Chapter 4.

Isolation and characterization of antibacterial compounds from *Terminalia sericea* leaves

4.1 Problem statement and aim

After having found the best extractant and selected *T.sericea* as the plant of choice, the aim in this chapter was to isolate the antibacterial compound(s) using bioassay-guided fractionation and to characterize the isolated compounds.

4.2 Materials and Methods

4.2.1 Extraction

The plant material was extracted by placing 500 g finely powdered leaf material in c. 2 *L* of acetone to fully immerse and wet the plant powder in a 2.5 *L* glass container with a closeable lid. The container was then vigorously shaken for 30 minutes on a Labotec shaking machine. The mixture was left to settle and the clear liquid decanted and filtered through a Büchner funnel into a clean container. The process was repeated three times on the same plant material and the filtrate combined. The extract was reduced to dryness using a rotary evaporator (Büchi, Germany) under reduced pressure at 50°C.

4.2.2 Group separation

The solid and liquid phases prior to liquid-liquid extraction was separated by vacuum filtration and centrifugation to ensure that no particulate matter was introduced in the separatory funnels.

Separation was undertaken with immiscible solvents to fractionate compounds with different polarities. The method employed was that developed by the National Cancer Institute and applied in the analysis of *Anthocleista grandiflora* by Eloff (1998a).

The group separation process followed is described below and illustrated in Figure 4.1.

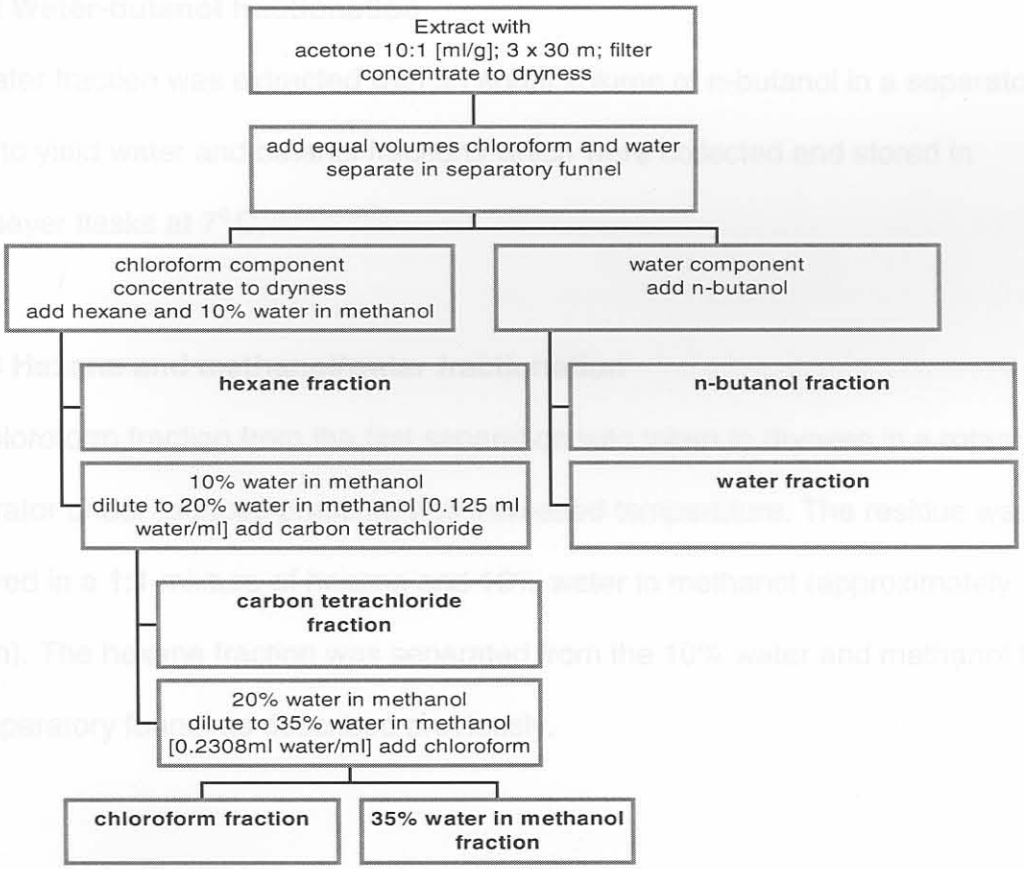

Fig. 4.1. Solvent-solvent fractionation process

4.2.2.1. Chloroform-water fractionation

Equal parts of chloroform (added first) and water was added to the 24.35 g of dried acetone extract. The minimum volume (about 100 ml) necessary to dissolve the extract was used, and after thorough mixing, the contents were poured into a separation funnel. Care was taken not to shake the contents excessively to avoid emulsions forming. The components were left to separate, resulting in the water fraction on top and the heavier chloroform fraction at the bottom. The fractions were collected separately in Erlenmeyer flasks that were then sealed with aluminium foil.

4.2.2.2 Water-butanol fractionation

The water fraction was extracted with an equal volume of n-butanol in a separatory funnel to yield water and butanol fractions which were collected and stored in Erlenmeyer flasks at 7° C.

4.2.2.3 Hexane and methanol/water fractionation

The chloroform fraction from the first separation was taken to dryness in a rotary evaporator under reduced pressure and increased temperature. The residue was then dissolved in a 1:1 mixture of hexane and 10% water in methanol (approximately 100 ml of each). The hexane fraction was separated from the 10% water and methanol fraction in a separatory funnel as described previously.

4.2.2.4 Carbon tetrachloride and methanol/water fractionation

The 10% water in methanol fraction was placed in a separatory funnel and further diluted to 20% water in methanol by adding 0.125 ml of water for every ml of the 10% water in methanol fraction. An equal volume of carbon tetrachloride was then added to obtain a carbon tetrachloride fraction and a methanol/water fraction after separation.

4.2.2.5 Chloroform and methanol/water fractionation

The 20% water in methanol fraction was further diluted to 35% water in methanol by adding 0.23 ml of water for every ml of the 20% water in methanol fraction. An equal volume of chloroform was added to the mixture, which was then partitioned to obtain a chloroform fraction and a 35% water in methanol fraction.

In all of the above separation procedures, the upper phase was re-extracted with fresh lower phase solvent two or three times, using about 10% of the original lower-phase volume, to ensure adequate separation. The fractions resulting from the solvent-solvent extraction process were concentrated by rotary evaporation. Assaying the components was the next important step in identifying the antibacterial active fractions. Following this, the extracts were subjected to TLC as described in 2.2.3 and bioautography to localize the antibacterial activity.

4.2.3 Vacuum liquid chromatography (VLC)

I decided to investigate VLC for large-scale preliminary fractionation of extracts as scaling up solvent-solvent extraction is labour-intensive and requires large quantities of solvents. The VLC served as a crude fractionation process beginning the search for single or "pure" components.

Antibacterial activity would be established by bioautography and MIC determination. A schematic summary of the procedure to be followed is shown in figure 4.2

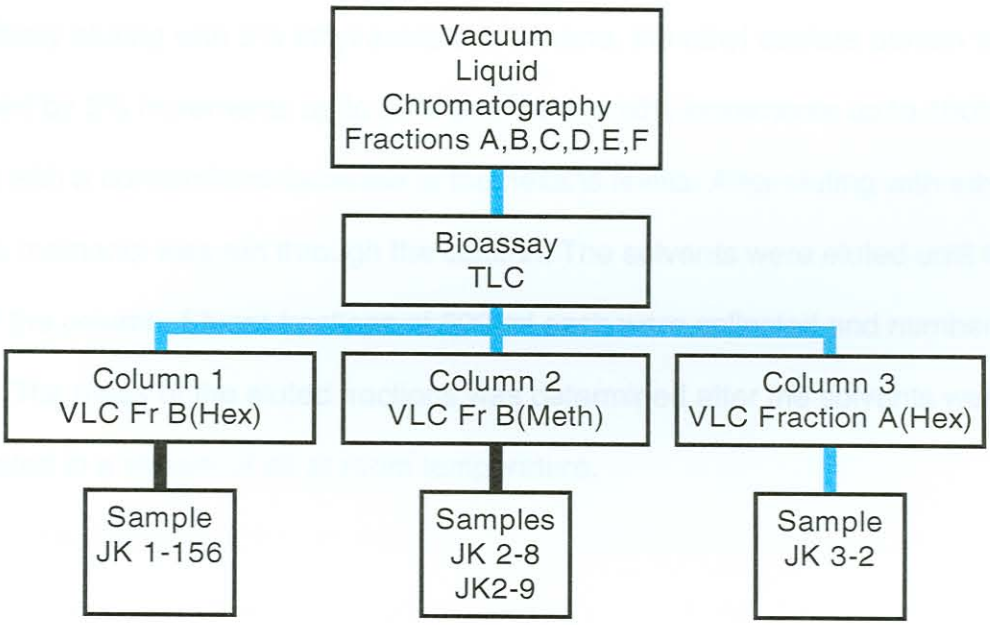

Fig. 4.2. Schematic summary of the fractionation process followed to obtain the final fractions for structure elucidation

One kilogram of finely ground *T.sericea* leaves was extracted in 2.5 L acetone by maceration overnight. The extract was dried in a Büchi rotary evaporator under reduced pressure to yield 149.75 g of residue. One hundred grams of this crude material was mixed with as small an amount as possible of silica gel (Merck) to form a dry slurry, which was then loaded onto a VLC column (diameter 9.5 cm and length 20 cm) filled with silica gel as stationary phase.

The column was eluted stepwise under vacuum with solvents of increasing polarity, ranging from a mixture of hexane and ethyl acetate, to pure ethyl acetate. To elaborate, after initially eluting with 5% ethyl acetate in hexane, the ethyl acetate portion was increased by 5% increments up to 50% and then in 10% increments up to 100% ethyl acetate with a concomitant decrease in the hexane levels. After eluting with ethyl acetate, methanol was run through the column. The solvents were eluted until it ran clear of the column. Eluant fractions of 500 ml each were collected and numbered from 1 to 37. The mass of the eluted fractions was determined after the solvents were evaporated in a stream of air at room temperature.

4.2.4 TLC and bioautography on VLC fractions

The fractions obtained from the VLC were analyzed by TLC, and fractions with a similar profile were combined. Aliquots of these fractions were reconstituted in acetone and analyzed for antibacterial activity using bioautography only as the initial search was for

single compounds with antibacterial activity. MIC would be determined once pure compounds were isolated. Machery-Nagel silica gel TLC plates were used to analyze the VLC fractions. The non-polar fractions (1 – 6) were separated with BEA, the samples with intermediate polarity (7 – 12) with CEF, and the most polar fractions (13 – 35) with EMW. Five µl of each sample was loaded on the TLC plates using the method described earlier. After development, the TLC plates were examined under UV light before spraying with vanillin spray reagent in an effort to detect the maximum number of compounds in each fraction.

Bioautography (as described in section 3.2.3) was performed using *Staphylococcus aureus* as test organism to indicate the antibacterial activity of the separated components. (Fig.4.3). The solvent system used was BEA, and the method as described, was followed. Antibacterial activity would be investigated by means of bioautography and MIC determination. A schematic summary of the procedure to be followed is shown in figure 4.2 as described previously.

Owing to the similarity of their composition determined by TLC analysis, fractions 1 and 2, fractions 3 to 13, fractions 15 to 20, fractions 24 and 25, fractions 26 to 29 as well as fractions 30 to 35 were combined in fractions A to F respectively. The masses of the combined fractions were determined to explore whether the selected fractions would be suitable for further investigation by column chromatography. Based on the results found with VLC I continued with these fractions only. The fractions obtained with solvent-solvent fractionation were not further investigated.

4.2.5 Column chromatography

Column chromatography was initially performed on sample B (VLC fractions 3 – 13) due to its high antibacterial activity and because of the apparently simple nature of the components as seen in TLC. Most of sample B was dissolved in methanol, and a small insoluble portion that remained was dissolved in hexane. Of the original 100 g "crude" extract, 52.6 g was used for the column. It was dissolved in 100 ml equal parts of hexane and chloroform in an ultrasonic bath until a soft paste was formed. The column was wet packed by suspending c. 250 g silica in hexane and pouring it into the column and leaving it to settle in a vertical position to prevent cracks and unevenness in the column. A problem arose that the paste was too sticky for the eluant to move it through the column and alternative ways and means were to be investigated to overcome the problem. The fraction was removed and dissolved in methanol and separated in column 2 and the rest that did not dissolve was dissolved in hexane and separated in column 1.

4.2.5.1 Column 1

This column was run using the hexane-soluble portion of sample B (0.101 gram). The column was coupled to an automatic fraction sampler (Isco Foxy Jr.) and standardised to 600 drops per fraction (c. 2ml/min) and 167 fractions were collected. The column was developed starting of with a 100% hexane as eluant (non-polar) and 5 % increments of dichloromethane (DCM) (more polar) was added while the hexane was incrementally lowered. The eluant mixture was run through the column each time until it was clear before the next increment would be used.

4.2.5.2 Column 2

Column 2 was run using the methanol-soluble component of sample B (1.495gram) in the same manner as that of column 1 and 187 fractions was collected.

Once single compounds were detected by TLC (Fig. 4.5) in various solvent systems, MIC values were determined for the isolated compounds. Bioautography was also performed using *S. aureus* as the test for bacterial species (Fig 4.3).

4.2.5.3 Column 3

Sample A (VLC fractions 1 – 2) was used to run a third column. Sample A was dissolved in 100 ml of equal parts of chloroform and hexane. The column was eluted using an initial mixture of 20% dichloromethane (DCM) and 80% hexane, gradually changing the concentration in 5% increments to 25% DCM: 75% hexane mixture etc. The column was finally eluted with a 30:70 DCM:hexane mixture each time until the eluant ran clear. The column was coupled to an automatic fraction sampler (Isco Foxy Jr.) and standardised to 600 drops per fraction (c. 2 ml/min). Fractions 1 – 26 were collected from the 15:85 ratio of DCM:hexane eluant mixture. From fraction 27 onwards the 20:80 mixture of DCM:hexane was used.

TLC using BEA, CEF and EMW was performed on every fifth sample to screen the compounds in the different fractions (Fig.4.6). The TLC plates were run in three stages (run for a third of the length of the plate, left to dry, run for another third of the plate and

the process repeated for the last third). This method was employed to enhance the separation of polar components on the plate.

Fractions 2 and 3 were combined to produce a sample containing a compound that appeared to be pure. The microplate dilution assay to obtain a MIC value was performed on this isolated compound which was named JK 3-2. The original crude acetone-dissolved fraction was also tested for its antibacterial activity (microplate dilution assay) in order to investigate the practical use of an acetone extract of the leaf material for use by rural people. A standard reference aminoglycoside antibiotic, neomycin, was used as a positive control to compare the MIC values obtained.

4.2.6 Chemical characterization of isolated compounds

Nuclear magnetic resonance (NMR) spectroscopy is a useful tool in elucidating chemical structures. ^{1}H- and ^{13}C- NMR spectra were obtained at the Medical University of South Africa (Medunsa) on a 300 MHz Varian NMR Machine (Oxford Instruments). Mass Spectrometry (MS) was performed on a VG70SEQ instrument at the Cape Technikon.

Samples JK 1-156, JK 2 – 8, JK 2 – 9, JK 2 – 12 were not pure enough according to NMR spectra for structure elucidation. Only sample JK 3 – 2 was found to be sufficiently pure to enable the elucidation of its structure (Fig 4.5).

4.3 Results and Discussion

4.3.1 Extraction and MIC values

One kilogram of ground *T. sericea* leaves yielded 149.75g of acetone extract. The 15% yield compares well with other data. (Eloff, 1998a)

From this a 100 mg was separated by solvent-solvent fractionation and the MIC values were determined as in table 4.1.

Table 4.1. MIC's of the solvent fractions in mg/ml

Solvent	Mass(mg)	MIC	TA
Butanol	19	0.12	158.
Water	44	1.00	44
Hexane	4	0.08	50
Carbon tetrachloride	5	0.18	278
Chloroform	14	0.50	28
35%Wat/MeOH	5	0.18	28

Hexane had the highest antibacterial activity and lowest MIC thus would be the best fraction for searching for single compounds with high antibacterial activity. Although it extracted the least mass (4 g), it had a high total activity. The largest mass was collected by the water fraction but it had the lowest antibacterial and second lowest total activity. The carbon tetrachloride fraction had the highest total activity. To determine if the same antibacterial compounds occur in the same fractions, bioautography was carried our on the fractions.

4.3.2 Bioautography

Bioautograms of the solvent extraction fractions are depicted in Figure 4.3.

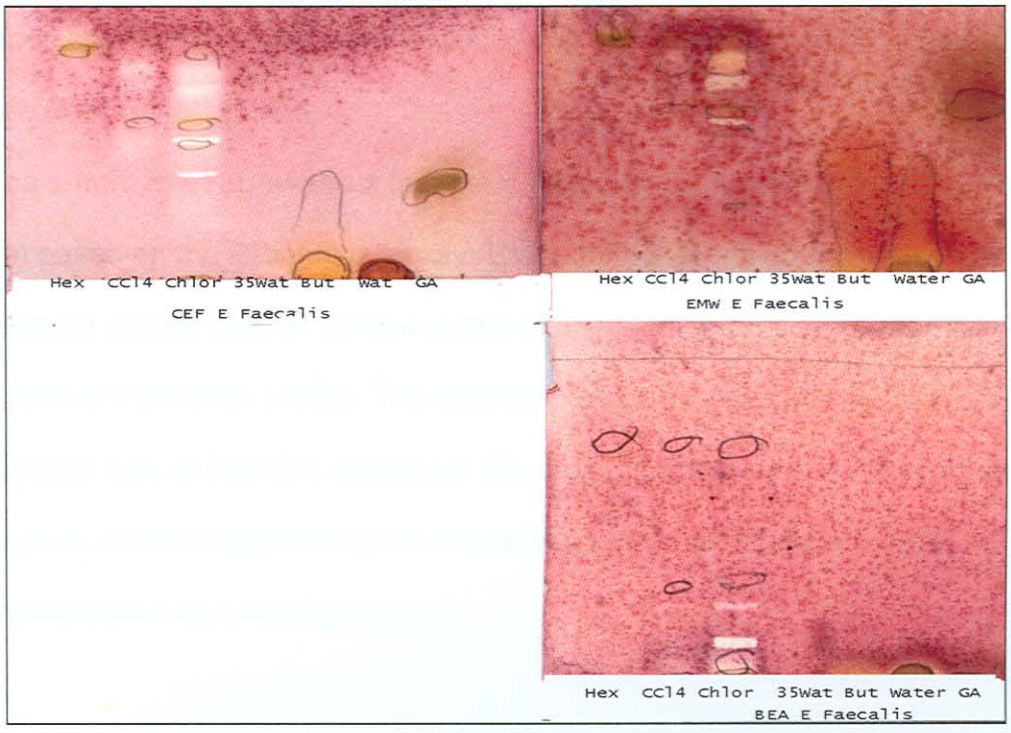

Fig. 4.3. Bioautograms of the solvent-solvent extraction fractions after TLC using different solvent systems (CEF, EMW and BEA) and spraying with *E. faecalis;* encircled areas identify fluorescent compounds where Hex = Hexane, CCL4= carbon tetrachloride, Chlor= chloroform, 35Wat = 35% water in methanol, But= butanol, Water = water fractions and GA= gallic acid.

It was surprising that the hexane fraction, which had the lowest MIC value (Table 4.1) did not have any antibacterial bands in the bio-autography. (Fig.4.3). The compound responsible for the low MIC may have been sufficiently volatile to have evaporated from the chromatogram during the drying stage prior to spraying with the test organism.

The chloroform fraction showed the highest number of antibacterial compounds (at least 3) with R_f values of 0.57, 0.71 and 0.85 and would therefore a promising fraction to continue with bioactive compound isolation and identification. Gallic acid was used as a reference compound as it is an anti-cancer and antibacterial compound found in *T. chebula* (Pettit *et al.*, (1988).

It appears from the TLC analysis that gallic acid may be present in the chloroform fraction based on the R_f value in vanillin/EMW but it was not one of the compounds with antibacterial activity against *S. aureus* based on autobiography (Fig.3.2). This questions the results of Petit *et al.* (1988). The quantity of material produced by liquid-liquid fractionation was so low that scaling up this procedure to isolate sufficient quantities for structure elucidation appeared to be impractical. The use of vacuum liquid chromatography was investigated next.

4.3.3 Vacuum Liquid Chromatography (VLC)

The mass of the different fractions obtained from the vacuum liquid chromatography procedure using the eluants ethyl acetate and hexane in a decreasing ratio varied from 0.183 to 1.342 g. (Table 4.2)

Table 4.2 Mass (g) of residue from different fractions of the ethyl acetate/hexane vacuum liquid chromatography fractionation process

FRACTION NUMBER	ETHYL AC/HEXANE RATIO	MASS (g)
1,2,3	0/100	0.632
4	5/95	0.599
5	10/90	0.291
6	15/85	0.183
7	20/80	0.605
8	25/75	1.392
9	30/70	0.679
10	35/65	0.685
11	40/60	0.787
12	45/55	0.438
13	50/50	0.29
14	60/40	0.171
15	70/30	0.159
16	80/20	0.25
17	90./100	10.075
18	100	0.58
Total		17.816

Table 4.3. Mass (g) obtained from fractions after VLC column eluted with ethyl acetate/methanol

FRACTION NUMBER	ETHYL ACETATE/ METHANOL RATIO	MASS (g)
19	100/0	0.394
20	95/5	0.209
21	90/10	0.167
22	85/15	1.131
23	80/20	1.832
24	75/25	2.006
25	70/30	1.731
26	65/35	1.479
27	60/40	0.871
28	55/45	0.709
29	50/50	0.554
30	45/55	0.391
31	40/60	0.369
32	30/70	0.333
33	20/80	0.249
34	10/90	0.224
35	0/100 – 1	0.091
36	0/100 – 2	0.272
37	0/100 – 3	0.119
Total mass		13.131

The mass of residue resulting from subsequent elution with ethyl acetate and methanol is shown in Table 4.2.The total mass recovered (Table 4.2 and 4.3) was 30.947 g of the original c 5225 g that was placed on the columns. Therefore only 58.8 % was recovered which means that 41.2% was possibly retained in the column or was volatile enough to have been lost during drying.

4.3.4 TLC and bioautography on VLC fractions

The fractions analyzed by TLC plates using CEF or EMW as eluants revealed up to three compounds present at high concentrations. No further compounds could be seen under UV light at 254 or 360 nm. Fractions 4 and 5 (eluted with BEA) yielded only one compound each. Fractions 1-3 contained several components. Fractions 4 to 22 showed only one component clearly visible under UV light at 254 nm. Fractions 23 – 35 also showed one component only whereas fractions 30 - 35 displayed multiple components. Bioautography revealed that there were quite a number of compounds with antibacterial activity against *S. aureus* (Figure 4.4). The most active components appeared to have intermediate polarity because they were moved from the origin by the highly non-polar eluant BEA.

Upon partial drying of the VLC fractions, crystals formed in the test tubes of fractions 4 and 5. The crystallization indicated the presence of a relatively pure compound in each of these fractions. In an attempt to further purify the crystals that formed, it was rinsed with hexane to remove possibly highly non-polar contaminants. The hexane rinse containing non-polar minor components was labelled A, e.g. 29A and 34A in Fig 4.4. The remaining crystals were labelled B e.g. 29B.

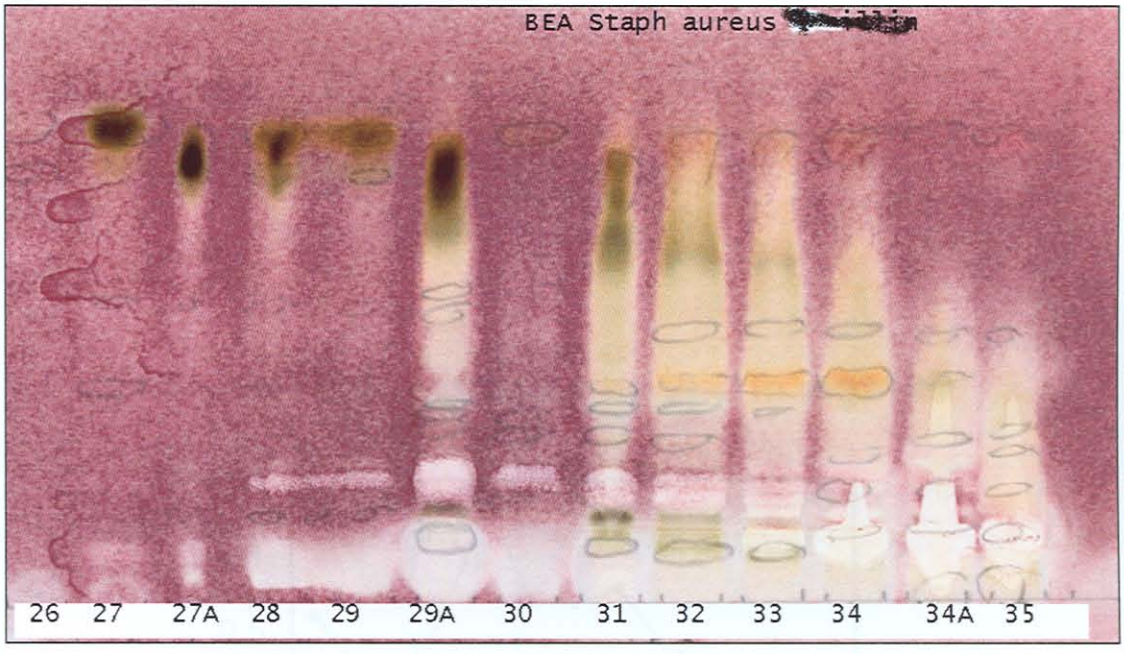

Fig. 4.4. Bioautography plate showing clear antibacterial zones of fractions collected after VLC using *S. aureus* as the test organism and BEA as the eluant. Encircled areas indicate fluorescent compounds.

Based on the TLC analysis, fractions were pooled to group fractions with similar chemical composition. It was decided to combine fractions 1 and 2(A), fractions 3 to 13(B), 15 to 20(C), 24 and 25(D), 26 to 29(E), as well as 30 to 35(F) because of the

similarity in their appearance on the TLC plate. (A indicates a hexane soluble fraction). Pooled fractions that were the most promising to use for isolating antibacterial compounds were subjected to further column chromatography using longer and narrower columns.

Table 4.4. Mass (mg) of pooled fractions resulting from Vacuum Liquid Chromatography

SAMPLE	COMBINED VLC FRACTIONS	TOTAL MASS (mg)
A	1 – 2	30
B	3 – 13	100
C	15 – 20	32
D	24 – 25	14
E	26 – 29	33
F	30 – 35	3

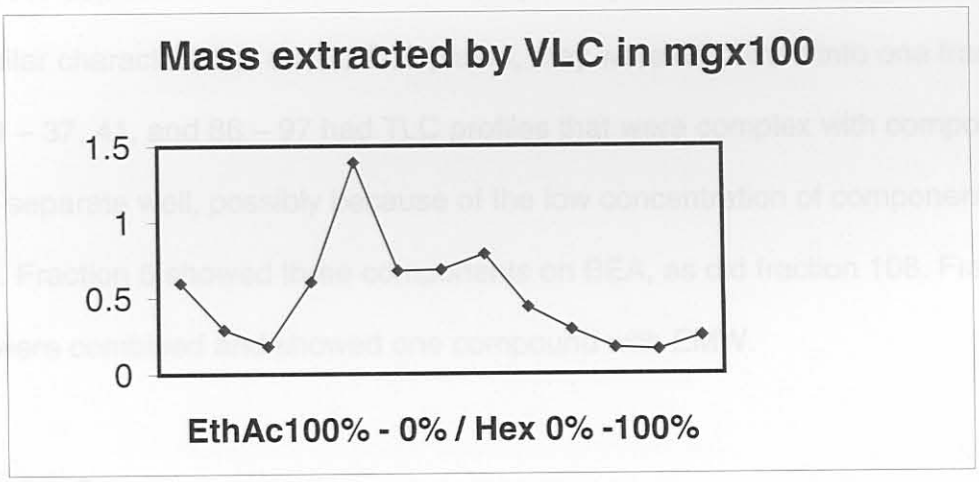

Fig.4.5 Mass (in mg x 100) of different fractions separated with gradients of ethylacetate/hexane ranging from a 100% ethylacetate in 0% hexane up to 0% ethylacetate in 100% hexane

4.3.5 Column chromatography

As discussed in section 4.2.5, the viscosity of the pooled VLC fractions was so high that it caused problems in the chromatography. Sample B of the VLC column was subsequently split into a hexane soluble fraction and fractionated in column 1. The balance that did not dissolve in hexane was dissolved in methanol and fractionated in column 2. Sample A of the VLC extraction was fractionated in column 3. These samples were selected because of the relatively simple chemical profile as well as the antibacterial activity it possessed.

4.3.5.1 Column 1

Selected fractions were analysed by TLC and, if found to possess only a single constituent, the adjacent fractions were also analyzed by TLC. Where adjacent fractions showed similar characteristics on the TLC plates, they were combined into one fraction. Samples 34 – 37, 41, and 86 – 97 had TLC profiles that were complex with compounds that did not separate well, possibly because of the low concentration of components in the fraction. Fraction 5 showed three components on BEA, as did fraction 108. Fractions 156 – 161 were combined and showed one compound with EMW.

4.3.5.2 Column 2

Fractions 00 – 7 were combined on the basis of their similarity on the CEF chromatogram. Fractions 8 – 14 were also combined, as were fractions 23 – 29, 30 – 41, 58 – 72 and 81 – 105. Fractions 106 – 181 were very polar and difficult to elute. A 20% methanol in chloroform mixture was used but even that showed poor results so it

was decided not to continue with these fractions, as their highly polar characteristics would have made them difficult to work with. The 10% water: 90% ethanol mixture as eluant showed some components. The TLC profile of fractions 81 – 105 is presented in Figure 4.5.

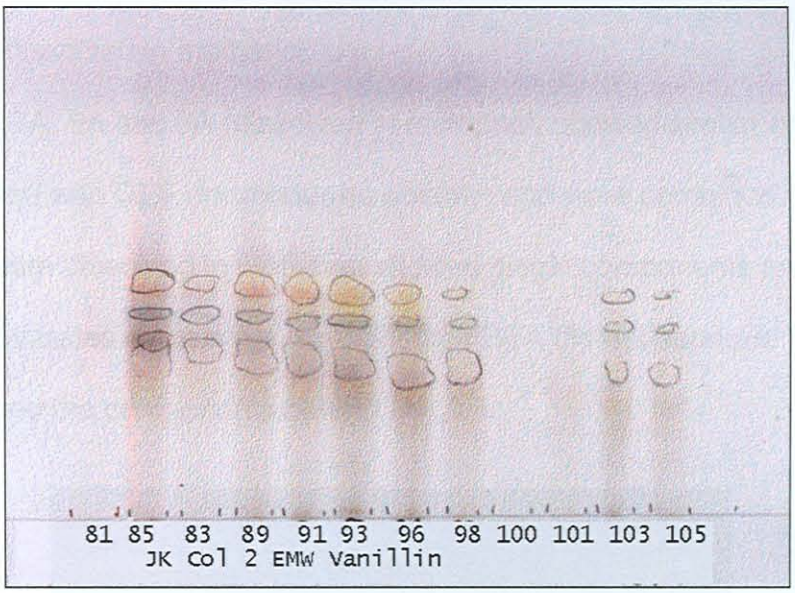

Fig. 4.6. TLC plate (sprayed with vanillin) presenting the complexity of fraction components eluted from fractions from column 2 using EMW as eluant. Encircled areas indicate fluorescent compounds under UV light

4.3.5.3 Column 3

Promising results were obtained with the third column separation in that some fractions showed only one component (for example fraction 52 which displayed a compound under 360 nm UV light). Fractions showing similar components were combined

In the TLC analysis of the initial column fractions, fractions 2 and 3 showed a single component (this was to be the fraction used for the final isolation and testing of compounds). Fraction 2 and 3 that were combined yielded a mass of 0.107g.

These fractions dissolved in hexane while an "A" fraction (the part that was insoluble in hexane) was dissolved in methanol.

Fractions 6A, 7A, 8A and 9A (dissolved in methanol) showed similar characteristics when separated with CEF (for moderate polarity) and were combined. Fractions 3A, 6A and 7A that were dissolved in methanol showed single components on TLC when eluted with EMW (separates more polar components). BEA did not separate fractions-33 – 37. EMW separated the components better.

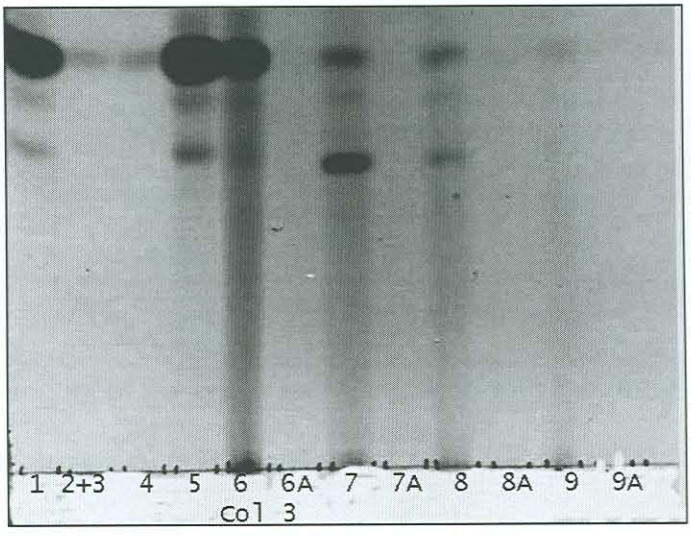

Fig. 4.7. Chromatogram (sprayed with vanillin) of fractions obtained from Column 3 by TLC with BEA as eluant and vanillin as spray reagent.

There was a single component present in fractions 2+3 (R_f value of 0.85). Much higher concentrations of a compound with a similar R_f value were present in fraction 4, but this fraction had a high concentration of contaminant. This compound was not the compound that inhibited growth of *E. faecalis.* (Fig. 4.3 c).

The MIC values obtained in the microplate dilution assay for the crude acetone extract and the isolated compound JK3-2 (Johann Kruger column 3, sample 2) are reported in Table 4.5.

Table 4.5. MIC values of the isolated compound and crude extract of *T.sericea*, compared to the reference antibiotic neomycin

COMPOUND	MIC (mg/ml)
JK3-2	0.33 mg/ml
Crude extract	1.56 mg/ml
Neomycin	0.003 mg/ml

The MIC of 1.56 mg/ml of the crude extract agreed with the results of Eloff (1999b) of 1.7 mg/ml for the same plant. The isolate (JK 3-2) at a MIC of 0.33 mg/ml showed reasonable inhibition of the *S. aureus* strain used, whereas neomycin had an MIC of 0.003 mg/ml.

Although inhibition of JK3-2 occurred at almost 100 times the concentration of the positive control, cost and availability factors indicate that both the crude extract and the isolated compound could possibly be useful as antibacterial remedies for use by rural

people. NMR spectra of apparently "pure" compounds – JK1-156, JK2-8, JK2-9 and JK2-12 indicated that these compounds more were complex than it appeared on the TLC plate and were not investigated further because only very small quantities were available. It is clear that there are more active compounds present in some of the other fractions (Fig. 4.7). This specific fraction was chosen because it appeared to be a single compound that could be isolated and identified. Further studies will have to be done on the other antibacterial compounds to determine their structure and characteristics.

4.3.5.4 Summary of column chromatography results

The origins of the different fractions obtained are presented in table 4.6.

Table 4.6 Sample identification and the fractions and columns from which it was taken.

Column from which sample was taken	Fractions from column that sample was taken from to be pooled	Sample ID
Column 1	Fractions 156 – 161	Sample JK 1-156
Column 2	Fraction 8	Sample JK 2 – 8
	Fractions 9 – 11	Sample JK 2 – 9
	Fraction 12	Sample JK 2 – 12
Column 3	Fraction 2 – 3	Sample JK 3 – 2

4.3.6 Chemical characterization of isolated compounds

4.3.6.1 Mass spectrometry data

Fast Atom Bombardment Mass Spectrometry (FABMS) gave a molecular ion at m/z 488 (2.5%) and a molecular formula of $C_{30}H_{48}O_5$ was deduced from this. Other prominent peaks were at *m/z* 470 (2%) $[M - H_2O]^+$, *m/z* 452 (6%) $[M - 2H_2O]^+$, *m/z* 442 (12.5%) $[M - HCOOH]^+$, *m/z* 248 (91%) $[M - C_{14}H_{24}0_3]^+$, *m/z* 249 (26.6%) $[M - C_{14}H_{23}O_3]^+$, *m/z* 203 (100%) $[M - C_{15}H_{23}O_5]^+$.

These fragments were strongly suggestive of a hydroxylated pentacyclic triterpene. There is an initial loss of water, then a Wagner-Meerwein rearrangement with a subsequent loss of another molecule of water (Geissman, 1959). The rearranged fragment then typically undergoes a retro-Diels-Alder (RDA) fragmentation (Djerassi *et al*, 1962; Karliner and Djerassi, 1966) (Fig. 4.9).

4.3.6.2 NMR data

The result of nuclear magnetic resonance spectroscopy experiment is presented in Fig.4.8

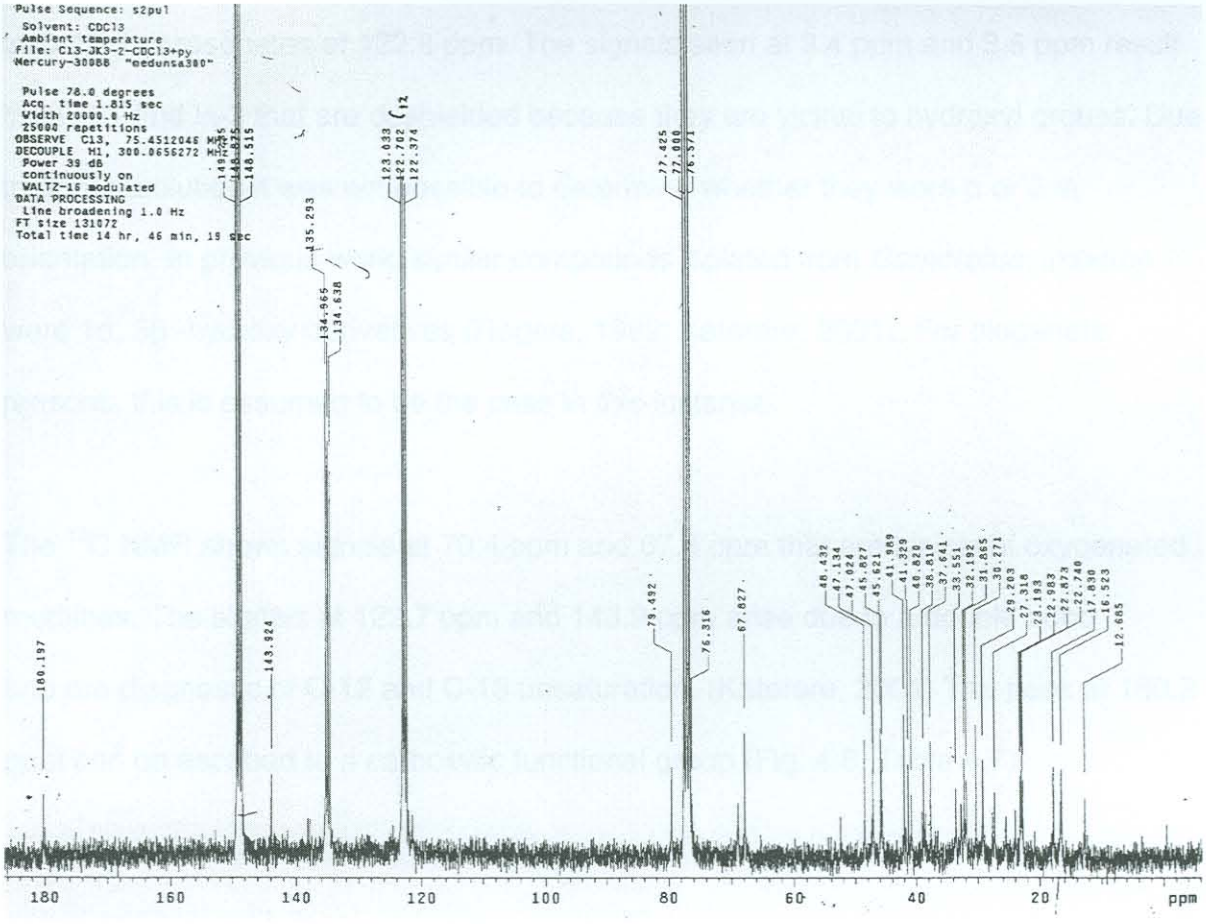

Fig. 4.8. An example of ^{1}H- and ^{13}C- NMR spectra of JK 3-2

The ^{1}H-NMR spectrum shows numerous peaks between 0.7 and 1.3 ppm. This is typical of methyl signals in a triterpenoid skeleton. There are seven methyl signals in this region. The carbon spectrum appears to confirm this.

A broad singlet occurs at 5.3 ppm and this is due to an olefinic proton. This is attached to C-12 that resonates at 122.8 ppm. The signals seen at 3.4 ppm and 3.6 ppm result from H-1 and H-3 that are deshielded because they are vicinal to hydroxyl groups. Due to poor resolution it was not possible to determine whether they were α or β in orientation. In previous work, similar compounds isolated from *Combretum imberbe* were 1α, 3β -hydroxy derivatives (Rogers, 1989; Katerere, 2001). For biogenetic reasons, this is assumed to be the case in this instance.

The ^{13}C NMR shows signals at 70.4 ppm and 67.8 ppm that are typical of oxygenated methines. The signals at 122.7 ppm and 143.9 ppm arise due to a double bond and are diagnostic of C-12 and C-13 unsaturation. (Katerere, 2001) The peak at 180.2 ppm can be ascribed to a carboxylic functional group (Fig. 4.8, Table 4.7).

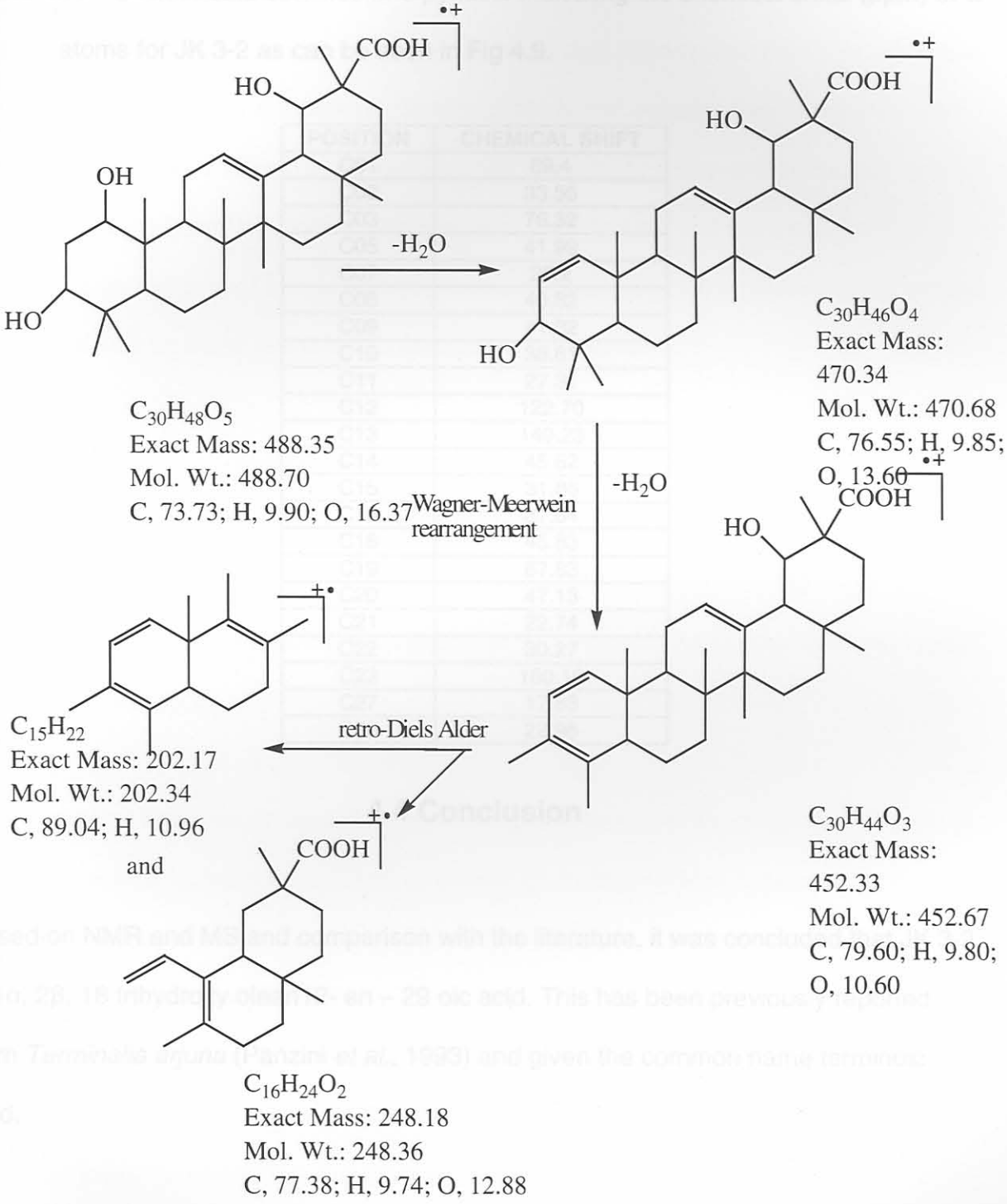

Fig. 4.9. The suggested fragmentation pattern of JK 3-2 (typical of a pentacyclic triterpenoid)

Table 4.7. ^{13}C NMR data obtained in d-pyridine indicating the chemical shifts (ppm) of C atoms for JK 3-2 as can be seen in Fig 4.9.

POSITION	CHEMICAL SHIFT
C01	69.4
C02	33.55
C03	76.32
C05	41.99
C07	29.2
C08	40.82
C09	41.32
C10	38.81
C11	27.31
C12	122.70
C13	149.23
C14	45.62
C15	31.86
C16	37.64
C18	45.83
C19	67.83
C20	47.13
C21	22.74
C22	30.27
C23	180.19
C27	17.83
C30	22.98

4.4 Conclusion

Based on NMR and MS and comparison with the literature, it was concluded that JK 3-2 is 1α, 2β, 18 trihydroxy olean12- en – 29 oic acid. This has been previously reported from *Terminalia arjuna* (Panzini *et al.,* 1993) and given the common name terminoic acid.

Triterpenoids have been attractive phytochemicals for research, not so much perhaps for their commercial or therapeutic importance, but for the relative ease with which they are available and amenable to purification (Mahato and Sen, 1997). NMR spectroscopy

is now almost invariably used for confirmation of a proposed structure by the assignment of a ^{13}C or ^{1}H spectrum, usually supported by NMR data from related model compounds. Triterpenoids, especially the pentacyclic triterpenoids that possess five fused rings and eight chiral centres, have attracted the attention of synthetic chemists for several decades (Mahato and Sen, 1997). Djerassi *et al.* reported in 1963 the mass fragmentation patterns of pentacyclic triterpenoids belonging to the oleanane and ursane classes as well as some rearranged oleananes and ursanes.

Triterpenoids are lipid-soluble secondary metabolites, which are biosynthetically derived from isoprenoid units. There are at least five classes of importance in the growth, metabolism and ecology of plants (Harborne, 1973). Triterpenoids are the most ubiquitous non-steroidal secondary metabolites in terrestrial and marine flora and fauna (Mahato *et al.,* 1992).

This is the first time that an olean12- en – 29-oic acid has been reported from *T. sericea.* The triterpenoids isolated from *Combretum* spp. of southern Africa appear to confirm close biogenetic, chemotaxonomic relationships. (Rogers and Subramony, 1988). Katerere (2001) also proposed that the presence of 1α, 2β-dihydroxy pentacyclic triterpenoidal acids in *T. stuhlmanii* similar to those from some *Combretum* species confirms a close relationship with *Terminalia* species. This study provides further chemical evidence of the taxonomic proximity of the two genera.