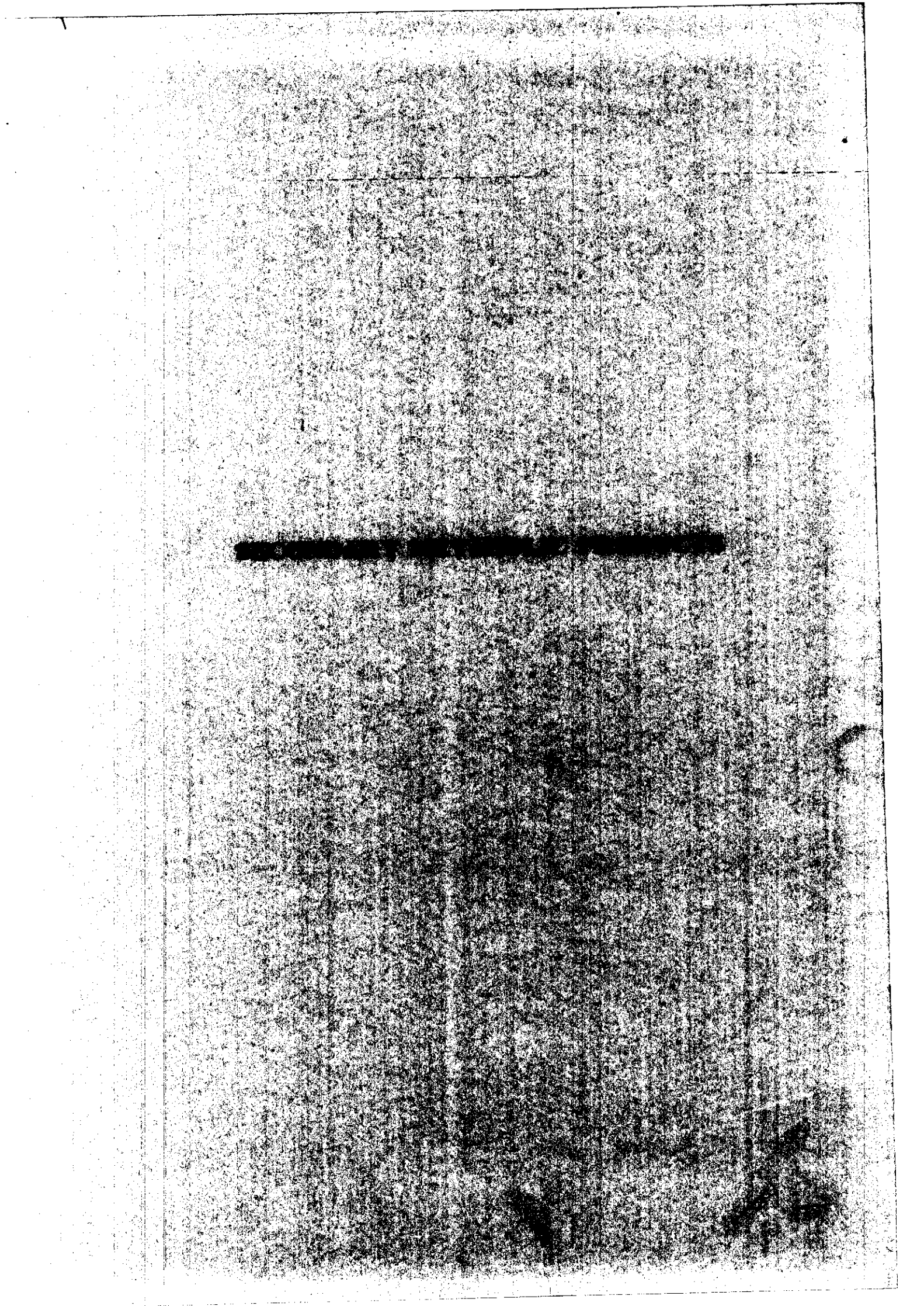


Secondary Metabolic Products

A- Preliminary Phytochemical Screening

- Preparation of the extract:

A known weight of the powdered air-dried plant material (leaves, stems and roots) obtained from each habitat was separately mixed with 50 ml of 80% ethyl alcohol, refluxed for twenty hours and filtered. The remaining powder was then washed several times with hot ethyl alcohol. Each of the obtained filtrate was concentrated under vacuum and then subjected to the following tests.

1- **Test of tannins:**

Few ml of the alcoholic extract of each sample was evaporated, each of the residue obtained was dissolved in few ml of distilled water. The presence of tannins was detected by addition of $FeCl_3$ or gelatin or NaCl solutions, where a greenish colour in case of $FeCl_3$ or white precipitate in case of gelatin and NaCl indicate the presence of tannins.

2- **Test for sterols** (Libermann-Burchard test):

According to Fieser and Fieser (1959) and Wall *et al.*

(1964), few ml of the alcoholic extract of each sample was evaporated and the resulted residue was dissolved in 2 ml chloroform and filtered. The filterate was then subjected to Libermann-Burchard test by adding one ml of acetic anhydride followed by one or two ml, dropped carefully down the side of the tube, of conc. H_2SO_4 to form a separate layer. The formation of red ring at the junction between the two layers confirms the presence of unsaturated sterols.

3- <u>Test for terpenes</u>: Solkowaski's reaction: Brieskorn (1961):

Few ml of the alcoholic extract of each sample was evaporated and the resulted residue was dissolved in chloroform, to each of which conc. H_2SO_4 was added slowly down the side of the test tube. The formation of yellow coloured ring that changes to bloody red colour indicates the presence of terpenes.

4- <u>Test for flavonoids:</u>

Five grams of each plant material (leaves, stems and roots) collected from the two habitats were defatted with petroleum ether. Each of the defatted plant material was extracted with ethyl alcohol. Test for flavonoids was carried out by adding conc. HCl dropwise to one ml of

alcoholic extract containing fragments of magnesium ribbon, Shinoda (1928). The formation of pink colour indicates the presence of flavonoids.

5- **Test for alkaloids:**

Few grams of the defatted plant material (leaves, stems and roots) collected from the two habitats were mixed with few mls of 28% ammonium hydroxide solution and about one gram of sodium carbonate. The mixture was then percolated with a mixture of ether-chloroform (3:1), then evaporated and the residue obtained was extracted with dilute HCl. Detection of alkaloids was carried out by testing the extract by treatment with Mayer's Wagner's and Dragendorff's reagents. The formation of precipitate or colour indicates the presence of alkaloids.

6- **Test for glycosides:** (Molish's test):

Few ml of each of the concentrated alcohol extract of leaves, stems and roots of *Kanahia laniflora* collected from the two habitats, were tested for glycosides using Molish's reagent. A violet colour appearing at the junction of the two layers indicates the presence of glycosides.

7- **Test for saponins:**

a) **Foam test:**

Ten ml of the alcoholic extracts of leaves, stems and roots of the two habitats were separately evaporated to dryness. The residue of each extract was dissolved in 4 ml of distilled water, then filtered. The filtrate was vigorously shaken where a voluminous forth was developed indicating the presence of saponins.

b) **Haemolysis:**

Few mls of both of the alcoholic extracts were separately evaporated to dryness, the remained residue was then dissolved in 2 mls of distilled water and the aliquot obtained was added to 1 ml of 1:40 suspension of erythrocytes in physiological saline solution, Wall *et al.*, (1964). Haemolysis was occurred indicating the presence of saponins.

8- **Test for resin:** (Fahmy, 1933)

Five grams of each of dry powdered plant material (leaves, stems, and roots) obtained from the two habitats were separately boiled with fifty ml of 25% alcohol on water bath for twenty minutes and filtered. On addition of two hundred ml of distilled water to the filtrate, a white precipitate indicates the presence of resins.

9- **Test for chlorides and sulphates:**

One gram of each dried powdered leaves stems and roots obtained from the two habitats was extracted with ten ml of hot distilled water, then filtered. Few ml of each water extract was subjected to test for chlorides and sulphates using silver nitrate and barium chloride solutions respectively. The formation of white precipitate indicates the presence of chlorides and sulphates respectively.

The results obtained in Table (16) indicated the presence of tannins, sterols, saponins, glycosides, resins, chlorides and sulphates in leaves, stems and roots of *K. laniflora*. Terpenes were detected in the leaves and stems but not in the roots. Meanwhile, flavonoids were detected in the leaves only alkaloids were not detected in any plant organ of plants grown in the two habitats.

Table (16) Preliminary chemical screening of leaves stems and roots of *Kanahia laniflora*.

Test	Leaves		Stems		Roots	
	R.H.	C.W.S.H.	R.H.	C.W.S.H.	R.H.	C.W.S.H.
Test for flavonoids	+	+	-	-	-	-
Test for terpenes	+	+	+	+	-	-
Test for tannins	+	+	+	+	+	+
Test for glycosides	+	+	+	+	+	+
Test for saponins	+	+	+	+	+	+
Test for resins	+	+	+	+	+	+
Test for alkaloids	-	-	-	-	-	-
Test for chlorides and sulphates	+	+	+	+	+	+

B- Investigation and identification of tannins separated from *Kanahia laniflora*:

1) Preparation of tannins:

A known weight of each of the air dried powdered leaves, stems and roots of *Kanahia laniflora* collected from the two habitats was defatted with petroleum ether (b.p. 40-60°C), then dried and extracted with hot water. The aqueous extract obtained was filtered, cooled and centrifuged. Tannins were precipitated from the clear aqueous filtrate with 5% gelatine solution and recovered by complexing with aqueous acetone solution.

2) Paper chromatographic investigation of tannins:

The aqueous extract was concentrated and spotted on Whatman No. 1 filter paper. The ascending one dimensional technique was adopted using N-butanol-acetic acid-water 4:1:5 V/V for 24 hours (Smith, 1962).

After complete development the chromatograms were dried at room temperature and detected with 0.1% ferric chloride solution (Roberts and Wood, 1951) where dark blue spot with R_f value 0.55 was detected.

3) **Purification of tannins:**

Following Boudet and Gadal (1965), the tannins acetone complex was purified by cation exchange resin (Dowex 50 H^+ and Dowex $2OH^-$) then evaporated in vacuum.The residue was amorphous, yellowish, soluble in water, methanol and ethanol but insoluble in benzene, chloroform, ether and petroleum ether.

The obtained aqueous extract was then subjected again to paper chromatographic analysis as mentined before where one spot with the same R_f value (0.55) was detected, Fig. (32).

4) **Hydrolysis of tannins:**

Tannins were hydrolysed in order to separate glycone part from aglycone one.

According to El-Sisi and Saleh (1964) one gram of each of the residue obtained after concentration of aqueous solution was refluxed sepaarately for 4 hours with 2.5% H_2SO_4. After cooling $Ba(OH)_2$ solution was added till neutralization was obtained. The solution was filtered and the filtrate was tested for the abscence of sulphate. The pure filtrate was shaken with 80% ethyl alcohol and evaporated to form a thick syrup. The latter was subjected

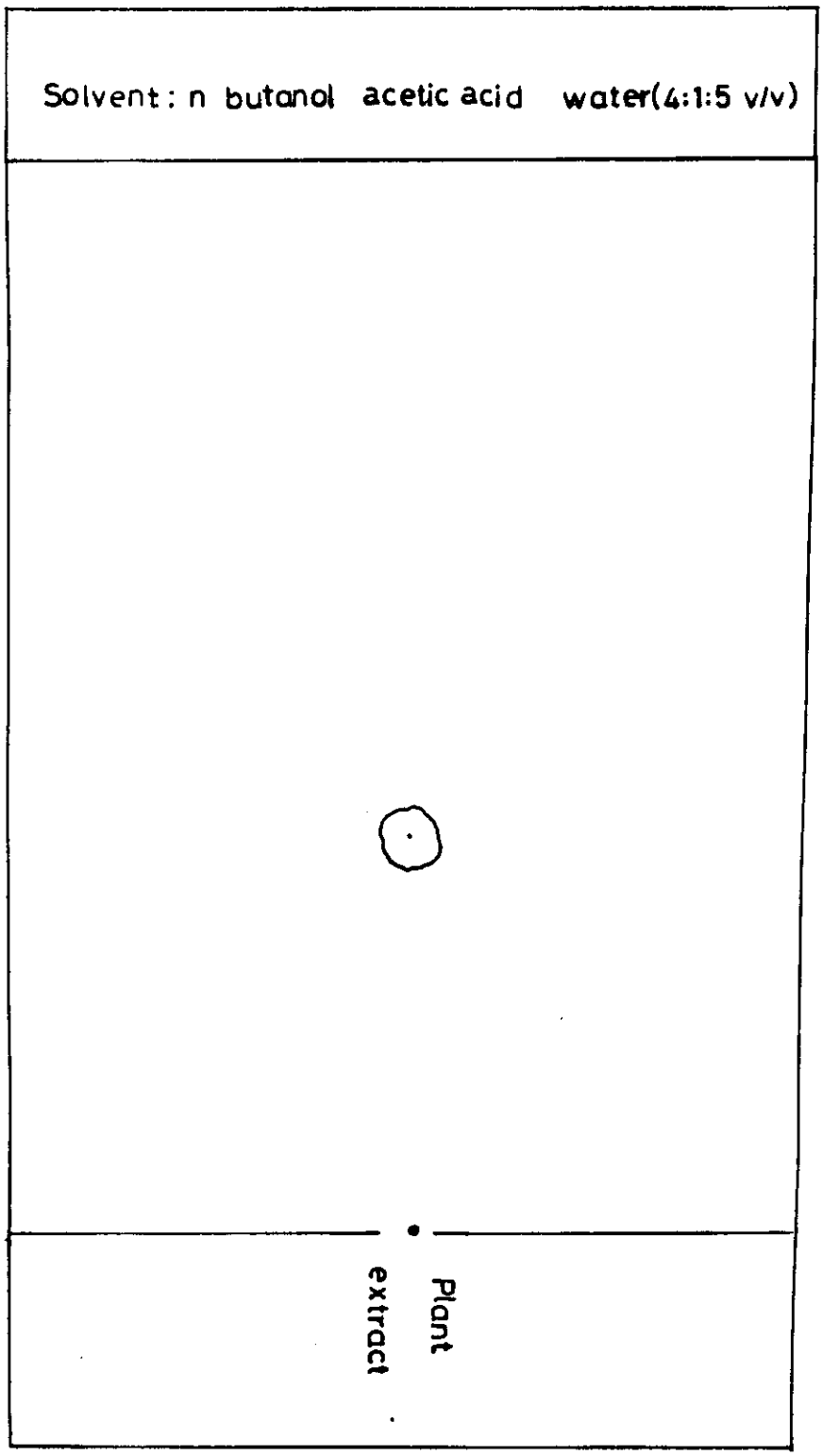


Fig .(32) : Ascending paper chromatogram tannin separated from water extract of Kanahia laniflora

to column chromatography using aluminium oxide as adsorbent.

Elution was carried out using 200 ml acetone (the eluent was yellow) follwed by 200 ml of ethanol where the eluent became colourless.

5) Chromatographic investigation of the obtained eluents:

Acetone and ethanol eluents were concentrated under reduced pressure, then subjected to paper chromatographic investigation.

The acetone extract was spotted on Watmann No. 1 filter paper as well as an authentic sample of gallic acid, while the alcohol extract was spotted on another chromatogram as well as authentic solution of glucose. The chromatograms were supported in an air tight jar which had an atmosphere saturated with the upper organic layer of the solvent system n-butanol-acetic acid-water (4:1:5 v/v) which was used as a mobile and stationary phase for 20 hours.

After development, the first chromatogram (acetone eluents) was dried in air and sprayed with ferric chloride solution which gave dark blue spot with R_f value (0.682) identical to that of gallic acid. This may indicate the

presence of gallic acid in the acetone eluents of the leaves, stems and roots of *Kanahia laniflora* plants. The second chromatogram (alcohol eluents) was sprayed with aniline phthalate reagent to detect the presence of sugars, then dried in an oven at 105°C where brown spot with R_f value similar to authentic glucose was detected, Fig. (33).

From the above investigation, it is clear that tannin contents of the leaves, stems and roots of *Kanahia laniflora* plant may be of gallotannin groups. This can be confirmed by studying the properties of the separated aglycone part (gallic acid).

6) **Properties of the separated gallic acid:**

Crystallization followed by purification of acetone eluents obtained before was carried out from acetone where needle shaped crystals were obtained. These were soluble in alcohol and acetone, while sparingly soluble in water and ether.

The separated compound had a melting point of 218°C undepressed on admixture with authentic gallic acid. It gave blue black precipitate with ferric chloride solution and dark red colour with sodium or potassium hydroxide solutions.

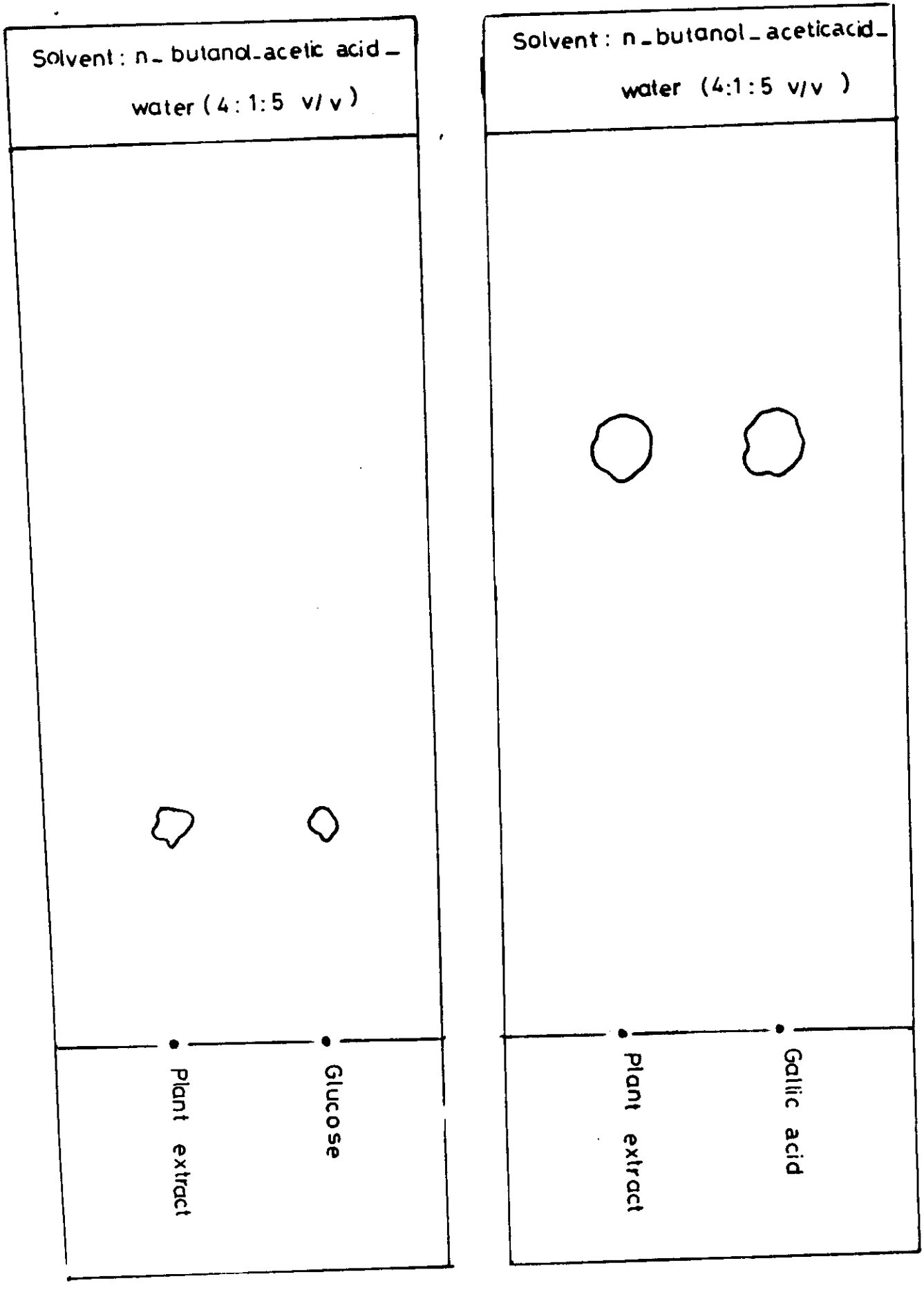


Fig.(33): Ascending paper chromatograms for glucose and gallic acid present in tannin extract of Kanahia laniflora

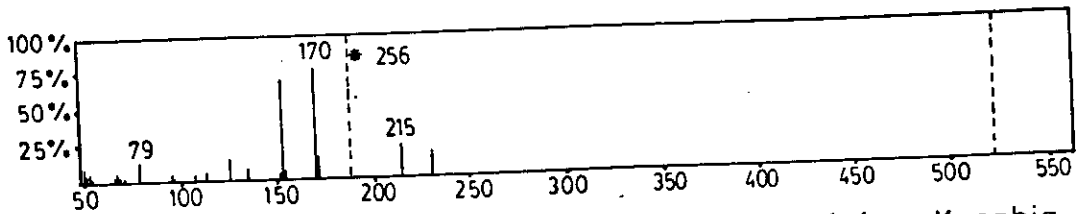
Elemental analysis of the isolated compound reveals only (C, 52.2% and H, 6.1%).

Mass spectrum analysis (Fig. 34-a) shows molecular ion (M^+ = 230) and accordingly the moleuclar formula is $C_{10}H_{14}O_6$.

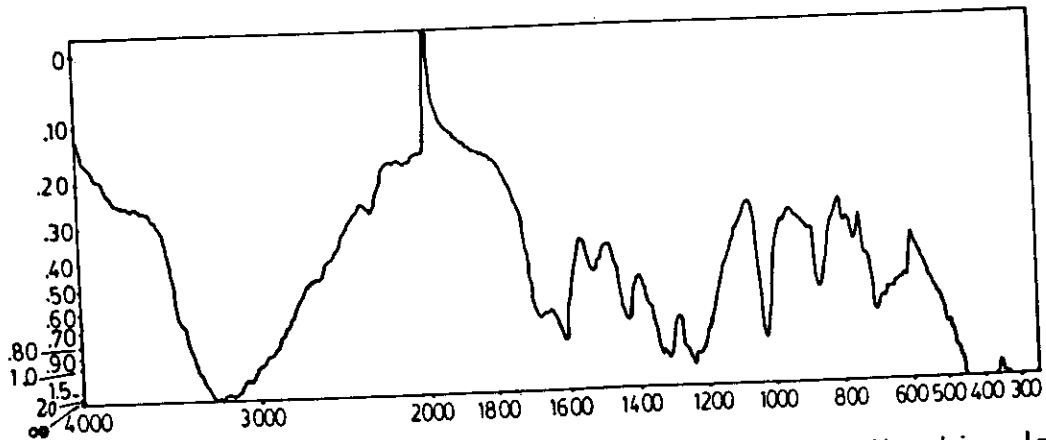
The infra red spectrum (Fig. 34-b) shows absorption bands indicating the presence of chelated OH group of carboxylic acid at a range of 3280-3150 cm^{-1}, aromatic and aliphatic c-H at 3050 and 2850 cm^{-1}, carbonyl group of acid at 1700 cm^{-1} and C=C for aromatic system at 1600 cm^{-1}.

The ultra violet spectrum analysis (Fig. 34-c) shows max at 220 and 270 which support the aromatic nucleus.

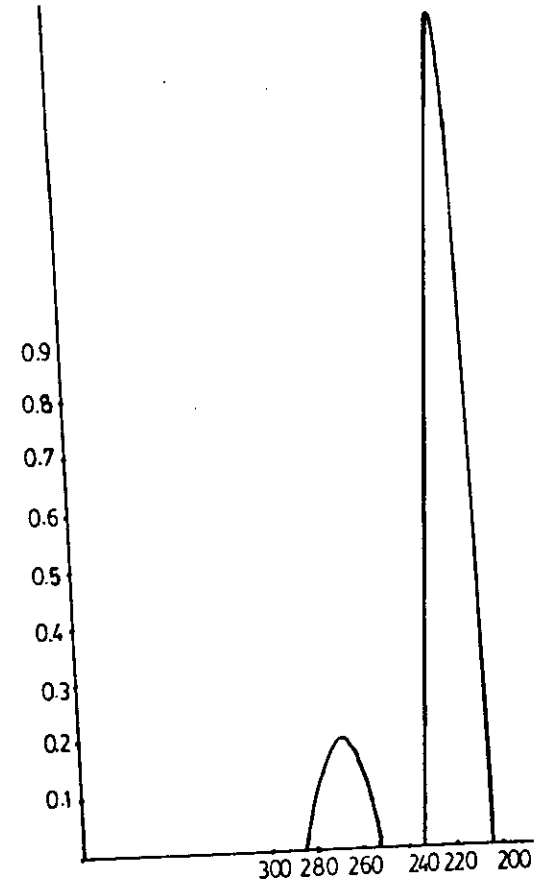
Mass spectrum shows the molecular ion peak at (M^+= 230) with percentage abundance (20%). This ion loss methyl group (M^+-15) to give ion 215 with percentage abundance (40%). The base peak appears at (70 and 100%) corresponding to gallic acid nucleus. Also there are some peaks at 153 (80%), 126 (16%) and 79 (18%) for aromatic moeity. So the probable structure of this compound may be as follows:



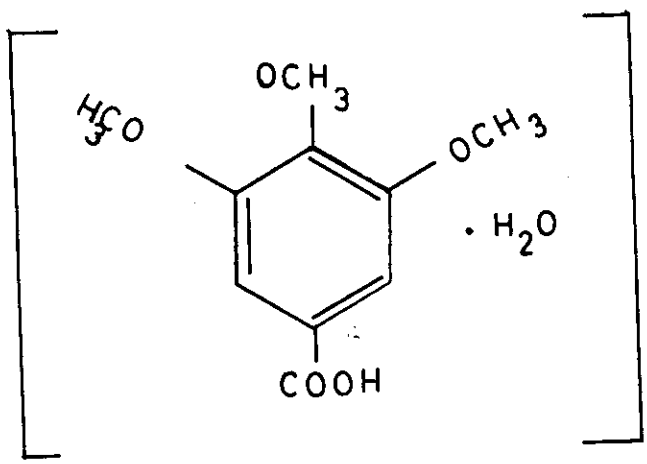
Fig(34a) Mass spectrum analysis of Tannin separated from Kanahia laniflora



Fig(34-b) Infra red spectrum of Tannin separated from Kanahia laniflora



Fig(34-c) UV absorpation spectrum of Tannin separated from Kanahia laniflora



7) **Quantitative estimation of gallotannin:**

Following the method described at Ph. Helv. V. (1962) the tannin content of the leaves, stems and roots of *Kanahia laniflora* was determined as follows:

1. Five grams of each of the air dried powdered leaves, stems and roots of the plants collected from the two habitats were refluxed with 250 ml of distilled water for ten minutes, then boiled for half an hour with continuous shaking. After cooling at room temperature, it ws then filtered and the filtrate obtained was then centrifugated. Fifty mls of the clear filtrate were placed in a previously wieghed crucible, placed in an oven at 105°C until complete evaporation of the solution and reweighed. The weight

of the residue was determined (T_1).

2. Six grams of skin powder were added to eighty ml of the main filtrate with continuously shaking for half an hour and filtered. Fifty mls of the obtained filterate were evaporated at 105°c till constant weight (A_2). The residue was burnt at 650°C in muffle furnace for two hours and weighed to constant weight (T_2).

The percentage of gallotannins was determined according to the following equation:

$$\% \text{ of gallotannin content} = \frac{500\ (T_1 - A_1 - T_2 + A_2 + H)}{P}$$

where;

H = the difference in weight of fifty mls of the filtrate at 105°C and the weight at 650°C.

P = weight of the powder.

the results obtained revealed that the percentage of gallotannins was 6.11 and 8.9 in case of leaves, 9.9 and 11.8 in case of the stem and 8.3 and 8.92 in the roots of plants of rocky and coarse wadi sediments habitats respectively.

C) Investigation and identification of saponins in *Kanahia laniflora* leaves:

The phytochemical screening of the leaves of *Kanahia laniflora* revealed the presence of saponins. Consequently investigation of saponins become indispensable so as to annonate such compound of pharmaceutical value.

1- Extraction of saponins:

A known weight of each of the defatted powdered leaves of plants collected from the two habitats was extracted with 80% ethanol in soxhlet apparatus for eight hours. Lead acetate solution was added and filtered. The obtianed residue was suspended in 50% ethanol, then current of hydrogen sulphide gas was passed to precipitate the excess of lead (Pb^{++}) ions, and filtered. The filtrate was boiled to get rid of the excess gas, then evaporated unde reduced pressure till dryness. Each of the residue obtained (9.75 and 8.68 gm from plants of rocky and coarse wadi sediments habitats respectively) was dissolved in the least volume 50 ml of hot absolute alcohol, then poured into cold acetone with continuous stirring for one hour where yellow precipitate was obtained by filteration. Each of the obtained precipitate was separately extracted several times with absolute ethyl alcohol. For each extract, the combined solution was filtered, concentrated

and each of the residue obtained was kept for further investigation.

2- Chromatographic investigation of the separated saponins:

i- Paper chromatographic investigation:

Paper chromatographic investigation of each of the residue obtained after dissolving in ethanol was carried out using Whatman No. 1 filter paper and the solvent system n-butanol-acetic acid-water (4:1:1.5 v/v). The solvent used was allowed to run for eighteen hours. After complete development the chromatograms were air dried and sprayed with antimony trichloride reagent (Huneck, 1962), where three spots with R_f values 0.57, 0.42 and 0.13 were obtained.

ii- Thin layer chromatographic investigation:

The concentrated absolute alcohol extract was spotted on the precoated silica gel G plate, developed by the solvent system n-butanol-acetic acid-water (4:1:1 v/v). After complete development, the plates were raised, dried in air and sprayed with antimony trichloride reagent where three spots were also detectd, Fig. (35 a and b).

In either paper and T.L.C. technique spot No. 1 was the major one.

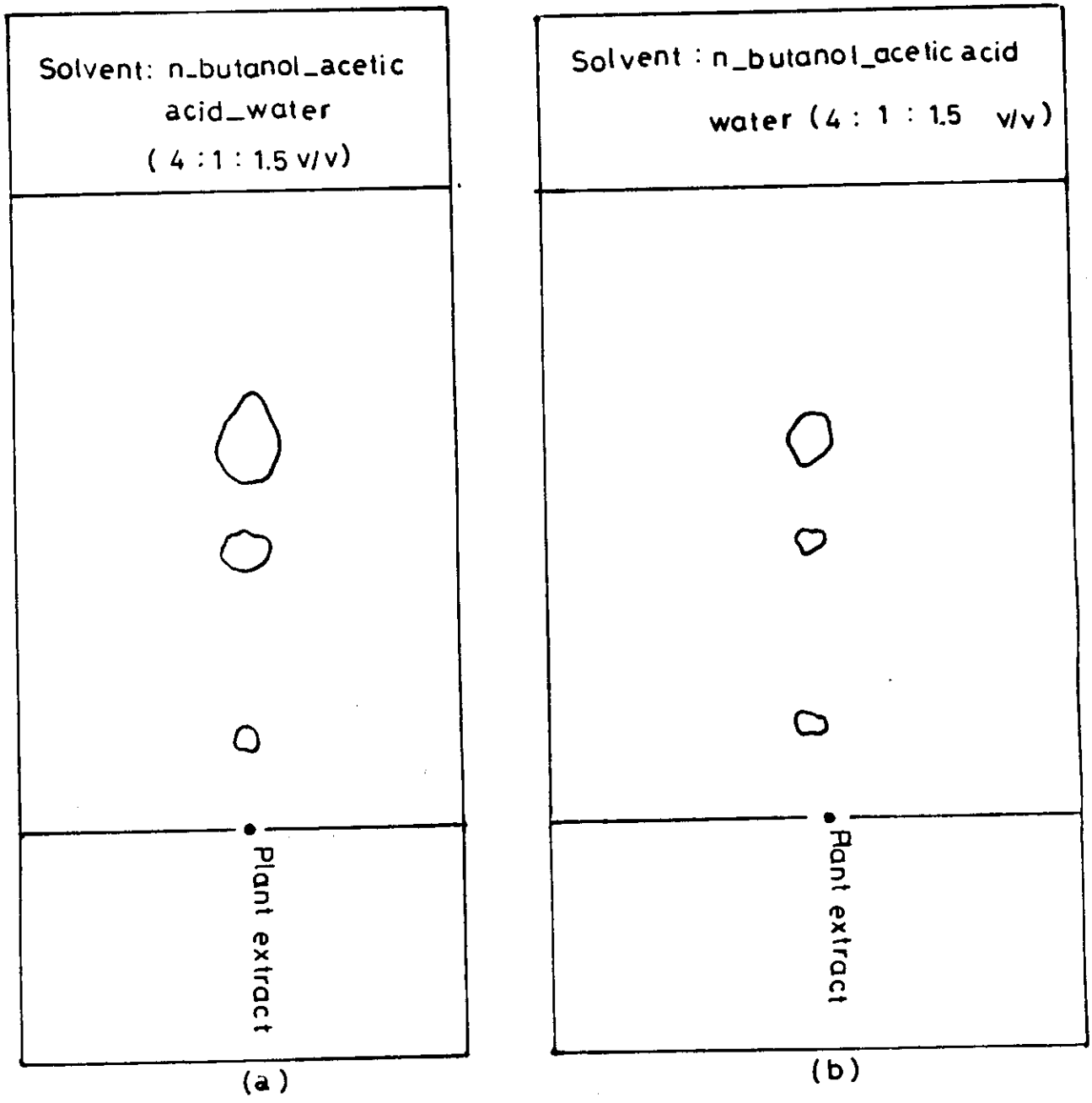


Fig.(35_a , b) Paper chromatogram(a) and thin layer chromatogram(b) for the separated saponin from Kanahia laniflora

iii- Column chromatographic separation of saponins:

Each of the crude saponin obtained was dissolved in absolute ehtanol, concentrated and subjected to separate by using column chromatography (column 50 cm long and 2 cm diameter). The extract was added on top of a column. Elution was carried successvely using 50 ml of 80% ethyl alcohol which became brown in colour, followed by 120 ml of 80% ethyl alcohol where greyish solution was obtained. With another 250 ml of 80% ethyl alcohol the eluent was grey in colour and proved to contain the major saponin fraction, i.e. present with high amount in both extracted fractions from the plant of the two habitats.

The fraction was concentrated and dried under vacuum where an amorphous substance was obtained. On crystallization from ethanol followed by purification a pale yellow powdered material was obtained. It gave positive results for either foam, haemolysis and Molish's tests and negative result with Fehling's solution.

3- Hydrolysis of the isolated saponin:

A known weight of each of the separated saponin was hydrolysed separatly by adding 5% H_2SO_4 and on a water bath for five hours and the solution was cooled. Barium hydroxide solution was added for neutralization followed by

filteration. The filtrate was then shaken several times with distilled ethyl acetate using a separating funnel. The ethyl acetate phase contained the aglycone part of the separated saponin, while the aqueous layer phase contained the glycone.

4- **Identification of the glycone fraction:**

After separation of the aglycone part, the remaining aqueous solution was concentrated and subjected to paper chromatographic analysis using Whatman No. 1 filter paper and the solvent system n-butanol-acetic acid-water (4:1:1.5 v/v) for twenty hours.

The chromatogram was revealed by spraying with aniline phthalate, then dried in air and placed in oven at 105°C for few minutes,then replaced for five mintues at 120°C. The detected spots were identified as glucose, galactose and fructose (Fig. 36-a).

5- **Identification of the aglycone fraction:**

The obtained aglycone fraction was then dried over anhydrous sodium sulphate, concentrated and subjected for thin layer chromatographic investigation using the solvent system n-butanol-acetic acid-water (4:1:1 v/v) and phosphomolybdic acid in 95% ethanol as detection reagent

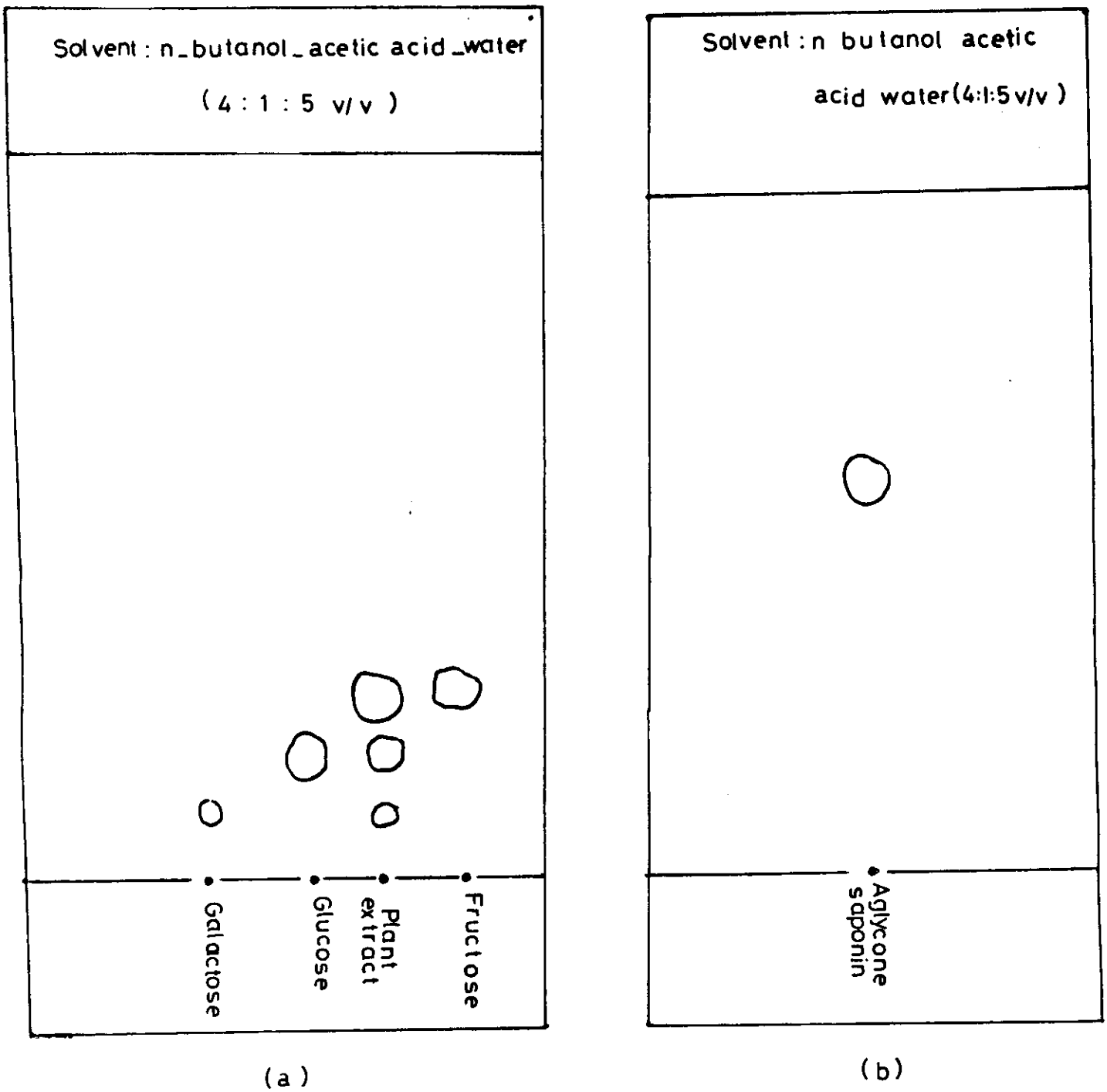


Fig.(36_a&b) Chromatograms for glycone(a) and aglycone(b) fractions of the separated saponin from Kanahia laniflora.

where one spot with R_f value 0.57 was detected, (Fig. 36-b).

The process of hydrolysis was repeated using large quantities of the main saponin. All the amount of aglycone part was crystallized form ethanol and then subjected to different analysis.

6- Properties of the aglycone (sapogenin):

The separated sapogenin was soluble in methanol, 80% ethanol and isopropanol, but insoluble in petroleum ether, ether, chloroform and acetone with melting point 247°C.

Analysis of the separated compound indicates that it contained neither nitrogen nor sulphur, phosphorus and halogen.

Elemental analysis of the separated sapogenin reveals the following percentages (C; 47.25% and H; 9.0%).

Mass spectrum, Fig (37-a) shows molecular ion at ($M^{\cdot+}$ 426) and so the molecular formula will be $C_{27}H_{38}O_4$.

The infera red spectrum (Fig. 37-b) shows absorption bands indicating the presence of hydroxyl groups at a range of 3380 to 3340 cm^{-1}, aliphatic C-H at 2900 cm^{-1} and C=C at 1600 cm^{-1}.

The ultra violet structure shows λ max at 210 nm which supports the absence of diene system or aromatic

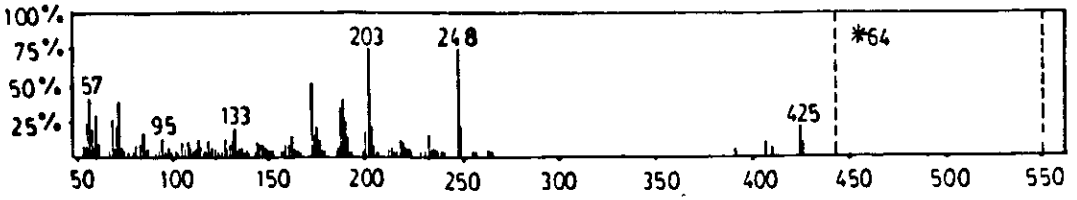


Fig (37-a) Mass spectrum analysis of Saponin separated from Kanahia laniflora

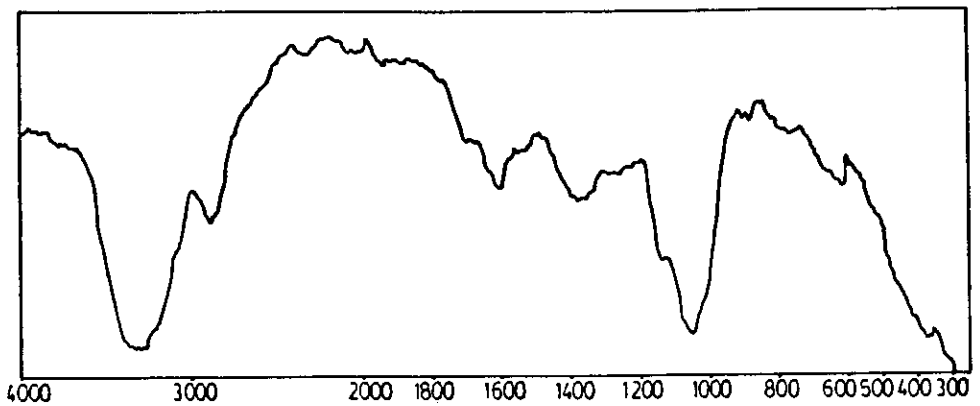


Fig (37-b) Infra red spectrum of Saponin separated from Kanahia laniflora

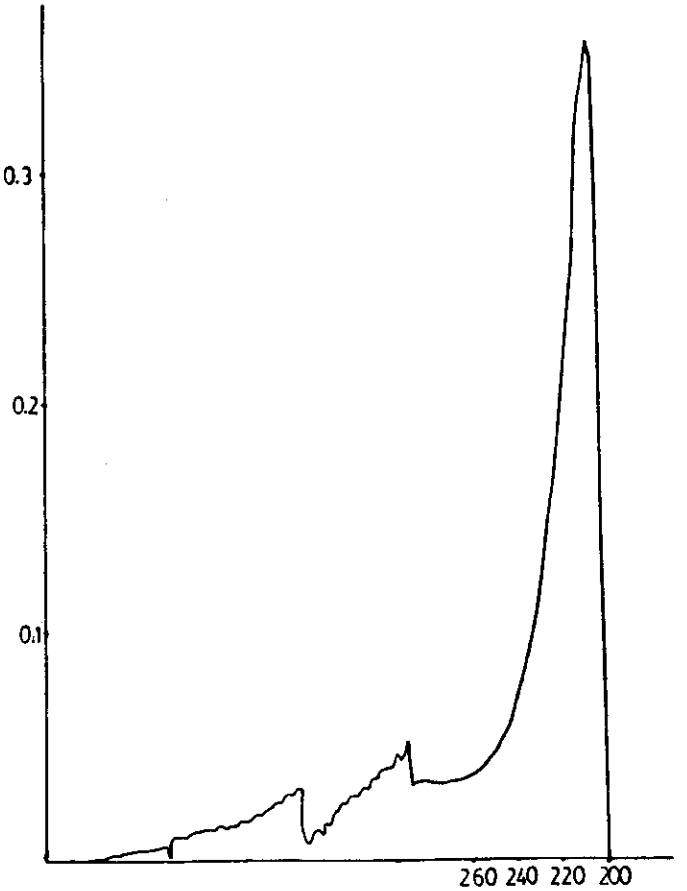


Fig (37-c) UV absorpation spectrum of Saponin separated from Kanahia laniflora

moeity (see Fig. 37-c).

Mass spectrum analysis (Fig. 37-a) shows molecular ion peaks at ($M^{\cdot+}$ = 426) and ($M^{\cdot+}$ −1 = 425) with percentage abundance as shown in Table (17).

Table (17) m/z values and percentage abundance for saponin separated from the leaves of *Kanahia laniflora*.

m/z	% abundance	m/z	% abundance
426	17	188	60
425	25	185	17
248	100	178	25
247	13	133	30
221	18	95	20
203	98	57	60

The molecular ion (M^{+} = 426) undergoes fragmentation to two ions. The former with m/z = 248 which is the base peak (100%) while the latter (m/z= 178) with percentage abundance 25%. The fragmentation of this compound is illustrated by chart (1).

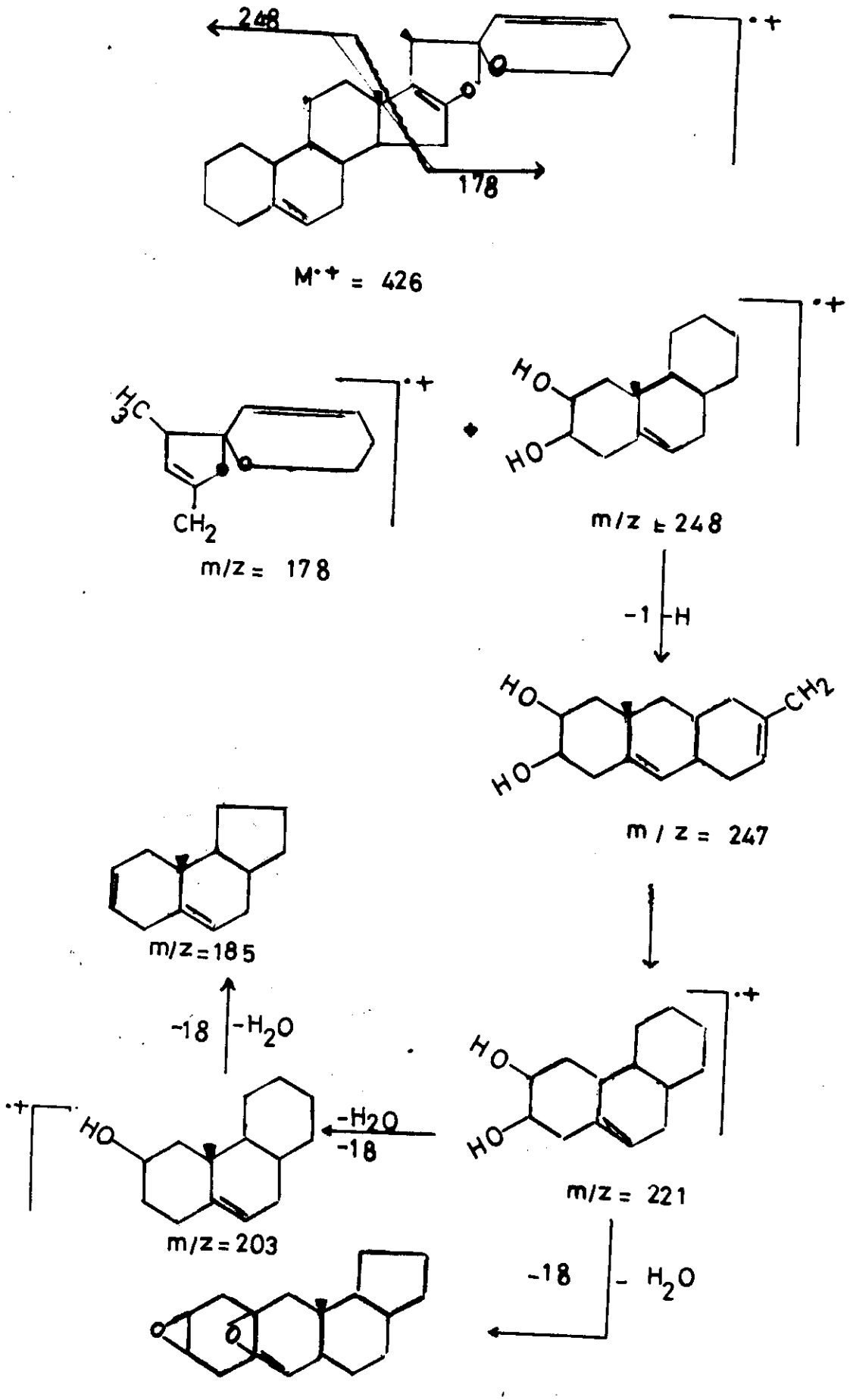


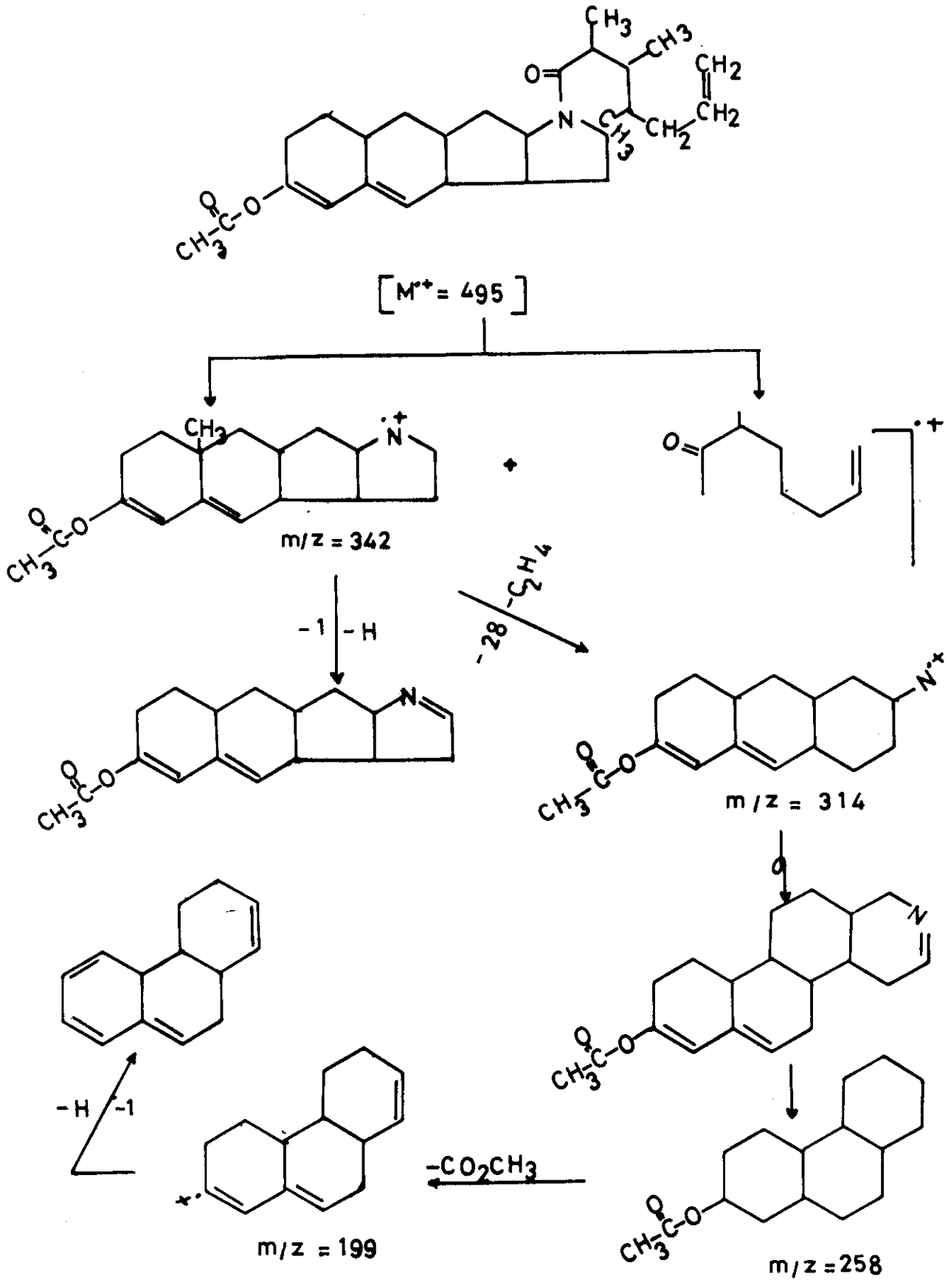
Chart (1) Structure of the separated sapogenin

Mass spectrum analysis shows molecular ion ($M^{\cdot+}-1$ = 494) which undergoes fragmentation via two routes, the first route suffers subsequent degradation of the side chain (c.f. Chart 2 and Table 18) to give ions (m/z's 454, 426, 398, 370 and 342) followed by degradation of cyclic skeleton to give ions (m/z's 342, 341, 314, 258, 199 and 198) respectively.

The second route of fragmentation is the side chain (m/z = 153) and the cyclic skeleton fragment as shown in the Chart (2) and Table (18).

Table (18) m/z values and percentage abundance of compound "A" separated from the leaves of *Kanahia laniflora*.

m/z	% abundance	m/z	% abundance
494	10	358	25
454	22	199	6
426	60	198	5
398	36	153	12
370	70	139	20
342	100	97	70
341	100	69	22
314	22	57	60



Chart(2) Structure of compound (A) separated from Kanahia laniflora

2- **Separation of compound "B":**

The residue remained from the decolorized methylene chloride was dissolved in methanol and the solution poured in 1% NaCl solution. The suspension formed was extracted with ethanol and methylene chloride (1:1 v/v) and recrystallized from methanol where a white crystalline compound with melting point 210°C was obtianed. It gave positive reaction with 14% anizaldehyde in acetone indicating the cardinolide characteristics.

- Properties of the separated compound "B":

The micro-analysis indicates that this compound consists of C; 76.8, H; 77% and N; 4.7%.

Mass spectrum Fig. (39-a) shows molecular ion (M^+ = 297) and accordingly the molecular formula will be C_{19} H_{23} NO_2.

The infra red spectrum Fig. (39-b) shows bands of phenolic OH, at 3600 cm^{-1}, aliphatic and aromatic C-H, 3030-3000 cm^{-1}.

The ultraviolet spectrum Fig. (39-c) shows absorption bands at λ max 208 and 284 which indicate the presence of aromatic system.

Mass spectrum Fig. (39-a) shows molecular ion $M^{\cdot+}$ = 297 and $M^{\cdot+}-1$ = 296 with percentage abundance as shown in Table (19), these too lose methyl radical to give ions

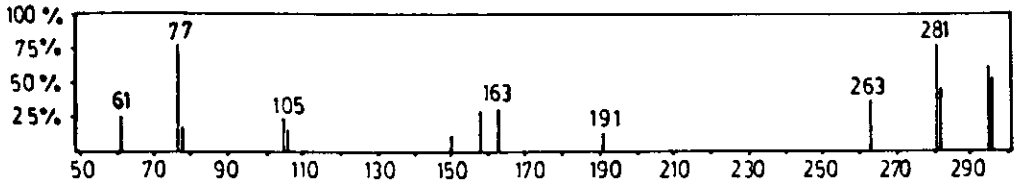


Fig.(39-a) Mass spectrum analysis of the compound (B) separated from Kanahia laniflora

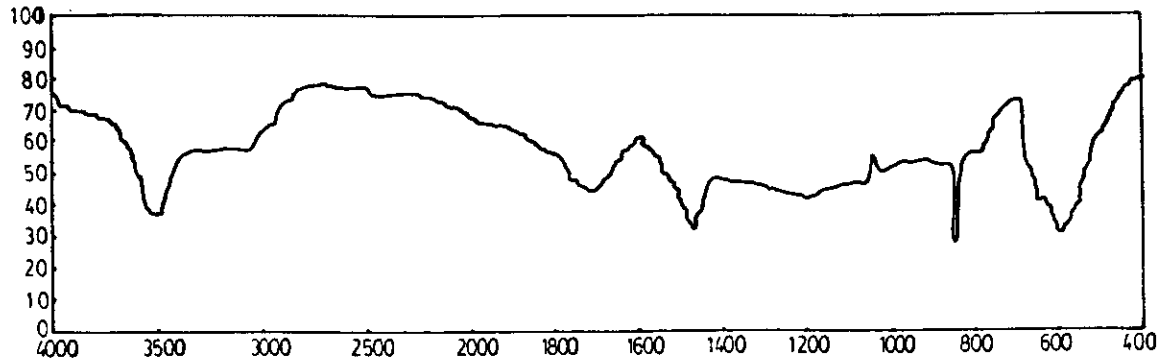


Fig.(39-b) Infra red spectrum of compound (B) separated from Kanahia laniflora

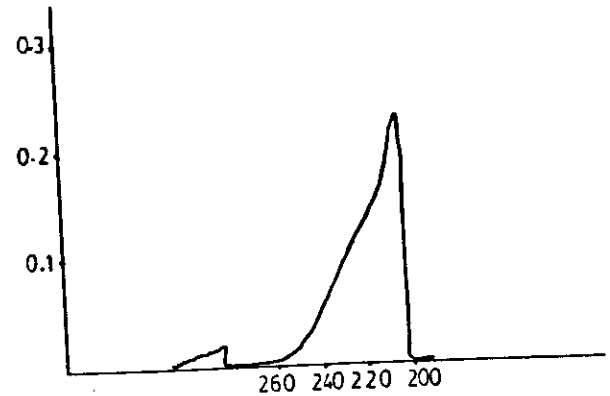


Fig.(39-c) UV absorpation spectrum of compound (B) separated from Kanahia laniflora

with m/z's = 280 and 281 respectively.

Table (19) m/z values and percentage abundance of compound "B" separated from the leaves of *Kanahia laniflora*.

m/z	% abundance	m/z	% abundance
297	75	158	20
296	75	106	20
282	60	105	35
281	100	78	28
263	50	77	98
191	20	61	30
163	40	-	-

The molecular ion (M^+ =297) undergoes fragmentation to two ions (m/z's 191 and 161). Both of these ions suffered from another usual fragmentation for seven membered ring and phenolic compounds to give ions 163, 105 and 77 respectively (see Chart 3).

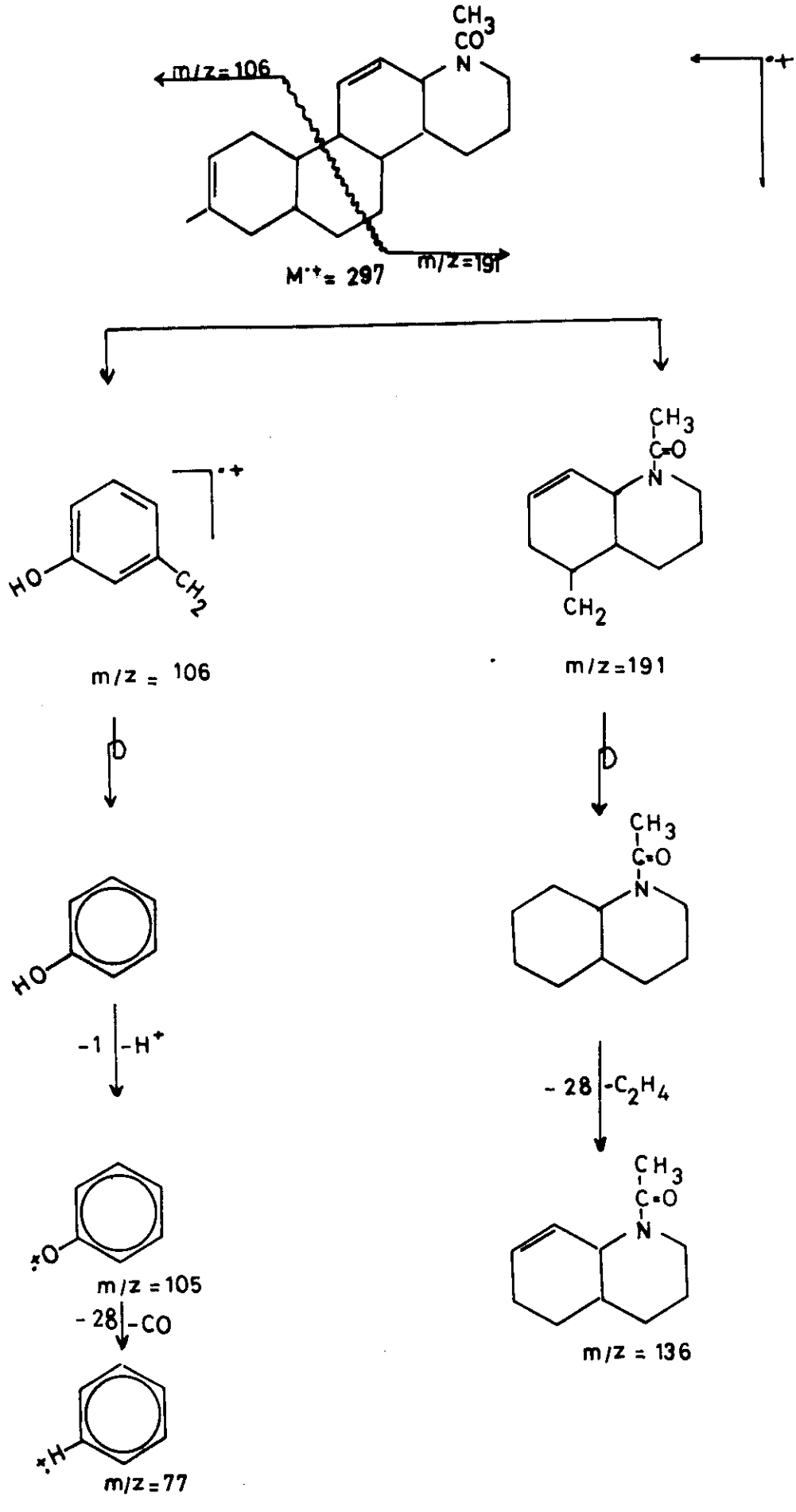


Chart (3): Structure Of compound (B) separated from Kanahia laniflora

3- **Separation of compound "C":**

The methylene chloride remained was dissolved in chloroform and poured onto the top of column chromatography containing silica gel G for column. Elution was carried out using 0.5% methanol in chloroform followed by 0.1% methanol and left to evaporate at room temperature to a very small volume. cold ethanol-methylene chloride (1:1 v/v) was added where an amorphous substance was obtained. Crystallization followed by purification processes were carried out where a white powdered substance with melting point 203°C was obtained.

- **Properties of the separated compound "C":**

The microanalysis indicated that the compound consists of C; 72%, H; 7.7%.

Mass spectrum analysis Fig. (40-a) revelaed molecular weight= 482. Accordingly the molecular formula is $C_{29}H_{38}O_6$.

Infra red spectrum Fig. (40-b) indicates the presence of C-H aliphatic at 2900 and 2830 cm^{-1}, three carbonyl groups at 1800 cm^{-1} for carbonyl of lactone, at 1730 cm^{-1} for carbonyl of saturated ester (acetoxy ester) and at 1720 cm^{-1} for carbonyl of unsaturated ester. Also it showed C=C at 1600 cm^{-1}.

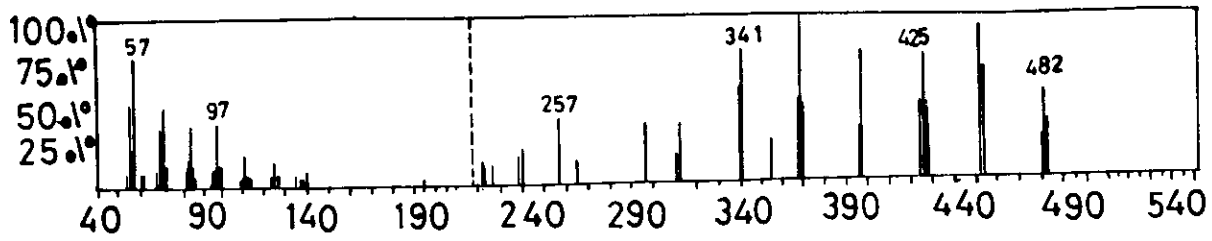


Fig.(40_a) Mass spectrum of compound(C) separated from Kanahia laniflora

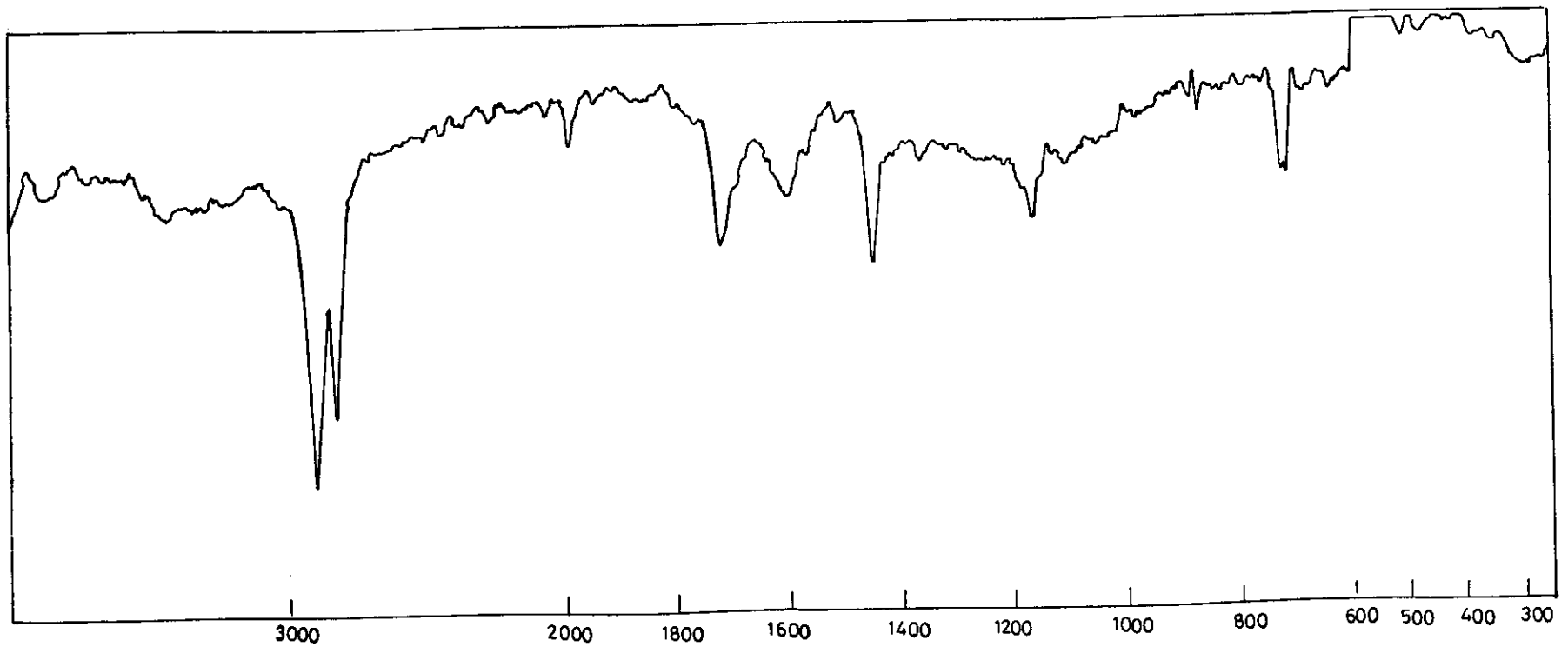


Fig.(40_b) Infra red spectrum of compound(C) separated from Kanahia laniflora

Mass spectrum showed molecular ion ($M^{\cdot+}$=482). This molecular ion undergoes fragmentation via three routes (C.F. Fig. 39a, Table 20 and Chart 4).

Route (A)

The molecular ion ($M^{\cdot+}$=482) loses two hydrogen atoms to give ion (m/z= 480), this ion fragmented by two ways. The frist is to give ions with m/z= 257 and m/z= 223. The latter ion (m/z= 223) loses carbon monoxide followed by acetyl group giving ions with m/z= 195 and m/z= 152 respectively. The second way of fragmentation of the ion (m/z=480) takes place according to Mclefferty rearrangement to give ion (m/z= 426) and butadiene (m/z= 54).

Route B:

The moleuclar ion ($M^{>+}$=482) simultaneously loses carbon monoxide and one molecule of formaldhyde giving ions with m/z's= 454 and 424 respectively.

Route C:

The molecular ion ($M^{\cdot+}$= 482) loses C_5H_9Coo group to give ion the base peak at (m/z= 369, 100%). This ion loses carbon monoxide to give ion (m/z= 341). This ion loses hydrogen atom followed by acetyl group to give ions with (m/z= 340 and 297) respectively.

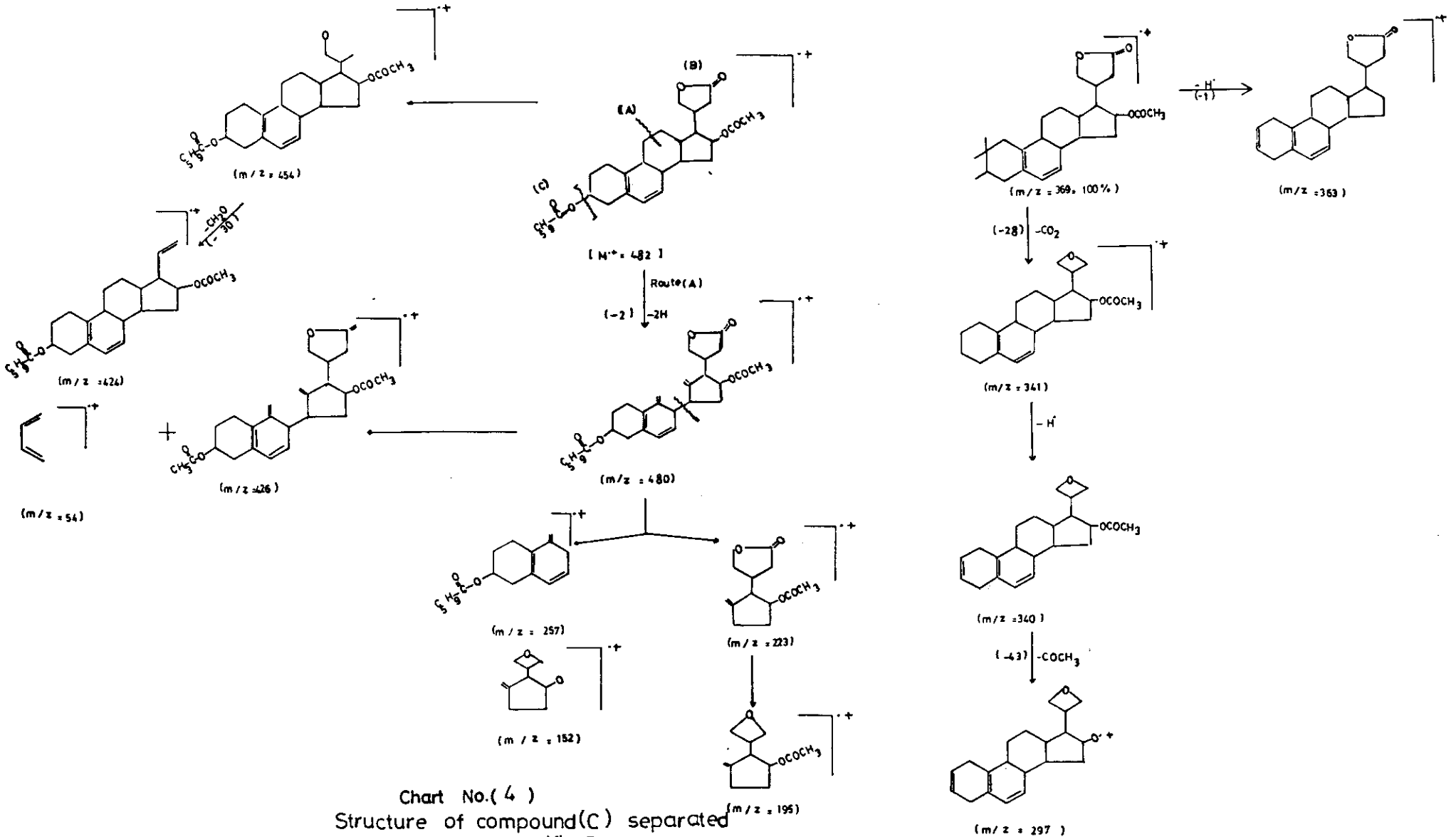


Chart No.(4)
Structure of compound(C) separated from Kanahia laniflora

Table (20) m/z values and percentage abundance of compound "C" separated from the leaves of *Kanahia laniflora*.

m/z	% abundance	m/z	% abundance
482	50	340	60
480	30	297	33
454	95	257	40
426	48	223	12
424	80	195	3
369	100	152	3
368	45	114	4
341	85	-	-