petroleum-ether (60-80°), and the defatted plant material was then treated exhaustively with aqueous sodium chloride (2% - 400 ml) for 8 hours.

The aqueous sodium chloride (2%) extract (100 ml) was heated with hydrochloric acid (6N, 25 ml) for 20 hours, on a steam-bath. The hydrolysate was then taken in a porcelain dish and the mineral acid was completely removed. The hydrolysate was then extracted with absolute alcohol.

Descending paper chromatographic technique$^{11}$ (Whatman filter paper No.1) was employed for the characterisation of amino-acids using n-butanol: acetic acid:water (4:1:5 ; v/v-organic layer, 20 hrs.) as the developing solvent system$^{11}$. After developing the chromatogram was air-dried and then sprayed with ninhydrin in acetone (0.1%). Thereafter, the chromatogram was heated at 55° for 10 mts., and the amino-acids were located as coloured spots using simultaneously the authentic specimens of the various amino-acids. Glycine, arginine, valine and asparagine were thus identified.

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