CHAPTER IV

Oldenlandia diffusa

Experimental

General:

Melting points were obtained on a Fisher-Jones apparatus and were uncorrected. Elemental analyzes were done by Department of Science, Ministry of Industry and Australian Microanalytical Service, Division of Applied Organic Chemistry, CSIRO, University of Melbourne. Infra-red spectra were recorded on a Hitachi Model EPI-G2, Japan and a Pye Unicam SP 200G spectrophotometer. The spectra of solids were obtained by incorporating the sample into a pellet of potassium bromide. The bands at 3083, 1602, and 907 cm^{-1} in a polystyrene film (0.05 mm.) were used as reference peaks. Nuclear magnetic resonance spectra were obtained by using a Perkin-Elmer model R12 and a Varian A60D spectrometer. Tetramethylsilane was used as the internal standard and TFA-D & $CDCl_3$ as solvent. Mass spectral data were obtained using a Perkin-Elmer model 270, or a Hewlett-Packard model 5930 mass spectrometer. Atomic absorption data were measured with a Varian Techtron AA-5 spectrometer and air-acetylene was used as ionizing flame. Optical rotation was recorded on an automatic sugar polarimeter, Carl Zeiss.

Physical Separation:

Column chromatography (wet and dry column chromatography) was performed on a glass column by using E. Merck's standardized alumina 90, neutral alumina 90, and silica gel (70-325 mesh ASTM, E Merck, Darmstadt) as the solid support. All reagents were purified and dried according to the standard procedures.(37, 38)

Thin layer chromatography (TLC) was carried out by using E. Merck (Darmstadt) silica gel-G and PH 254, coated (20 x 20 cm. and 5 x 20 cm.) glass plates. Chromatoplates were prepared by using Desaga spreader with thickness of 0.25 mm. for identification. The plates were activated at 120°C for one hour. The solvent system was $CHCl_3$, ether-petroleum ether (50:50) and methanol-chloroform (1:19) unless otherwise stated and concentrated sulphuric acid was used as detecting agent.

Colour Tests:

4.1 Liebermann-Burchard test.

To a solution of the sample to be tested (2-3 mg.) in chloroform (0.5 $cm.^3$) was added a few drops of acetic anhydride, followed by one drop of concentrated sulphuric acid. Development of the colour after a few minutes suggests the presence of steroid or triterpenes.

4.2 Bromine in carbon tetrachloride

In this test 0.01 g. of compound to be tested was added to 2 $cm.^3$ of carbon tetrachloride, and a 5 % solution of bromine in carbon tetrachloride was added drop by drop (with-shaking)

until the bromine colour persisted. This reagent was widely used to test for the presence of an olefinic or acetylenic linkage. A positive test for unsaturation was one in which the bromine colour was discharged without the evolution of hydrogen bromide gas.

4.3 Potassium permanganate solution

To 2 $cm.^3$ of ethanol was added 0.01 g. of the compound to be examined. Then 2 % potassium permanganate solution was added drop by drop with shaking until the purple colour of the permanganate persisted. A solution of potassium permanganate was decolourized by compounds having ethylenic or acetylenic linkages. This is known as Baeyer's test for unsaturation.

4.4 Shinoda test:

To an alcoholic solution (1 $cm.^3$) of the sample (2-3 mg.) was added a few pieces of magnesium turning and 1-3 drops of concentrated hydrochloric acid. Any colour developed within a few minutes.

4.5 Barfoed's reagent:

This reagent may be used as a general test for monosaccharides. A test-tube containing 1 $cm.^3$ of the Barfoed's reagent and 1 $cm.^3$ of a dilute solution of sample was heated in a water bath . If orange-red cuprous oxide was formed within 2 minutes, it would indicate the presence of monosaccharide

4.6 Benedict's reagent:

To 5 $cm.^3$ of Benedict's solution was added 0.4 $cm.^3$ of a 2 % solution of sample. The solution was boiled for 2 minutes and allowed to cool spontaneously. If a reducing sugar was present,

the solution would contain a red cuprous oxide.

4.7 Fehling's reagent:

5 cm.^{3} of Fehling's solution were placed in a test-tube and heated to gentle boiling. A solution of 0.01 g. of sample in 2 cm.^{3} of water was added and it was continued to boil gently for two minutes. An orange-red precipitate of cuprous oxide indicated the presence of a reducing sugar.

4.8 Molisch's test:

This is a general test for carbohydrates. 5 mg. of the sample were placed in a test-tube containing 0.5 cm.^{3} of water and it was mixed with 2 drops of a 10 % solution of β-naphthol in alcohol. 1 cm.^{3} of concentrated sulphuric acid was dropped down the side of the inclined tube so that the acid formed a layer beneath the aqueous solution without mixing with it. If a carbohydrate was present, a red ring appeared at the common surface of the liquid, the colour quickly changed on standing or shaking, resulting in a dark purple solution.

4.9 Tollen's reagent:

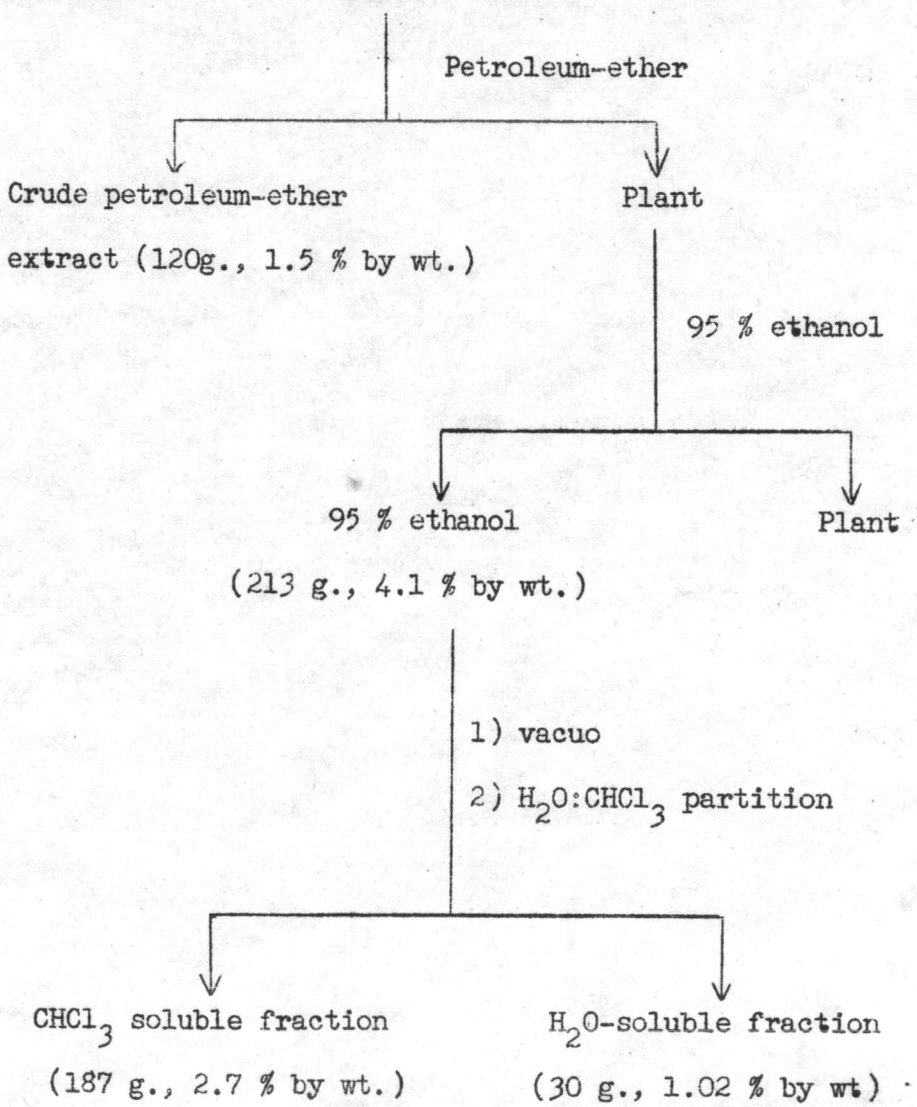
A few drops of a dilute solution of the sample was added to 2-3 cm.^{3} of a freshly prepared Tollen's ammoniacal silver nitrate reagent in a clean test-tube. A silver mirror was deposited on the walls of the tube either in cold or upon warming in the beaker of boiling water.

Extraction and Separation

The ground sun-dried O. diffusa was extracted by soaking in light petroleum ether (b.p. 60-80°C) for five days at room temperature (120 g, 1.5 % by wt.). The soaking was repeated ten times. The ground plant material was then filtered off and similarly extracted with 95 % ethanol for six days (213 g., 4.1 % by wt.). After evaporation of the ethanol solution in vacuo, the residue was then distributed between chloroform and water. Evaporation, in vacuo, of the chloroform gave 187 g. of crude material (2.7 % by wt.). The aqueous layer was concentrated under reduced pressure to yeild 30g. of a reddish syrup. (1.02 % by wt.). The various extracts were processed as follows:

Chart I

Procedure for Extraction of the O. diffusa



The light petroleum ether (b.p. 60-80°C) extract was concentrated under reduced pressure. It gave a dark sticky liquid of 120 g. (1.5 % by wt. from O. diffusa 7.6 kg.)

Table II

Crude Petroleum ether Extraction

time	Dry ground wt. of O. diffusa (g.)	petroleum ether (l.)	wt. of crude extraction (g.)	% by wt. from O. diffusa
1	1400	10	21	1.5
2	2200	15	32	1.45
3	4000	25	67	1.67

Thin layer chromatography (solvent system: light petroleum ether: ether 1:1 or chloroform) of the concentrated extract revealed at least two major components. A portion of the concentrated extract (30 g.) was dissolved in a minimum volume of light petroleum ether (50 $cm.^3$), and chromatographed on a alumina 90 column, using light petroleum ether as the initial eluting solvent (2.5 $dm.^3$), changing to diethyl ether by gradual introduction of the latter. Finally, the column was stripped with methanol. Each eluted fraction (table III) was monitored by TLC using concentrated sulphuric acid as a spraying agent and equivalent fractions were combined.

All waxes, oils and tars, including the unseparated mixture were discarded.

Compound I

The combination of eluted fractions No. 10 to No. 20 (Table III) on standing deposited colourless amorphous crystals which were filtered off and washed with a little cold light petroleum ether. On recrystallization from petroleum ether-benzene, white amorphous crystals (120 mg.,0.00162 %) was obtained, m.p. 87-88°C. It gave negative tests with Liebermann-Burchard reagent, potassium permanganate solution and bromine in carbon tetrachloride solution.

Spectroscopic data:

IR (KBr pellet) $\gamma_{max.}$ 3350, 2915, 2845, 1475, 1460, 1060, 735, 720 cm^{-1}

Analytical data: found: C 82.34 % H 14.48 %

calc. for $C_{30}H_{62}O$: C 82.11 % H 14.24 %

Table III

Column Chromatography of the Light Petroleum-Ether Extract

Eluting solvent	Eluted fractions*	Remark
Petroleum-ether	1-3	waxes
	4-5	-
5% ether	6-9	tarry oil
10 % ether	10-20	Compound I
		(120 mg., 0.00162 %)
20 % ether	21-28	tarry oil
40 % ether	29-31	tarry oil
	32-42	Compound II
		(478 mg., 0.00645 %)
ether	54-62	tarry oil and
		a little compound
methonol	63-75	tar

* About 500 $cm.^3$ of each fraction were collected

N.B. The percentage yields of the extracted compounds were based on the dried ground weight (7.6 kg.)

Preparation of Compound I Acetate

Compound I (50 mg.) was mixed with acetic anhydride (1 $cm.^3$) and a few drops of pyridine. The mixture was heated on a water steam bath, with occasional shaking, for two hours. The cold reaction mixture was then poured, with vigorous stirring, into 5 $cm.^3$ of ice water. Stirring was continued until the excess acetic anhydride was hydrolyzed. The crude precipitated acetate (65 mg.) was filtered off, washed thoroughly with cold water until no more pyridine was left and then purified by crystallization from petroleum-ether as a colourless amorphous solid (48 mg., 87,6 %), m.p. 74-75°C.

Spectroscopic data:

IR (KBr pellet) $\gamma_{max.}$ 1740, 1230, 1030 cm^{-1}

Preparation of Compound I Benzoate

0.05 g. (50 mg.) of the compound I, 2 $cm.^3$ of pyridine and 1.5 $cm.^3$ of redistilled benzoyl chloride were mixed in a 50 $cm.^3$ flask, and heated under reflux, with occasional shaking, for one and a half hours. A 25 $cm.^3$ of 5 % sodium hydrogen carbonate solution were added to the cold reaction mixture and cooled in an ice-bath until the precipitate solidified. The precipitate was filtered and washed with cold water until no more pyridine was left (65 mg.) and it was recrystallized from petroleum-ether for several times. The yield of pure myricyl benzoate, m.p. 67-68°C was 46 mg. (74 %).

Spectroscopic data:

IR (KBr pellet) $\gamma_{max.}$ 2950, 2870, 1725, 1608. 1585, 1465, 1290, 1130, 700 690 cm^{-1}.

Compound II

The combined eluted fractions No. 32 to No. 42 (Table III) on standing deposited colourless needle-shaped crystals which were collected and washed with a little cold light petroleum ether. It was recrystallized from methanol or petroleum ether as white needle-shaped crystals (478 mg.,0.00645 %). It gave a deep green colour with Liebermann-Burchard reagent, a positive reaction for unsaturated hydrocarbon by using potassium permanganate solution and bromine in carbon tetrachloride. It gave a precipitate on the addition of 0.5 % digitonin in 95 % ethanol. With chloroform as the developing solvent, compound II had an Rf value of 0.54 on TLC (silica gel G).

Spectroscopic data:

IR (KBr pellet) $\gamma_{max.}$ 3400-3200, 3100-300 1450-1350, 1060, 970 960, 830, 790 cm^{-1}

NMR ($CDCl_3$) δ 5.4 (1H, broad triplet, $>C=CH-CH_2-$), 5.14 (2, broad multiplet like structure, $>CH-CH=CH-CH<$), 3.3-3.8

(1H, broad multiplet, $>$CH-OH),

1.57 (1H, singlet, $>$CH-OH), 1.2

(3H, doublet, CH_3-CH$<$, J=6Hz.)

1 (3H, singlet, $CH_3-\overset{\|}{\underset{|}{C}}-$), 0.87

(3H, triplet, CH_3-CH_2-, J=6Hz.)

0.82 (6H, doublet, $\begin{matrix}CH_3\\CH_3\end{matrix}>$CH-, J-6Hz.)

0.7 (3H, singlet, $CH_3-\overset{|}{C}-$)

M.S. m/e M^+ 412, base peak 55, also major peak 397 ($M^+ - CH_3$), 394 ($M^+ - H_2O$) 273 (M^+ - R, R-side chain), 271 (M^+ - R - 2H), 255 (M^+ - R - H_2O) 253 (M^+ - R - 2H - H_2O), 213 (M^+ - R - H_2O - 42)

Analytical data: found: C 83.98 %, H 11.92 %

calc. for $C_{29}H_{48}O$: C 84.4 %, H 11.72 %

Preparation of Compound II Acetate

Compound II (100 mg.) was mixed with acetic anhydride ($2\ cm.^3$) and a few drops of pyridine. The reaction mixture was refluxed on a steam bath for four hours, with occasional shaking. The cooled solution was then poured, with vigorous stirring, into $30\ cm.^3$ of ice water. Stirring was continued until the excess acetic anhydride was hydrolyzed. The precipitated acetate (118 mg,) was collected by filtration, washed thoroughly with ice-cold water, and purified by crystallization from methanol or petroleum ether as colourless plates (102 mg., 92.7 %), m.p. 143-144°C.

Spectroscopic data:

IR (KBr pellet) $\gamma_{max.}$ 1742, 1270-1250 cm^{-1}

NMR ($CDCl_3$) δ 5.37 (1H, doublet, $>C=CH-CH_2-$), 5.1 (2H, triplet like structure, $>CH-CH=CH-CH<$), 4.9-4.3 (1H, broad multiplet, $>\underline{CH}-OAc$) 2.01 (3H, singlet, $\underline{CH_3}-\overset{O}{\overset{\|}{C}}-O$), 1.01 and 0.7 (6H, singlet, $CH_3-\overset{|}{\underset{|}{C}}-$), 1.03 (3H, doublet, $\underline{CH_3}-CH<$, J=6Hz.), 0.85 (6H, doublet, $\begin{matrix}CH_3\\CH_3\end{matrix}>CH-$, J=6Hz.), 0.84 (3H, triplet, CH_3-CH_2, J=6Hz.)

M.S. m/e M^+ 454 was not observed, base peak 81, and major peak at 394 (M^+-AcOH), 379 (M^+-AcOH-Me), 255 (M^+ - R - AcOH, R = side chain), 253 (M^+ - R - AcOH -2H), 213 (M^+ - R - AcOH - 42)

Hydrogenation of Compound II

Under Normal Pressure Condition

In a 100 $cm.^3$ suction flask were placed 100 mg. of compound II, 30 $cm.^3$ of absolute ethanol and 20 mg. of 10 % Pd/C as a catalyst. Hydrogen was introduced into the reaction flask and the solution was stirred with magnetic stirrer under normal pressure and temperature until there was no further change in volume of hydrogen, the mechanical stirring was continued for further two hours. The reaction product was filtered through two filter papers supported on a Buchner funnel, and the alcoholic solution was evaparated to dryness on a water bath. The residue (98 mg.) had m.p. 135-136°C, the m.p. was uneffected after crystallization from 95 % ethanol. It gave a negative test for unsaturation by using potassium permanganate solution and bromine in carbon tetrachloride.

Under Pressure Condition (2.06×10^5 newtons per square meters)

20 mg. of 10 % Pd/C catalyst were placed in the hydrogenation vessel and then a 100 mg. of compound II in 30 $cm.^3$ of absolute alcohol were introduced. The solution should completely cover the catalyst, the stopper was lubricated with an inert grease and inserted into the vessel. The hydrogen gas was introduced into the reaction mixture with mechanical shaker, under 2.06×10^5 $n/m.^2$ pressure at such a rate that the temperature did not rise above 25°C. The mixture was shaken for further four hours. The reaction product was then filtered through two filter papers supported on a Buchner funnel. The filtrate was evaporated to dryness on a water bath and the residue was purified by crystallization from 95 % ethanol as white colour plates (101 mg., 91.8 %), m.p. 135-136°C.

Spectroscopic data

IR	(KBr pellet)	$\gamma_{max.}$	3400-3200, 2915, 2880 1475, 1460, 1385, 1060 cm^{-1}
NMR	($CDCl_3$)	δ	3.55 (1H, broad multiplet >CH-OH), 1.63 (1H, singlet, >CH-OH), 0.84 (9H, doublet, CH_3-CH < , J = 6Hz.), 0.83 (3H, triplet, CH_3-CH_2-, J = 6Hz.)

M.S. m/e M^+ 416, base peak 81, and also the major peak 401 (M^+ - Me), 383 (M^+ - H_2O - Me), 275 (M^+ - R, R = side chain), 257 (M^+ - R - H_2O), 255 (M^+ - R - H_2O - 2H), and 215 (M^+ - R - H_2O - 42)

The Chloroform Fraction of O. diffusa

The chloroform fraction obtained from the crude ethanol fraction was extracted by partition between chloroform and water. After evaporating the chloroform in vacuo, the crude residue was preliminary tested with the reagents as followed. It gave red to deep violet colour with Shinoda's reagent, yellow colour with aqueous sodium hydroxide solution, and reddish-orange colour with concentrated sulphuric acid. The crude chloroform soluble fraction was then chromatographed on a standardized alumina 90 (activity II-III), a neutral alumina 90 (activity I), and a silica gel (size 70-325 mesh, ASTM) column, respectively. 40 % chloroform in petroleum ether was used as the initial eluting solvent and it was changed to chloroform by gradual introduction of the latter. Finally, the column was stripped with methanol. Each eluted fraction was monitored by TLC using concentrated sulphuric acid for detection.

The basic extract of crude chloroform soluble part was taken by dissolving in the ether solvent and then extracted by using 5 % sodium hydrogen carbonate, 20 % sodium carbonate, 1 % sodium hydroxide, and 5 % sodium hydroxide, respectively. Each basic extracted fraction was acidified with hydrochloric acid and then re-extracted with ether. Each ether re-extracted fraction, after being concentrated under reduced pressure, was checked by TLC plates using concentrated sulphuric acid as a spraying reagent.

The aqueous soluble part, after being equilibrated with chloroform, was concentrated under reduced pressure and then washed with methanol for several times. The precipitate was obtained as inorganic salts. The chemical test showed that it mainly consisted of sodium chloride and potassium chloride. The atomic absorption spectrum shows that it was chloride salts of sodium 28.9 %, potassium 15 %, magnesium 0.00425 %, manganese 0.0009 %, and traces amount of copper, cobalt, zinc, calcium and lead.