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Oleanic acid : its isolation and derivatisation to potential antimicrobial compounds

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OLEANOLIC ACID – ITS ISOLATION AND DERIVATISATION TO POTENTIAL ANTIMICROBIAL COMPOUNDS

by

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ABSTRACT

An increasing number of natural products possessing the oleanolic acid moiety have been shown to demonstrate a wide spectrum of biological activity.

This thesis deals with the extraction and isolation of oleanolic acid from *Syzigium aromaticum* and the examination of its stereochemistry and crystal structure by X-ray diffraction.

The synthetic routes used for converting functional groups on the oleanolic acid molecule to afford derivatives are described in Chapter 5.

Oleanolic acid and its derivatives were evaluated for antimicrobial activity. Three different procedures viz. Kirby-Bauer, Broth dilution and Tetrazolium salt chemosensitivity were used. Acceptable results were obtained from the last method and these were used to arrive at conclusions regarding this study.

ABBREVIATIONS

Calc.	calculated
d	doublet
dd	doublet of doublets
DCM	dichloromethane
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
dt	doublet of triplets
EtOAc	Ethyl acetate
^{1}H NMR	proton nuclear magnetic resonance
Hz	Hertz
IR	infrared
J	coupling constant
Lit.	Literature
M^+	molecular ion
m.p.	melting point
m/z	mass-to-charge ratio
PCC	pyridinium chlorochromate
PDC	pyridinium dichromate
s	singlet
t	triplet
t.l.c.	thin layer chromatography
δ	chemical shift

EXPERIMENTAL

GENERAL

1. All ^1H NMR spectra were recorded on a 200MHz Gemini 2000 Varian NMR spectrometer at ambient temperature in deuterochloroform.

2. Mass spectra were recorded on a modified AEI analyser (902) at 70 eV and an ion source temperature of between 180°C and 220°C

3. Infrared spectra were measured for nujol mulls on Perkin-Elmer FTIR spectrometer SPECTRUM 1000, while melting points were determined on a Fischer-John melting point apparatus and are quoted uncorrected.

4. *Column chromatography was carried out on dry-packed columns with* Merck Kieselgel 60 (70-230 mesh as adsorbent, while thin-layer chromatography (t.l.c.) was carried out on aluminium plates coated with Merck Kieselgel 60 F_{254}.

5. Hexane refers to the fraction of boiling point 60 - 80°C derived from a petroleum ether raw material. Most organic solvents and liquid reagents were distilled prior to use.

6. The phrase "residue obtained upon work-up" refers to the residue obtained when the organic layer was separated (using an appropriate

organic solvent), backwashed with water, dried (using anhydrous magnesium sulphate) and the solvent (after filtration) evaporated under reduced pressure.

OBJECTIVES OF THE STUDY

The objectives of this study were:

(i) To extract oleanolic acid from the dried buds of *Syzigium aromaticum* (clove).

(ii) To corroborate the stereochemistry of the extracted oleanolic acid molecule by X-ray diffraction methods.

(iii) To synthesise the oleanolic acid derivatives **(2) – (11)**.

(iv) To evaluate the derivatives **(2) – (11)** for antimicrobial activity *in vitro*. The first step in this regard was to find a method which was not affected by the hydrophobic nature of the selected compounds.

CHAPTER 1

INTRODUCTION

Oleanolic acid, OA **(1)** (3 β-hydroxy-olean-12-en-28-oic acid) is a pentacyclic triterpenoid present in many species of the plant kingdom. It exists in almost 190 species of medicinal herbs and plants in the form of the free acid or aglycones for triterpenoid saponins. It is a compound that appears to have multiple and diverse pharmacological activities in animals and humans.

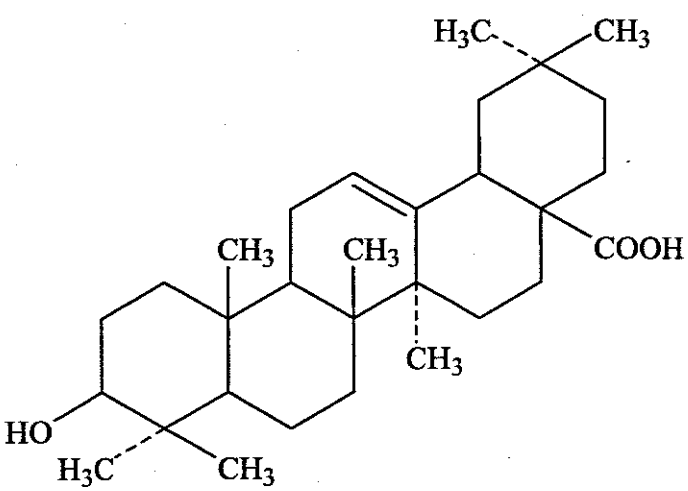


Figure 1.1 : Oleanolic acid (1)

Perilla frutescens, a famous traditional Chinese medicinal herb, is used to treat diseases such as depression, anxiety, tumour, cough, bacterial and fungal infections, allergy, intoxication and some intestinal disorders[1]. Oleanolic acid, one of the components of the leaves of this plant, has been ascertained to be anti-inflammatory[2], possess hepatoprotective[3] and anti-ulcer activities[4].

A second Chinese medicine is the panacea, ginseng, from the root of the arialaceous plant, *Panax ginseng*, which stimulates the body's defence system[5]. Ginseng saponins (ginsenosides) were separated and isolated from the *Panax ginseng* plant. Oleanolic acid saponins are one of the main constituents of the plant. Ginseng has been used to treat diabetes, hepatitis, and cancer.

The anti-inflammatory activity of oleanolic acid was displayed when rats and mice with induced oedema were treated by orally and intraperitoneally dosing them with oleanolic acid[6]. The acid for this study was extracted from the seeds of *Luffa cylindrical* using methanol.

Here in the Western Cape the effect of oleanolic acid on sperm motion characteristics and fertility have been studied on male Wistar rats. Mdhluli and Van der Horst carried out this study at the University of the Western Cape[7]. Reversible sterility without adverse effects on *libido* was shown after a 30-day administration of 16 mg/Kg body weight.

It has also been reported that the medicinal activities include anti-HIV [8, 9], antiviral[10] and anti-tumor[11].

Oleanolic acid commonly occurs in plant species with another triterpene, ursolic acid.

The clove

The clove is the dried flower bud of *Syzigium aromaticum* (Family: Myrtaceae), also known as *Eugenia caryophyllata*, an evergreen tree 10 - 20m in height indigenous to India, Indonesia, Zanzibar, Mauritius and Sri Lanka[12, 13]. The finest cloves come from Molucca (Indonesia) and the island of Pemba (Tanzania). Pemba is covered with clove gardens and the island can be smelled on any ship approaching it. The cloves have strong aromatic and very intense fragrance. It gives a sensation of burning when eaten.

Despite the wide existence of oleanolic acid in the plant kingdom, its presence in dried cloves appears to be high enough to afford a relatively uncomplicated extraction procedure.

Cloves are used as a flavouring and condiment in many types of cooking. On repeated distillation, clove oil is obtained. The essential oil in cloves of a good quality may exceed 15%. The oil is dominated by eugenol (70% - 85%), eugenol acetate (15%) and β-caryophyllene (5% - 12%), which make up 99% of the oil. Eugenol is used in dentistry as an anaesthetic and a powerful antiseptic as well as a stimulating expectorant in phthisis and bronchial ailments. Cloves contain about 2% of the triterpene oleanolic acid.

Many plant materials such as olive skins, almond hulls, grape pomace and grape skins are known to contain oleanolic acid. It has also been isolated from the fruit barks of *Periploca laevigata* (Asclepiadaceae)[14] and from the seeds of *Luffa cylindrical*[6].

Oleanolic acid in South African plants

In South Africa, OA is found in the root bark of *Melianthus comosus*[15] which is a multi-branched shrub of up to three metres in height. It is also named the "kruidjie-roer-my-nie" or in Zulu "ibonya". All parts of the plant produce a strong unpleasant smell when touched or bruised. Leaf poultices and decoctions are used to treat septic wounds, sores, bruises, backache and rheumatic joints, and it is a snakebite remedy from the Khoi healing culture. Root infusions are used for treating cancer. The medicinal value of *Melianthus* is partly due to the triterpenoids in the leaves and roots[16, 17, 18].

Derivatives of oleanolic acid

Many chemical derivatives of oleanolic acid have been shown to have pharmacological activities.

Hichri et al.[19] isolated oleanolic acid from the fruit barks of *Periploca laevigata.* This was treated with acetic anhydride in pyridine to give 3-O-acetyloleanolic acid **(2).** Moderate activities were shown against *Staphylococcus aureus, Eschericia coli* and *Pseudomonas aeruginosa* bacteria both for **(1)** and **(2)**.

Our decision was to use **(1)** and 18β-olean-12-ene-3β, 28 diol **(5)** as starting materials for the synthesis of other derivatives as shown in schemes 1 – 3 in Chapter 5 (pages 32-34).

In a recent study Huang *et al.*[20] determined the anti-tumour and differentiation-inducing effect of the 3-oxo derivative, 3-oxo-olean-12-en-28-oic acid **(10)**. The

evaluation of **(10)** by Huang *et al.* showed that it might be a useful anti-cancer agent for melanoma, as inhibition by differentiation of highly malignant mouse melanoma B16-BL6 cells occurred. In scheme 3 (page 34), **(1)** was oxidised with PDC in CH_2Cl_2 to produce **(10)**.

The gastroprotective activity of oleanolic acid derivatives had been assessed on gastric ulceration in rats by Astudillo et al.[21] Intraperitoneal administration of OA in mice did not cause toxicity or mortality at doses up to 600 mg kg^{-1} whereas the protective effect of OA had been shown at 200 mg kg^{-1}. The activity of methyl oleanolate **(3)** and 3-acetyl-17-methyl oleanolate **(4)** against the gastric lesions were similar to OA, **(2)** was slightly less, but the oxidation of the hydroxyl group to give **(10)**, reduced the activity of the triterpene.

CHAPTER 2

SAPONINS

Saponins are glycosidic compounds often referred to as "natural detergents" because of their foamy texture. These are compounds composed of a steroid (C27) or triterpenoid (C30 such as oleanolic acid) saponin nucleus, the aglycone, with one or more carbohydrate branches[22].

The saponins extracted from *Anemone obtusiloba* were named obtusilobinin and obtusilobin[23]. Obtusilobinin (Fig.2.1), $C_{47}H_{76}O_{16}$, yielded a genin content of 50.7% oleanolic acid with one unit each of D-glucose, L-rhamnose and L-arabinose. The sugars were not present in ester combination with the –COOH group, but were linked as a trioside unit to the –OH at C-3.

Obtusilobin (Fig.2.2), $C_{41}H_{66}O_{11}$, gave on hydrolysis oleanolic acid at 61.5 %, L-rhamnose and L-arabinose. The C-1 of the L-arabinose moiety is linked to C-2 of L-rhamnose and C-1 of L-rhamnose is linked to C-3 OH of oleanolic acid. Both sugars were involved in the formation of α-glycosidic linkages.

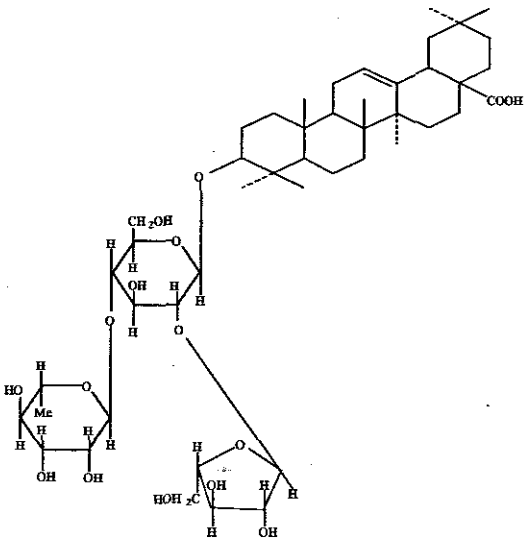


Figure 2.1 : Obtusilobinin

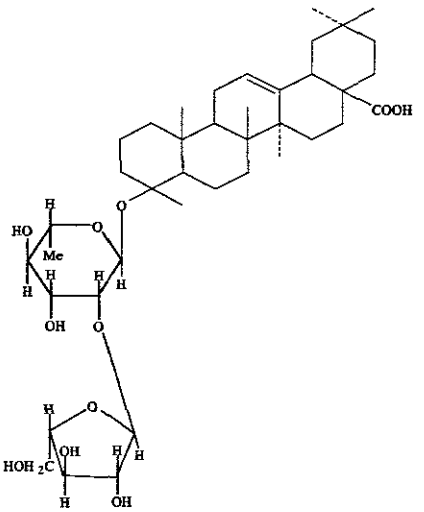


Figure 2.2 : Obtusilobin

Two saponins have been extracted from the seeds of *Achyranthes aspera* which is an annual herb growing throughout India[24]. The seeds are used in Indian medicine as a cure for renal dropsy. Previously the alcoholic extract had been hydrolysed and the genin characterised as oleanolic acid. The sugars were identified as galactose, glucose, rhamnose and xylose.

Two homogeneous compounds were obtained, the two saponins A and B. On complete hydrolysis saponin A gave oleanolic acid methyl ester, showing that in the parent saponin the –COOH at C17 of the genin was not esterified by any sugar. The sugars were D-glucose, L-rhamnose and D-glucuronic acid. In the saponin B the oleanolic acid aglycone existed in ester combination with sugars D-glucose, D-galactose, D-glucuronic acid and L-rhamnose.

Saponin glycosides have an anti-inflammatory and anti-oedema action. In South Africa an indigenous plant that contains oleanolic acid in the form of a saponin is *Phytolacca dodecandra*. The saponin is oleanoglycotoxin A which is toxic to snails and is used to combat bilharzia in Africa[16] (Fig.2.3).

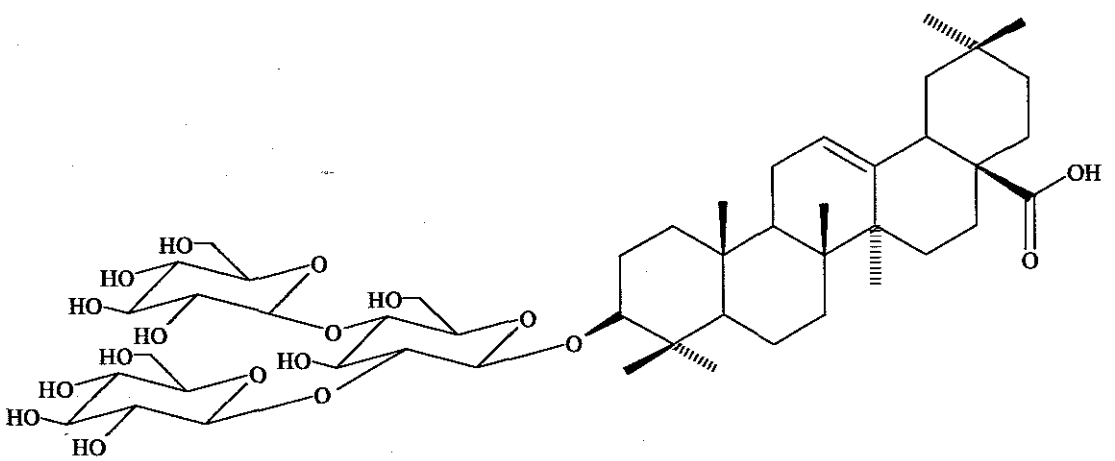


Figure 2.3 : Oleanoglycotoxin A (triterpenoid saponin)

It was shown in a study based in Japan that oleanolic acid glycosides inhibited the increase of glucose levels in oral glucose-loaded rats[25]. Oleanolic acid itself did not exhibit activity but 3-*O*-glucuronide strongly inhibited the increase in serum glucose levels. The 3-*O*-glucuronide appears to have affected glucose absorption in the gastrointestinal tract.

From the above information about a small number of plant species, it becomes clear that many saponins containing oleanolic acid or derivatives thereof are of wide occurrence. Extracts from various plants have been used as animal and human treatments for various diseases. The uses of oleanolic acid and its

derivatives for antimicrobial activity will be studied to show whether or not they are strong contenders as antibiotics.

Studies in Canada[22] have indicated that dietary sources of saponins offer chemo preventative assistance in lowering the risk of human cancers. Saponins appear to inhibit or kill cancer cells without affecting normal cells.

In plants, saponins form their active immune system. They function as a "natural antibiotic" for plants and scientists are attempting to establish them in the fight of humans against fungal and yeast infections, microbes and viruses.

CHAPTER 3

EXTRACTION OF OLEANOLIC ACID FROM CLOVES

Figure 3.1 : Clove flowers

As mentioned above, oleanolic acid is present in many different plant species, either as the free acid or as the aglycone in saponins. Oleanolic acid and some of its derivatives have been extracted from plants, and various biological activities have been reported in the literature. The extraction of oleanolic acid from olive pumice was initially considered but after many attempts in the laboratories at UWC, it did not prove to be a viable proposition. The method considered for this study, was extraction from the dried buds of cloves, which contain a relatively high amount of oleanolic acid.

Figure 3.2 : Dried buds of cloves

Experimental

Clove oil was removed from clove powder (ground cloves) (1 Kg) in a water (2L) slurry by steam distillation for 4h as shown in Fig.3.3. The mixture was prevented from dehydration by the periodic addition of water (± 500ml). The oil and water residue was yellow, not dark coloured.

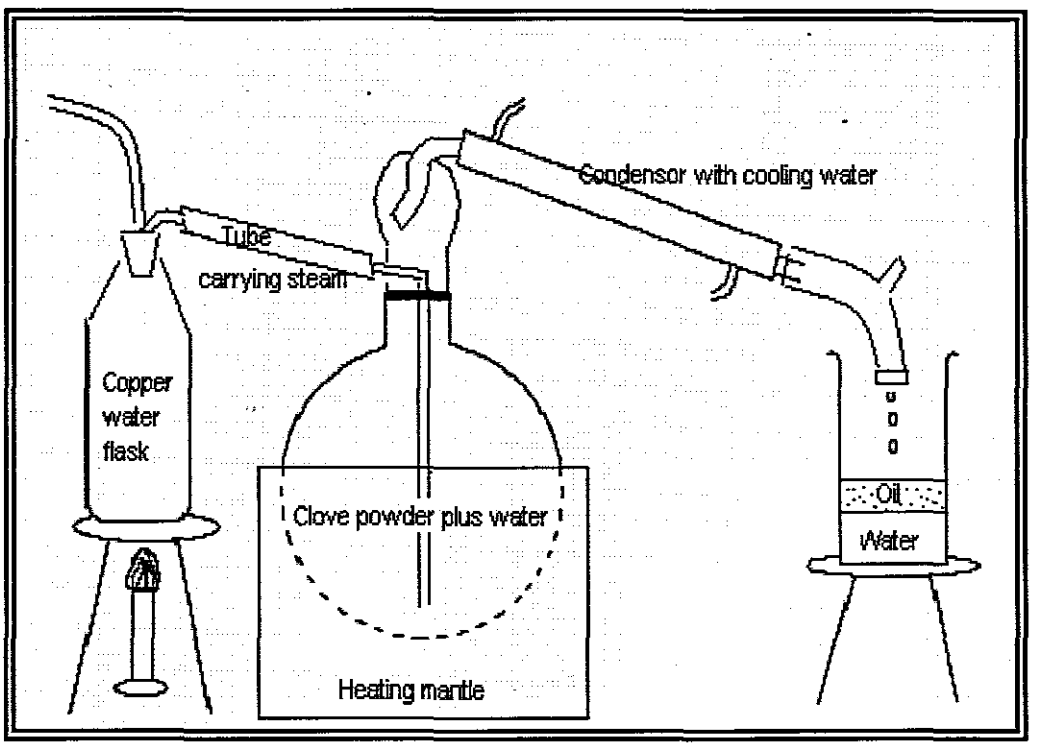


Figure 3.3 : Extraction of oil by steam distillation

The oil-free clove powder was air-dried for two weeks on top of a laboratory oven. Soxhlet extraction of the powder for 48 to 60h (MeOH (4L)) until the extract was colourless followed. Clove pwder was supported in a Soxhlet apparatus with cotton wool (Fig.3.4).

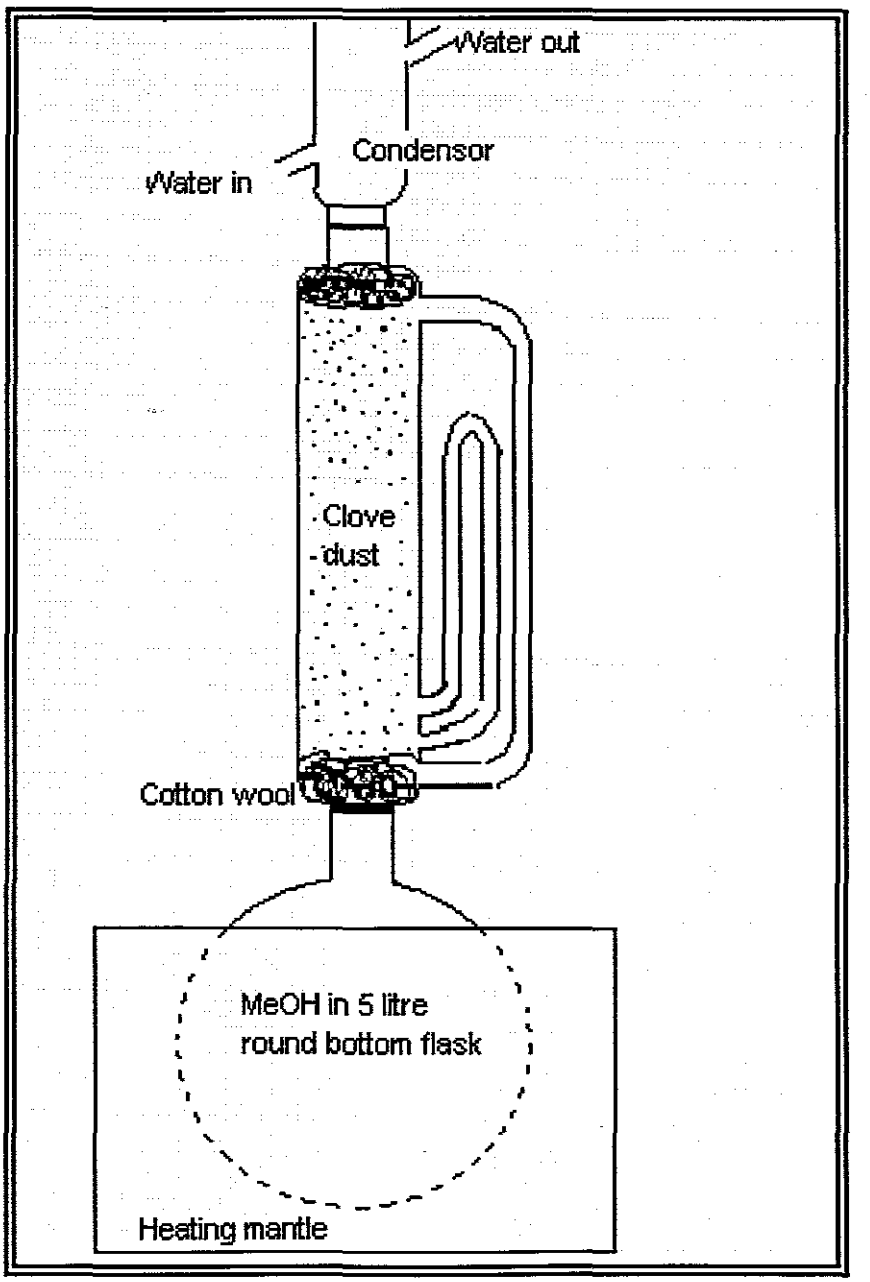


Figure 3.4 : Soxhlet extraction

The volume of the extract was reduced to 400 ml by evaporation of organic solvent, and treated with H_2SO_4 (dil.) until the mixture was acidic. The OA was extracted into the DCM organic phase, leaving alkaloids in the acidic aqueous phase. The residue was dried, filtered and the organic solvent evaporated.

The presence of OA was verified by t.l.c. analysis using EtOAc:hexane (1:4) mobile phase. The OA spot appeared as a long tailing spot in the clove extract compared with a non-tailing spot for the standard.

The best eluent ratio for separating OA was determined by t.l.c. Column chromatography with EtOAc:hexane (1:4) mobile phase was initially used. The mobile phase was changed to EtOAc:hexane (9:11) and fractions 9 to 15 collected. After removal of solvent by evaporation, the crude oleanolic acid residue (12g; 1.2%) was obtained.

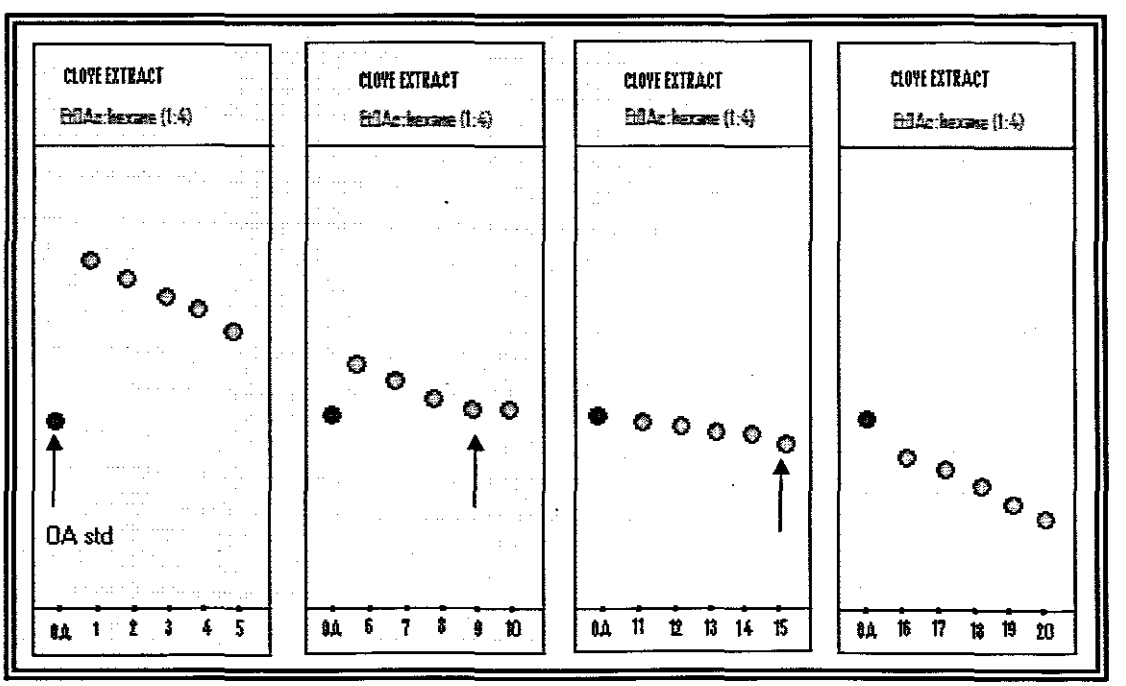


Figure 3.5 : TLC plates

The residue was recrystallised (isopropanol) by standing for a few days until "white balls" of crystals were formed. Upon filtration, the white crystals were scraped from the filter paper after the solvent had evaporated. Re-

crystallisation was repeated until a pure material (8g; 0.8%) had been obtained.

The compound extracted was identified and characterised by IR, NMR and MS spectroscopic means as well as by HPLC[1] and X-ray crystallography.

CHAPTER 4

THE STRUCTURE AND STEREOCHEMISTRY OF OLEANOLIC ACID

The stereochemistry of the pentacyclic triterpenoids was initiated in 1938 by Giacomello[26]. He had concluded from an X-ray analysis of certain members of the β-amyrin group, that all five rings were fused trans-anti-trans to each other. From a study of the rates of alkaline hydrolysis of β-amyrin acetate and epi-β-amyrin acetate as well as of molecular models, this configuration assignment was tentatively verified by Ruzicka and Gubser[27]. However, Giacomello's conclusions have been shown to be faulty.

Barton and Holness suggested two possible stereochemical formulae (Fig 4.1 and Fig 4.2) for the β-amyrin skeleton based on their chemical analyses[28].

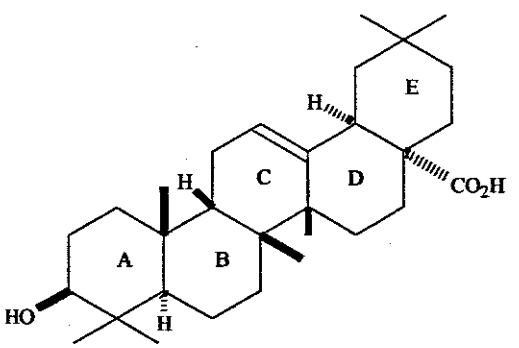


Figure 4.1

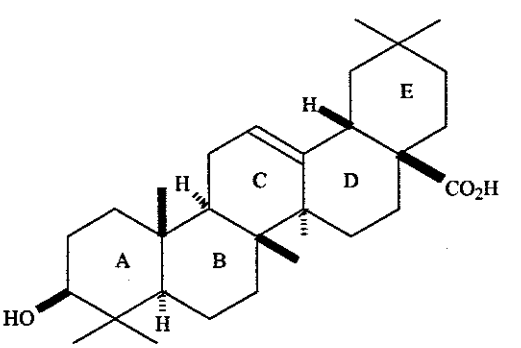


Figure 4.2

At Birkbeck College Crystallographic Laboratory, an X-ray investigation was performed by Abd el Rahim and Carlisle on crystals of methyl oleanolate iodoacetate using the "heavy atom technique". The distribution and weights of the peaks were used to determine that only the crystal structure of Fig 4.1 could fit the model. The rings A, B, C and D were outlined with good resolution. However ring E was seen in its "arm-chair or end-on view" which was interpreted as a cis fusion to the ring[29,30].

The natural compound oleanolic acid (Fig 4.2) has 8 asymmetric centres and can thus exist as 2^8 = 256 racemic configurations[31]. However, only one of these is synthesised in Nature.

Chemical procedures were used to reduce the problem of configurations to a choice between the two configurations (Fig 4.1 and Fig 4.2). In the X-ray crystallographic study by Abd el Rahim and Carlisle this was shown to correspond to Fig 4.3 and the planar structure (Fig 4.2).

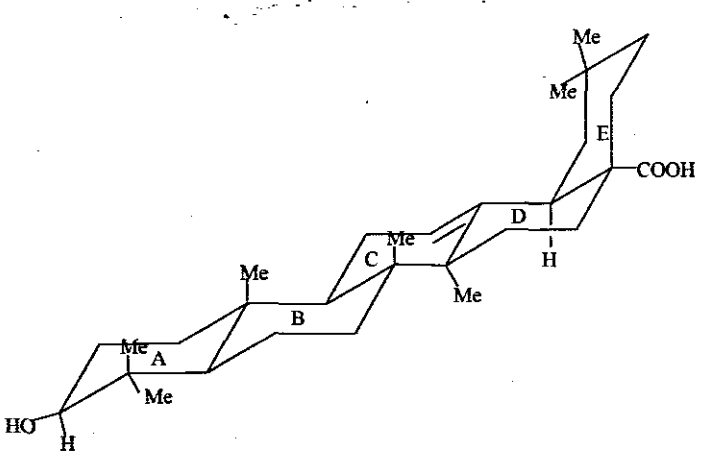


Figure 4.3

Experimental

The X-ray method has the advantage that it determines constitution, configuration and preferred conformation all at the same time. Despite the fine needle-like crystals the oleanolic acid forms on crystallisation in isopropanol, an attempt was made to grow crystals suitable for X-ray structure determination. An X-ray quality crystal should have a dimension of 0.2 – 0.4 mm in at least two of the three dimensions. The factors during crystal growth that affect the size of the crystals are solubility of compound in the solvent chosen for recrystallisation, the number of nucleation sites, mechanical agitation to the system and time.

The solvent chosen should be one in which the compound is moderately soluble and which does not form supersaturated solutions. These tend to give crystals which are too small in size. Ambient dust in the laboratory provides sites of nucleation and as one requires a small number of nucleation sites, dust should be excluded by covering the container. Crystals should be grown with a minimum of mechanical disturbance and should be left for a period of time longer than a week. Crystals recognise that patience is a virtue!

The following three methods were used to grow a crystal of oleanolic acid:

Slow evaporation method : A nearly saturated solution was made and this was covered and stored in a clean crystal-growing dish.

Slow cooling method : The saturated solution was heated then placed in a Dewar

flask filled with hot water and left for at least a week.

Low temperature method : The saturated sample was cooled in a refrigerator at 8°C and checked regularly for crystallisation [32].

Crystal growth was attempted in the following solvents: dimethyl suphoxide (DMSO), dimethyl formamide (DMF), m-xylene, tetrahydrofuran (THF), dioxane, iso-propanol and aniline.

In most of these solvents no crystallisation occurred, as the solvent was too volatile and a powdery residue formed before crystallisation could occur. In DMSO and DMF thin needle-like crystals formed. The solvated crystals in the DMF appeared to be large enough for an X-ray crystallographic analysis in the UCT laboratory. Despite the small size of the crystal, the data was collected and processed overnight to obtain the configuration and dimeric structure given in Figure 4.4 and Figure 4.5.

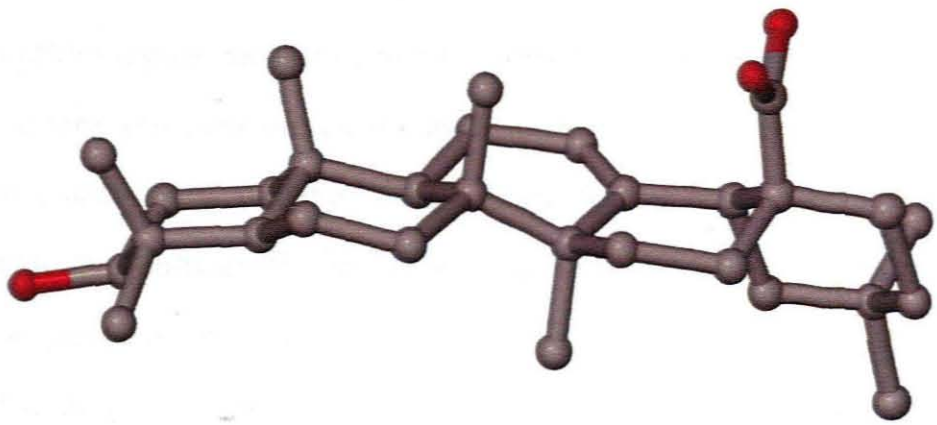


Figure 4.4: Configuration of Oleanolic acid by X-ray Crystallography

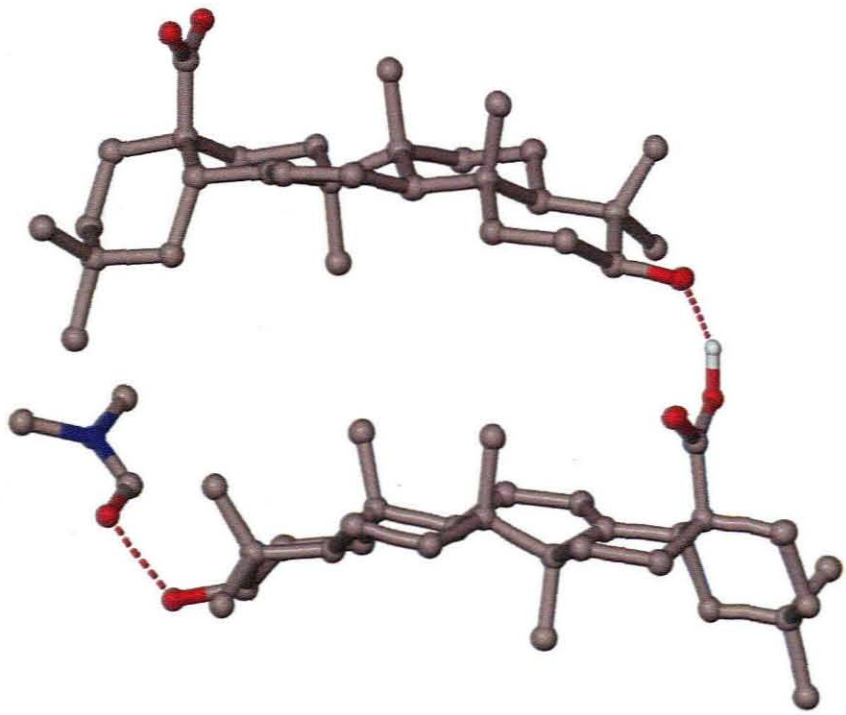


Figure 4.5: Dimeric structure of Oleanolic acid

Details of the crystal data are given in Table 4.1. Cell dimensions were established from the intensity data measured on a Kappa CCD diffractometer using graphite-monochromated MoK_α radiation. The strategy for the data collection was evaluated using COLLECT software[33] and for all structures the intensity data were collected by the standard phi scan and omega scan techniques and were scaled and reduced using the program DENZO-SMN[34] the structures were solved by direct methods using SHELX-86[35] and refined by full-matrix least-squares with SHELX-97[36], refining on F^2. The program X-Seed[37] was used as a graphical interface.

Discussion

Being a natural compound only one enantiomer was present and the compound appeared in a dimeric form with the molecule hydrogen bonded between the hydroxyl and carboxylic acid groups with a DMF molecule included, shown in Fig. 4.6.

Table 4.1: Crystal Data and Refinement Parameters

Formula	2(C30 H48 O3). C3 H7 N O
Crystal System	Monoclinic
Space Group	$P2_1$
a (Å)	11.520(2)
b (Å)	7.3320(15)

c (Å)	33.725(7)
α (°)	90
β (°)	97.99 (3)
γ (°)	90
V (Å^3)	2820.9 (10)
Z	2
Crystal Size (mm)	0.04 x 0.10 x 0.22
D_c (g cm^{-3})	1.161
No. of reflections collected	7600
No. unique reflections	3287
R_1 (I>2σ(I))	11.38%
wR_2	24.66%

There are two molecules of oleanolic acid in the asymmetric unit, labelled I and II.

The conformation of the molecules is described in terms of the asymmetry parameters, which are a function of the torsion angles of a given ring. These parameters are a measure of the degree of departure from the ideal symmetry of a given ring. For a six membered ring there are six possible idealised symmetries:

a) planar ; b) chair ; c) boat ; d) twist ; e) sofa (envelope) ; and f) half chair.

The asymmetry parameters may be defined as:

$$\text{Mirror } \Delta C_S = \sqrt{\frac{\sum_{i=1}^{m} (\phi_i + \phi_i')^2}{m}}$$

$$\text{Two-fold axis } \Delta C_2 = \sqrt{\frac{\sum_{i=1}^{m} (\phi_i - \phi_i')^2}{m}}$$

Related torsion angles, ϕ and ϕ', are compared in a way that will result in a value of zero for perfect symmetry. Mirror related torsion angles have the same magnitude but opposite sign and are added, while two-fold related torsion angles have the same magnitude and sign, and are subtracted [38].

The conformational parameters of the two oleanolic acid molecules, I and II, are very similar, and are summarised in Table 4.2. Rings A, B, D and E may be described as chairs, but the symmetry of ring C is distorted by the double bond (molecule I : C12-C13, molecule II : C42-C43) and is best described as a distorted envelope with C8 (molecule I) and C38 (molecule II) as the apex.

Rings A, B, C and D are all *trans*-fused, but rings D and E are *cis*-fused, with the torsion angle C18-C17-C18-C19 = -75.3° (molecule I) and
C46-C47-C48-C49 = -76.3° (molecule II).

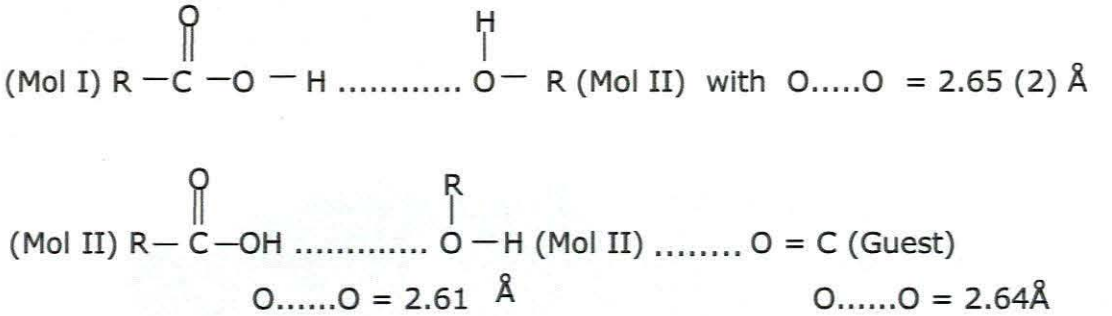
Table 4.2 Summary of Conformational Parameters of oleanolic acid

Molecule I	Ring	Molecule II
Chair ΔC_2 (1-10) = 0.9	A	Chair ΔC_2 (32-33) =2.3
Chair ΔC_S (5) = 2.7	B	Chair ΔC_S (35) =3.1
Envelope with C8 apex ΔC_S (8) = 7.4	C	Envelope with C38 apex ΔC_S (38) = 2.5
Chair ΔC_2 (13-14) = 2.4	D	Chair ΔC_2 (43-44) = 2.3
Chair ΔC_2 (17-18) = 2.7	E	Chair ΔC_2 (47-48) = 2.4

All bond lengths and angles are within expected values. The $C(sp^3)$-$C(sp^2)$ bond lengths in the two nuclei of the oleanolic acid range from 1.52(2) to 1.58(2) Å. The double bonds were confidently placed, with C12-C13 = 1.35(2) and C42-C43 = 1.34(2) Å. Similarly, it was possible to differentiate the bond lengths which occur in the carboxylic moiety (C=O = 1.23(2) Å, 1.22(2) Å; C-OH = 1.37(2), 1.33(2) Å for molecules I and II respectively).

The packing of the structure is shown in Figure 4.7, which is shown as a projection along [010].

The structure is stabilised by a series of hydrogen bonds linking the oleanolic acid, which form stacks parallel to a. There are three unique hydrogen bonds:



The guest molecules are located in channels running parallel to [010]. These are shown in Figure 4.7, in which the oleanolic acid is represented with Van de Waal's atomic radii and the guests are omitted for clarity. The cross sections of the channels are approximately 4.3 x 6.5 Å.

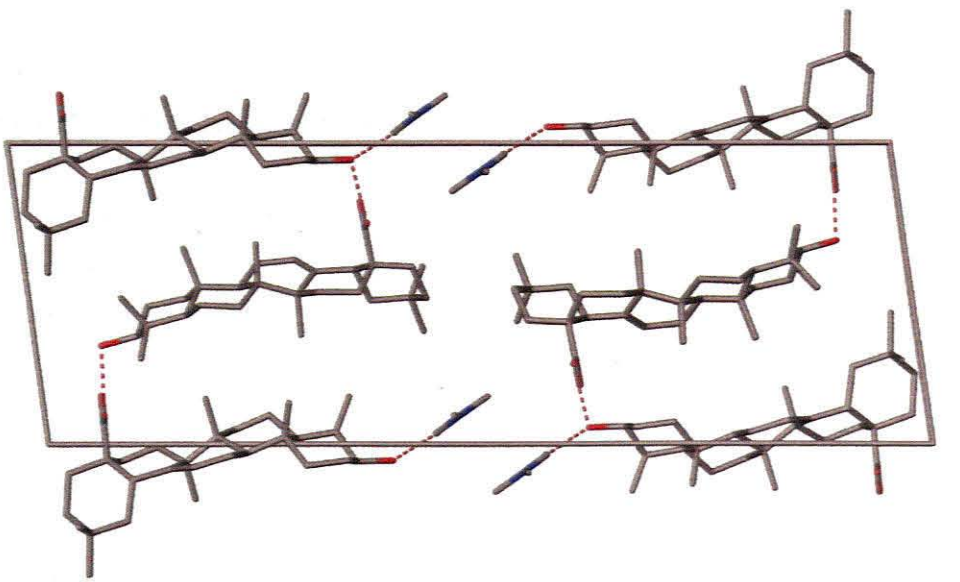


Figure 4.6: Packing diagram of oleanolic acid viewed along [010]

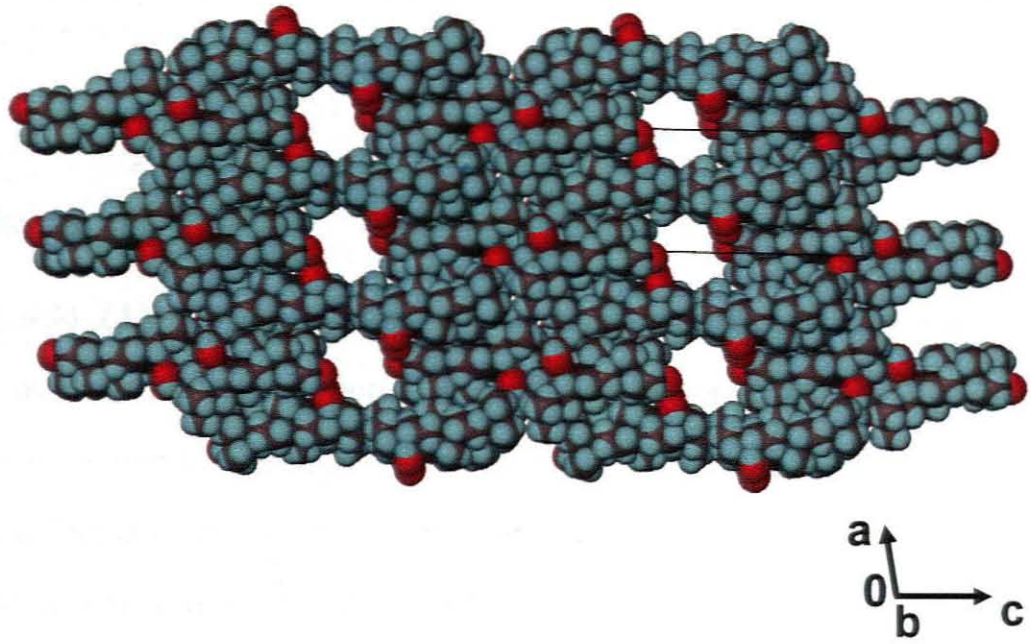


Figure 4.7 : Channels in oleanolic acid molecule viewed along [010]

CHAPTER 5

SYNTHESIS OF DERIVATIVES OF OLEANOLIC ACID

Introduction

Oleanolic acid **(1),** fig 5.1**,** has three functional groups, viz., a secondary hydroxyl at C-3, an olefinic bond at C-12 and a carboxylic acid at C-17. Synthesis of a number of derivatives of these functional groups has been carried out. The OA used in each of these syntheses was either purchased from Sigma and specified to be 97% pure, or the isolated OA was used.

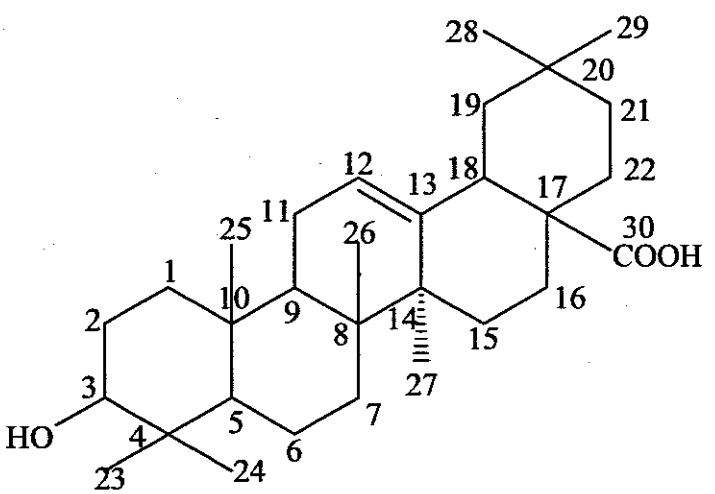


Figure 5.1 : Oleanolic acid, carbons numbered

The syntheses were carried out as depicted in Schemes 1, 2 and 3 on pages 32,

33 and 34.

The syntheses of 3-O-acetyloleanolic acid (**2**), methyl oleanolate (**3**), the "diester", 3-O-acetyl-17-methyl oleanolate (**4**) and the "diol", 3-hydroxy-17-hydroxymethyl olean-12-ene (**5**) are represented in Scheme 1 (Page 32).

Two of the functional groups of OA **(1)** were verified by the IR absorption bands at 3600 - 3300 (-OH) and 1705 (C=O) cm^{-1}. In the ^{1}H-NMR spectrum, protons on C-18 and C-3 were indicated by doublet of doublets at 2.82 (J 13.8 and 4.4) and 3.21 ppm (J 8.8 and 6.6) respectively, and on C-12 by a triplet (J 4.0) at 5.28 ppm due to the coupling with the two adjacent methylene protons. Agreement with literature values for ^{1}H-NMR and ^{13}C-NMR was established[39].

OA in anhydrous pyridine was acetylated with 100 equivalents of acetic anhydride to give 3-O-acetyloleanolic acid **(2)** in 92% yield. Acetoxy and carboxylic (1719 and 1668 cm^{-1}) carbonyl functions were shown by absorptions in the IR spectrum. The structure was confirmed by the signal at 2.04 attributable to CH_3 of the acetoxy group in the ^{1}H-NMR spectrum. The deshielding observed for H-3 from 3.21 to 4.49 ppm confirms its assignment due to the anisotropic field of the acetate carbonyl. The molecular mass (m/e) value of 498.36968 agreed with the calculated value for $C_{32}H_{50}O_4$ of 498.37091.

The COOH group at C-17 in OA was methylated in acetone by the addition of 10 equivalents of K_2CO_3 and iodomethane to form methyl oleanolate **(3)** with a yield of 84%. Absorptions due to –OH (3320 cm^{-1}), carbonyl groups (1728 and

1709 cm^{-1}) and –C-O-C (1252 cm^{-1}) functions were observed in the IR spectrum. The structure was confirmed by the ^{1}H-NMR singlet at 3.62 ppm attributable to the methoxy group. The HRMS of 470.37620 compared favourably with the calculated value for $C_{31}H_{50}O_3$ of 470.37600.

The diester **(4)** was obtained by acetylation of the OH at C-3 of the ester **(3)** in 86% yield. No hydroxyl absorption was present in the IR spectrum, but absorption of two carbonyl functions (1727 and 1696 cm^{-1}) were observed in the IR spectrum and the ^{1}H-NMR spectrum showed a singlet at 2.04 ppm ascribed to the acetate and 2.22 ppm ascribed to the methoxy group respectively. At 5.30 ppm a doublet of triplets was observed due to coupling of H-12. The compound was validated by the HRMS in which the M^+ had a value of 512.38534 ($C_{33}H_{52}O_4$ requires 512.38656).

The reduction of **(1)** gave a poor yield of diol **(5)** and thus attention was focussed on alternatively using **(4)** in diethyl ether with 10 equivalents of lithium aluminium hydride under nitrogen at 25°C to afford 18β-olean-12-ene-3β, 28 diol **(5)**. The yield was acceptable at 80%. The IR spectrum indicated the hydroxyl groups at 3350 cm^{-1}. The coupling between each of the diastereotopic H's on C-30 due to the chiral centre at C-18 were verified by the ^{1}H-NMR signals at 3.18 and 3.55 ppm each being a doublet (J 11.0). The molecular formula of $C_{30}H_{50}O_2$ was confirmed by a HRMS which gave a M^+ at 442.38022 (requires 442.38108).

Scheme 2 (Page 33) depicts the synthesis of 3 derivatives using diol **(5)** as the starting material viz., the "diester of diol", 3-acetoxy-17-methyl acetoxyolean-

12-ene (**6**), di-(3,17)-trifluoroacetoxy olean-12-ene (**7**) and 3-keto-olean-12-en-17-carbaldehyde (**8**).

Both hydroxyl groups on compound **(5)** in anhydrous pyridine were esterified with acetic anhydride to give the diester, 3-acetoxy-17-acetoxymethyl olean-12-ene (**6**) with a yield of 84% and melting point 176 – 178°C. IR spectrum peaks indicated the carbonyl group at 1735 cm^{-1} and the two C-O groups at 1240 and 1250 cm^{-1}. The ^{1}H-NMR spectrum had a singlet at 2.04 pm (6H) for the two acetate groups and two doublets (J 11.0) at 3.69 and 4.02 ppm for the two diastereotopic H's of the C-30 methylene group resulting from the influence of the chiral centre at C-18. The molecular formula of $C_{34}H_{54}O_4$ was confirmed by the HRMS which gave a M^+ at 526.40183 (requires 526.40221).

In a similar procedure as above, the diol **(5)** in anhydrous pyridine was esterified with trifluoroacetic anhydride to afford bis-trifluoroacetoxy oleanolic acid **(7)** with a 96% yield. A peak at 1785 cm^{-1} in the IR spectrum is ascribed to the ester carbonyl group. Two 1-proton doublets (J 11.0) at 4.02 and 4.27 ppm are ascribed to the diastereotopic C-30 methylene group and it is interesting to note that both signals are shifted upfield compared to the non-fluorinated analogue **(6)** due to the influence of the F atoms. The compound was validated by the HRMS in which the M^+ had a value of 634.3457 ($C_{30}H_{48}O_4F_6$ requires 634.34568).

Oxidation of the diol **(5)** with pyridinium chlorochromate in dichloromethane afforded the thick oily keto-carbaldehyde **(8).** The IR peaks indicated the ketone functional group on the molecule at 1708 cm^{-1}. Confirmation of the CHO

functional group was demonstrated in the ^{1}H NMR spectrum by the appearance of a 1-proton singlet 9.40 ppm.

Scheme 3 (Page 34) represents the synthesis of trifluoroacetoxy oleanolic acid (**9**), 3-keto oleanolic acid (**10**) and 12, 13-dibromo oleanolic acid (**11**).

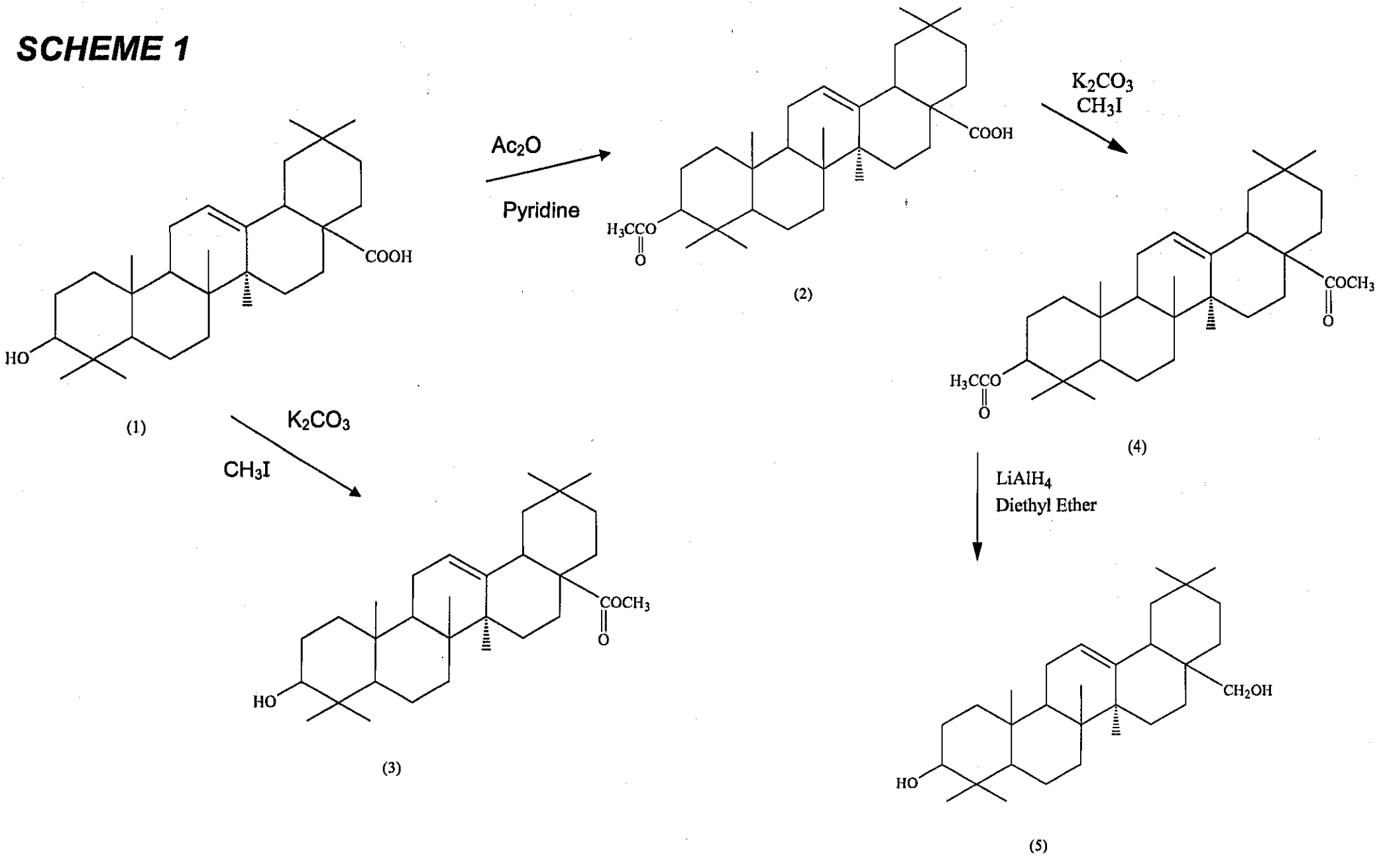
Trifluoroacetoxy oleanolic acid (**9**) was synthesised by acylating OA in anhydrous pyridine with trifluoroacetic anhydride with a 90% yield. The hydroxyl and the two carbonyl groups were observed in the IR spectrum at 3100-3300 (OH) and at 1820 and 1790 (C=O) $^{cm-1}$ respectively. The result for M^{+} of 552.34330 substantiated the mass for $C_{32}H_{47}O_4F_3$.

Oxidation of OA (**1**) with pyridinium dichromate under N_2 afforded the product 3-oxo-olean-12-en-28-oic acid (**10**). The IR peaks at 1718 and 1693 cm^{-1} indicated the two carbonyl groups (ketone and carboxylic acid) and at 3388 cm^{-1} the hydroxyl group was observed. The C=O was indicated by the ^{13}C resonance at 217.6 ppm and the mass of 468.32268 substantiated the formula of $C_{30}H_{44}O_4$.

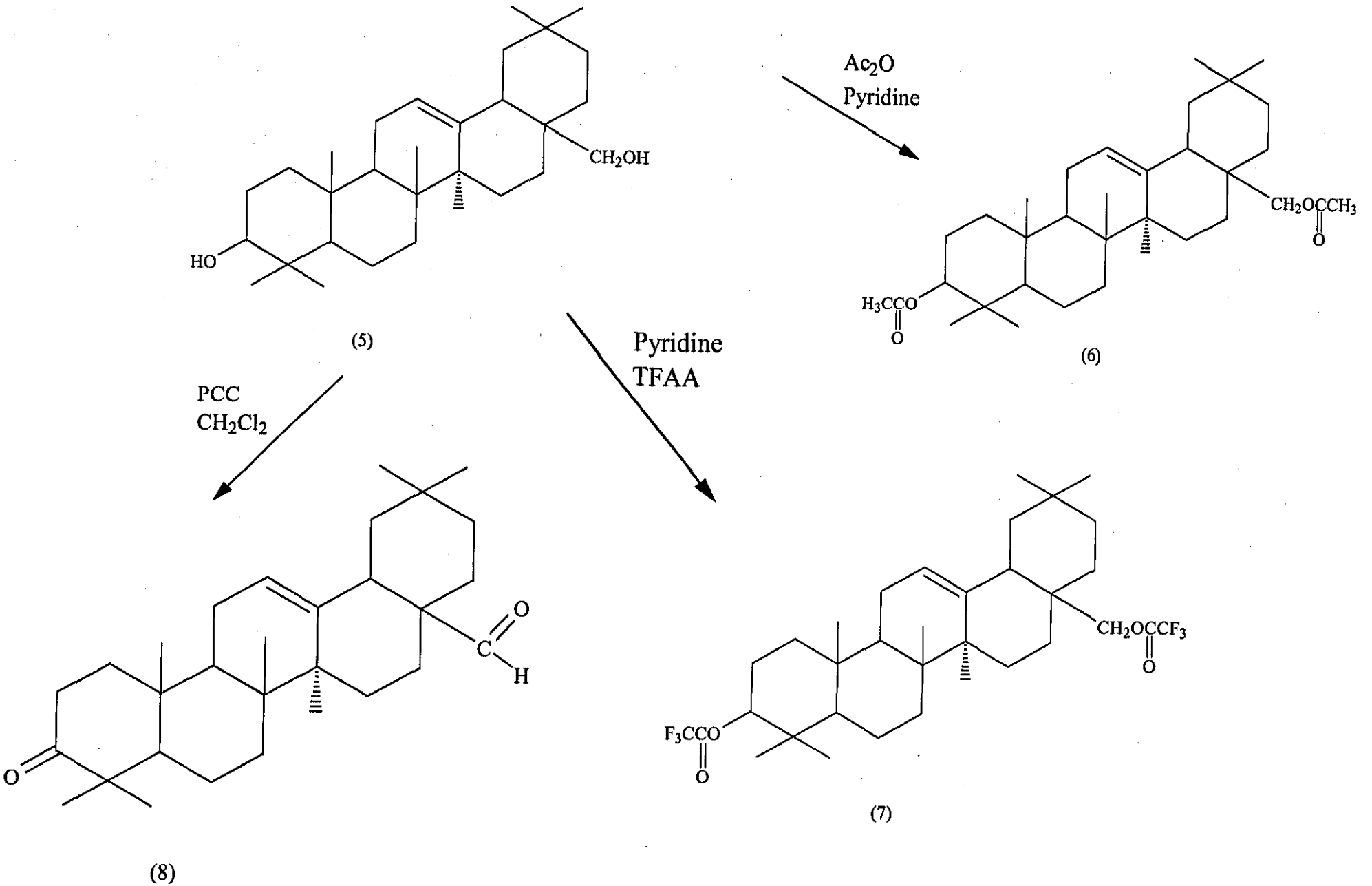
The olefinic bond between C-12 and C-13 was brominated by stirring OA (**1**) in bromine water. The hydroxyl and carbonyl groups were verified by the IR peaks. A mass spectrum signal at 613.1 gave indication of the $M\text{-}1^{+}$ ion.

OLEANOLIC ACID DERIVATIVE SCHEME

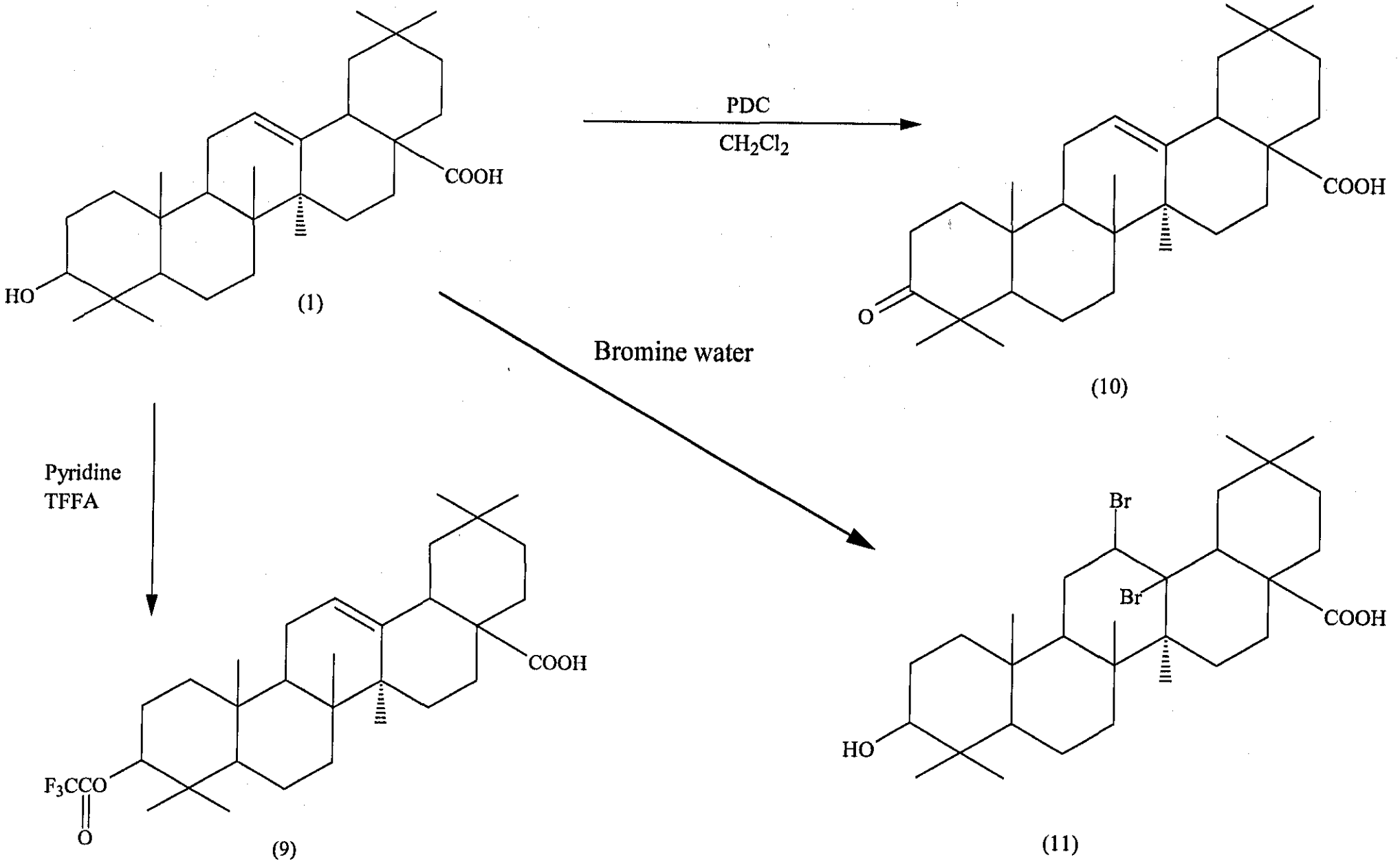
SCHEME 1



SCHEME 2

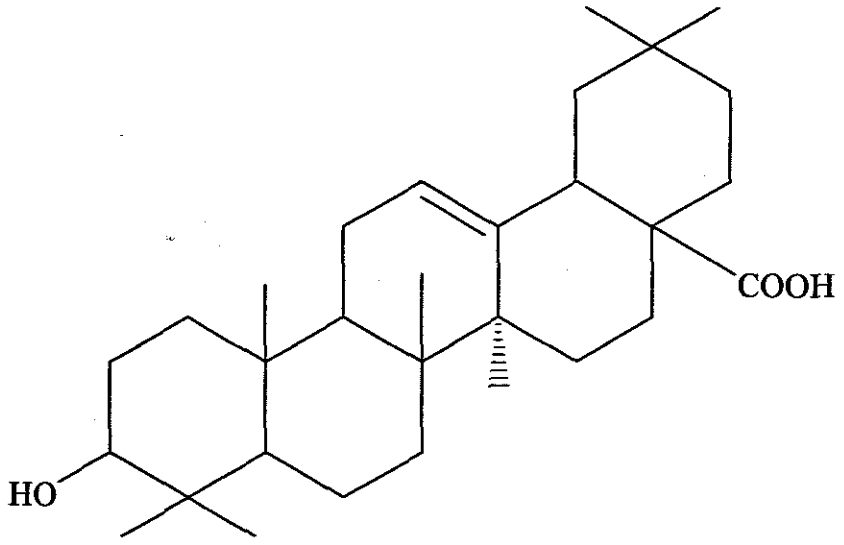

HO
CH2OH
(5)
Ac2O
Pyridine
CH2OCCH3
O
H3CCO
O
(6)
Pyridine
TFAA
PCC
CH2Cl2
O
C
H
O
(8)
CH2OCCF3
O
F3CCO
O
(7)

SCHEME 3


HO
COOH
(1)
PDC
CH_2Cl_2
O
COOH
(10)
Bromine water
Pyridine
TFFA
F_3CCO
O
COOH
(9)
Br
Br
HO
COOH
(11)

Experimental

Oleanolic Acid (1)



Extracted from dried clove buds, as described on pages 11 to 15, as a white powder. M.p. 310°C (from DCM:hexane) (Lit.[19,41] 310°C);

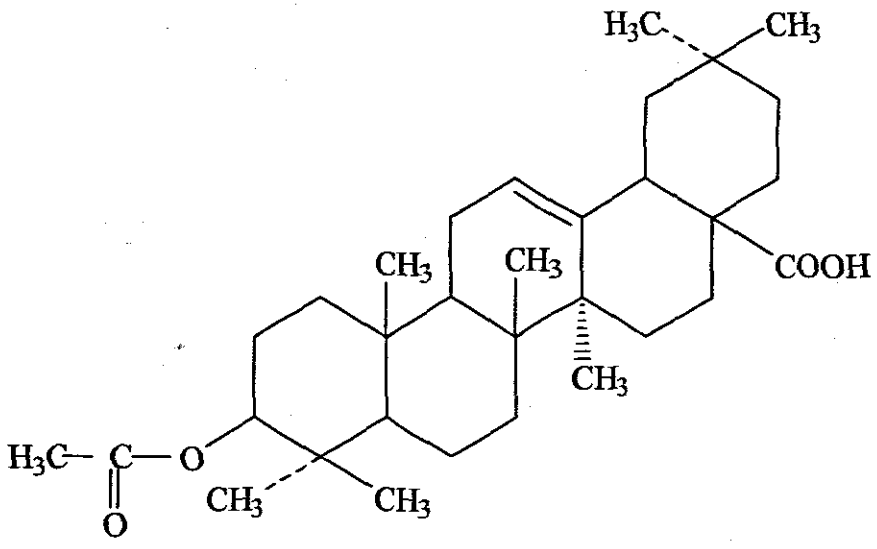
IR (cm^{-1}): ν_{max} 3600 - 3300 (OH) and 1705 (C=O);

^{1}H-NMR (200 MHz): δ ($CDCl_3$) 0.75, 0.77, 0.90, 0.91, 0.92, 0.98, 1.13 (7 x 3H, s, CH_3), 2.82 (1H, dd, J 13.3 and 4.4 Hz, H-18), 3.21 (1H, dd, J 8.8 and 6.6 Hz, H-3), 5.28 (1H, t, J 4.00 Hz, H-12), HO-3 and HO of carboxylic acid OH undetected;

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 38.9, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 79.2, 122.8, 143.7, 182.6;

Found: M^+, 456.36035; calc for $C_{30}H_{48}O_3$: M^+, 456.36035

3-O-Acetyloleanolic acid (2)



To a solution of oleanolic acid (242 mg; 0,531 mmol) in pyridine (1ml) was added acetic anhydride (5ml) and the reaction mixture was heated and stirred under reflux at 48°C for 1h. The cooled mixture was then treated with H_2O (50ml) and transferred to a separating funnel. The organic material was extracted into dichloromethane. Three drops of HCl (concentrated) were added to the dichloromethane extract which was subsequently washed with water (x2). The residue obtained upon workup was chromatographed using EtOAc:hexane (1:4) as eluent to afford the *product* (244 mg; 92%) as white crystals, m.p. 264°C (from DCM;hexane) (Lit. 268°C,[41]; 220-222°C, [19]);

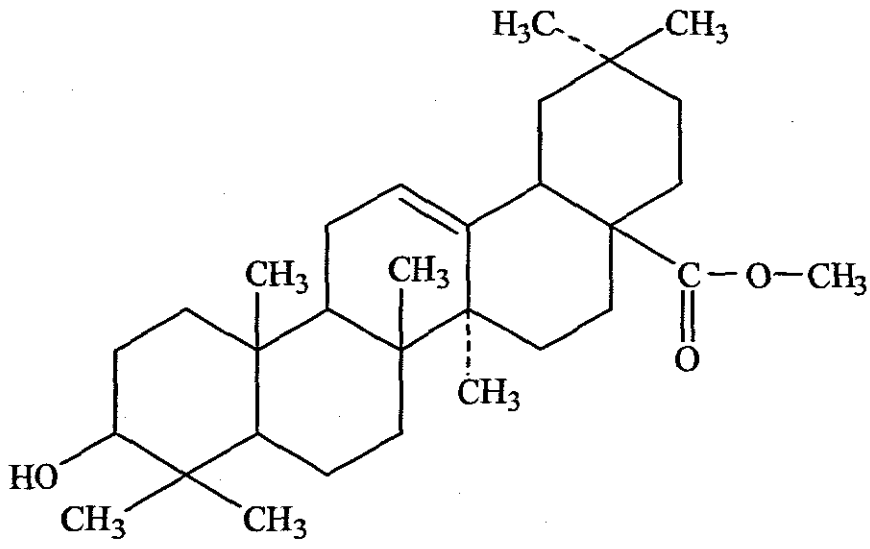
IR (cm^{-1}): ν_{max} 3180 (OH), 1719 and 1668 (C=O) and 1245 (C-O-C);

^1H NMR: δ ($CDCl_3$) 0.74, 0.84, 0.86, 0.90, 0.92, 0.93, 1.12 (7 x 3H, s, CH_3), 2.04 (3H, s, C-3 OAc), 2.81 (1H, dd, J 14.0 and 3.0 Hz, H-18), 4.49 (1H, dd, J 8.4 and 7.4 Hz, H-3), 5.27 (1H, t, J 3.6 Hz, H-12);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 81.1, 122.7, 143.7, 171.1, 182.6;

Found:M^+, 498.36968; Calc for $C_{32}H_{50}O_4$: M^+, 498.37091

Methyl oleanolate (3)



To a solution of OA (250 mg ; 0,548 mmol) in acetone (10 ml) were added K_2CO_3 (10 equivalents, 756 mg; 5,48 mmol) and iodomethane (5 ml; mmol). The reaction mixture was heated and vigorously stirred under reflux for 2.5h after which time no starting material remained (t.l.c.). The solid carbonate was filtered off, and the residue obtained upon removal of solvent was chromatographed using EtOAc:hexane (3:7) as eluent to afford the *product* (211 mg; 84%) as white crystals with m.p. of 199 - 201°C (from DCM:hexane). (Lit. 201°C[41])

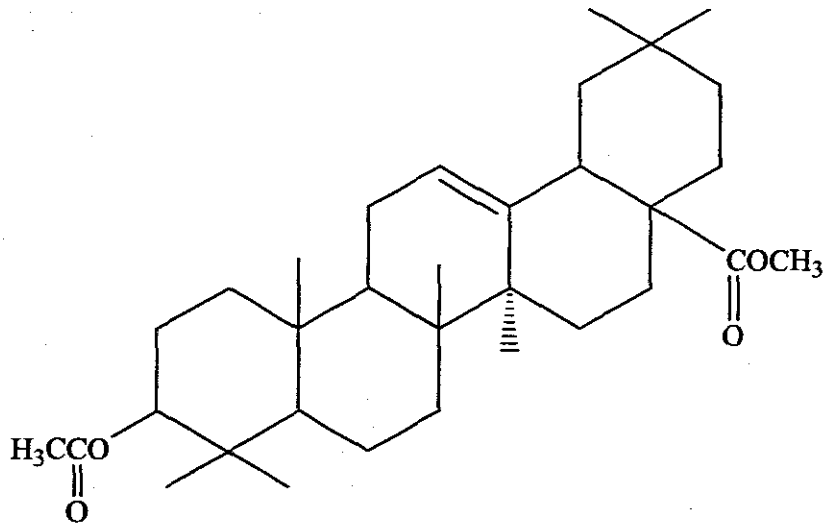
IR (cm^{-1}): ν_{max} 3320 (OH), 1728 and 1709 (C=O) and 1252 (C-O-C);

^{1}H NMR: δ ($CDCl_3$) 0.72, 0.78, 0.89, 0.90, 0.92, 0.98 and 1.13 (7 x 3H, s, CH_3), 2.85 (1H, dd, J 14.4 and 4.2 Hz, H-18), 3.21 (1H, dd, J 5.7 and 9.7 Hz, H-3), 3.62 (3H, s, -OMe), 5.27 (1H, t, J 3.5, H-12);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 79.1, 122.5, 143.9, 178.4;

Found: M^+, 470.37620 Calc for $C_{31}H_{50}O_3$: M^+, 470.37600.

3-O-Acetyl-17-methyl oleanolate (4)



To 3-acetoxy-OA (230 mg; 0,462 mmol.) was added K_2CO_3 (637 mg; 4,62 mmol.) and iodomethane (5 ml) and the resulting mixture was vigorously stirred and heated under reflux for 3h. The cooled mixture was filtered and the residue obtained by removal of solvent was chromatographed using EtOAc:hexane (1:4) to afford the *product* (206 mg ; 86%) m.p. of 203°C (from DCM:hexane). (Lit. 223°C[41]).

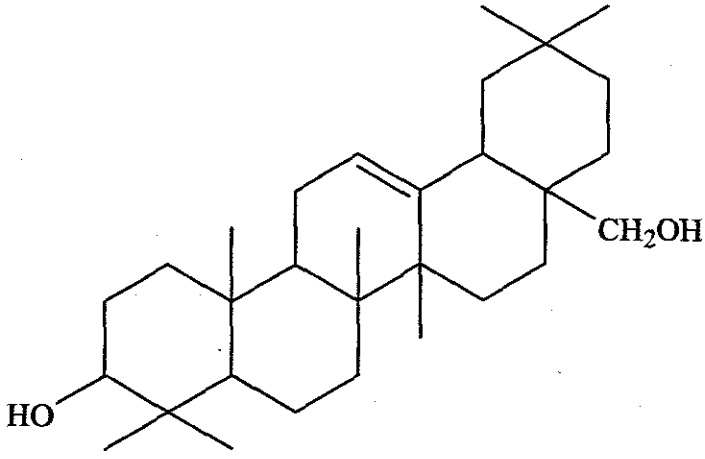
IR (cm^{-1}): ν_{max} 1727 and 1696 (C=O), 1247 (C-O-C);

^1H NMR: δ ($CDCl_3$) 0.71, 0.80, 0.85, 0.86, 0.89, 0.92, 1.14 (7 x 3H, s, CH_3), 2.04 (3H, s, CH_3CO), 2.22 (3H, s, CO_2CH_3), 4.49 (1H, dd, J 8.0 and 7.2, H-3), 5.30 (1H, dt, J 9.6 and 3.5, H-2);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 69.8, 79.1, 122.5, 143.7, 144.3, 182.3;

Found M^+, 512.38534, calc for $C_{33}H_{52}O_4$, 512.38656.

18β-olean-12-ene-3β, 28 diol (5)



Diester **(4)** (160 mg; 0,313 mmol) in ether (10 ml) was added dropwise to a slurry of LAH_4 (120 mg; 3.13 mmol) in diethyl ether (10 ml). Stirring was continued for 1.5h at 25°C under N_2. The reaction was quenched by the dropwise addition of saturated ammonium chloride solution. The mixture was dried, filtered and the residue obtained upon evaporation was chromatographed using EtOAc:hexane (1:4) to afford the *product* (0,249 mmol.; 80%) with m.p 223°C (from DCM:hexane) (Lit. 236 - 237°C, [40]).

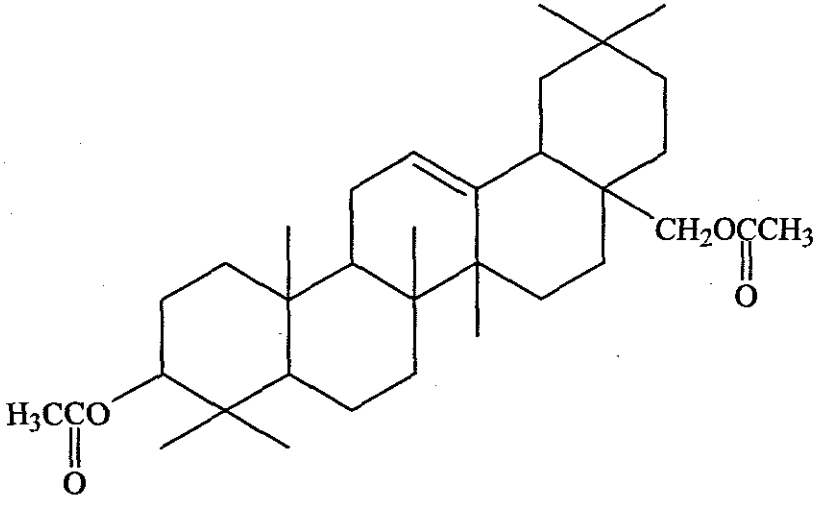
IR (cm^{-1}): ν_{max} 3350 (OH);

^1H NMR: δ ($CDCl_3$) 0.78, 0.87, 0.88, 0.92, 0.93, 0.99 and 1.16 (7 x 3H, s, CH_3), 3.18 (1H, d, J 11.0, CH_2OH), 3.23 (1H, t, J 5.2, H-3), 3.55 (1H, d, J 11.0, CH_2OH);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 81.0, 122.5, 143.7;

Found M^+, 442.38022, calc for $C_{30}H_{50}O_2$, 442.38108

3-Acetoxy-17- acetoxymethyl olean-12-ene (6)



To the diol **(5)** (187 mg; 0,422 mmol) in pyridine (1 ml) was added acetic anhydride (5 ml) and the resulting solution was heated with stirring at 48°C for 2h under reflux. The product was worked up using the same method described for **(2)**. After chromatography in EtOAc:hexane (1:4) the product (186 mg; 84%) with m.p. 176°- 178°C (from DCM:hexane) was isolated as white crystals.

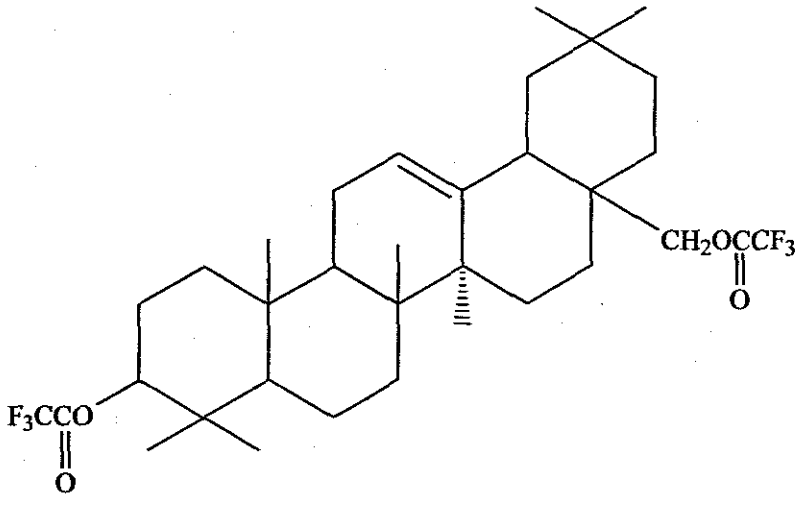
IR (cm^{-1}): ν_{max} 1735 (C=O) and 1240 and 1250 (C-O-C);

^1H NMR: δ ($CDCl_3$) 0.78, 0.87, 0.88, 0.92, 0.93, 0.99 and 1.16 (7 x 3H, s, CH_3), 2.04 (6H, s, 2 x CH_3CO), 3.69 (1H, d, J 11.0 Hz, CH_2OCOCH_3), 4.02 (1H, d, J 11.0 Hz, CH_2OCOCH_3), 4.49 (1H, dd, J 8.6 and 7.4 Hz, H-3), 5.19 (1H, t, J 3.2 Hz, H-12) ;

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 70.9, 81.0, 122.9, 143.7, 171.1, 171.4, 182.3;

Found M^+, 526.40183, calc for $C_{34}H_{54}O_4$, 526.40221.

Bis-trifluoroacetoxy oleanolic acid (7)



To the diol **(5)** (38 mg; 0.0855 mmol) in pyridine (1 ml) was added trifluoroacetic anhydride (5 ml) and the resulting solution was heated with stirring for 3h under N_2 at 48°C under reflux. The product was worked up using the same method as for **(2)**. The residue obtained was chromatographed using EtOAc:hexane (1:4) as eluent to afford the *product* (52 mg; 96%) as white

crystals with m.p. 188-190°C (from DCM:hexane).

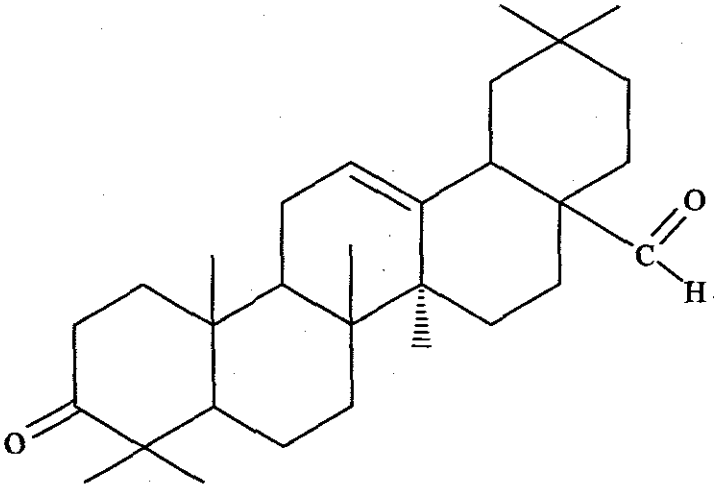
IR (cm^{-1}):ν_{max} 1785 (C=O);

^{1}H NMR: δ ($CDCl_3$) 0.88, 0.91, 0.92, 0.96, 0.99, 1.14, 1.18 (7 x 3H, s, CH_3), 4.02 (1H d, J 11.0 Hz, CH_2OOCF_3), 4.27 (1H, d, J 11.0, CH_2OOCF_3), 4.69 (1H, dd, J 11.4 and 5.6 Hz, H-3), 5.24 (1H, t, J 3.2 Hz, H-12);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 74.3, 86.3, 111.9, 117.7, 123.4, 143.1, 157.1;

Found M^+: 634.34530, calc for $C_{34}H_{48}O_4F_6$ 634.34568.

3-Keto-olean-12-en-17-carbaldehyde (8)



To the diol **(5)** (80.3 mg; 0.1816 mmol) in DCM (10 ml) was added PCC (587 mg; 2.725 mmol). This was stirred under N_2 in the fume hood for 12h. The solution was filtered and chromatographed using EtOAc:hexane (1:4) to afford the *product* as a thick oily substance (47.8 mg; 60.1%).

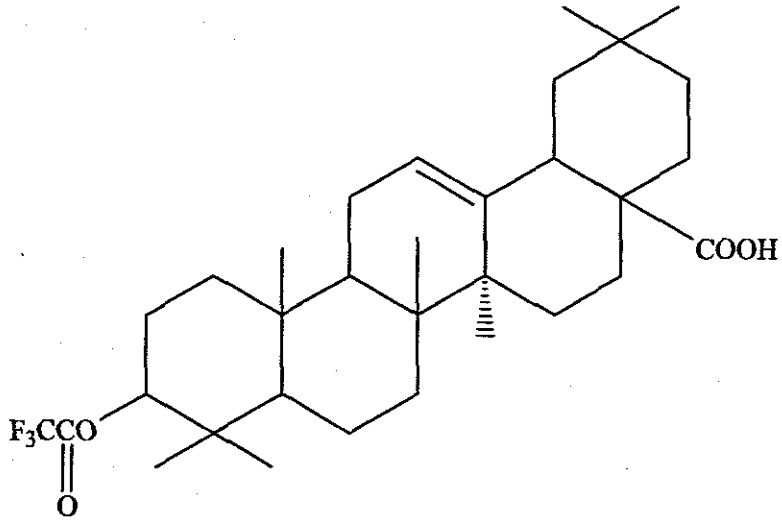
IR (cm^{-1}): ν_{max} 1708 (C=O);

^1H NMR: δ ($CDCl_3$) 0.78, 0.79, 0.91, 1.03, 1.07, 1.13, 1.24 (7 x 3H, s, CH_3), 5.35 (1H, t, J 3.6 Hz, H-12), 9.40 (1H, s, *CH*O);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 123.1, 143.1, 207.4;

Found M^+: no MS results obtained for $C_{30}H_{46}O_3$

3-Trifluoroacetoxy oleanolic acid (9)



Oleanolic acid **(1)** (203 mg; 0,445 mmol) in pyridine (1 ml) was treated with trifluoroacetic anhydride (5 ml) and the solution stirred at 48°C for 3h under reflux. Water (50 ml) was added to the solution and the organic material was extracted into DCM using a separating funnel. Hydrochloric acid (3M; 3ml) was added to neutralise the pyridine and the DCM washed twice with water. The solution was dried, filtered and the residue was chromatographed with EtOAc:hexane to afford the *product* (222 mg; 90%). M.p. 280-282°C (from

DCM:hexane).

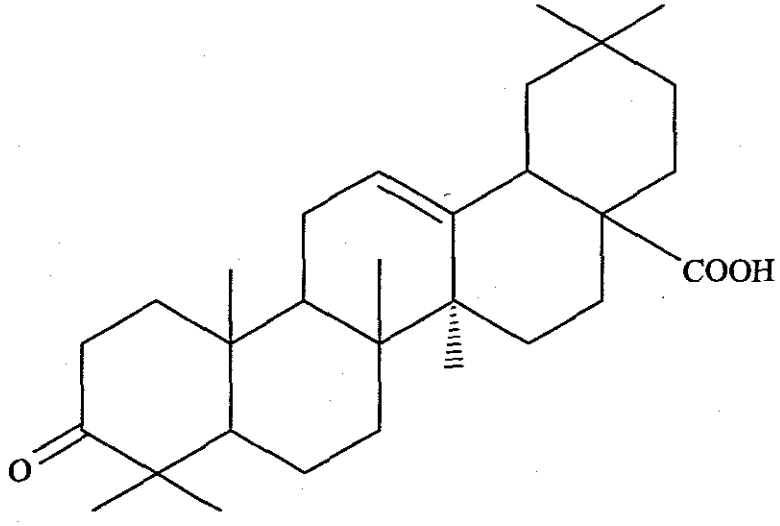
IR (cm^{-1}) ν_{max} 3100-3300 (OH), 1820, 1790 (C=O);

^1H NMR: δ ($CDCl_3$) 0.76, 0.81, 0.90, 0.93, 0.96, 1.13, 1.25 (7 x 3H, s, CH_3), 2.82 (1H, dd, J 14.4 and 4.2 Hz, H-18), 4.70 (1H, dd, J 11.0 and 6.0 Hz, 3-H), 5.28 (1H, t, J 3.2 Hz, H-12);

^{13}C-NMR : 15.4, 15.7, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 86.4, 122.5, 143.1, 180.6, 194.5;

Found M^+: 552.34330, calc for $C_{32}H_{47}O_4F_3$ 552.34265.

3-Oxo-olean-12-en-28-oic acid (10)



To a solution of oleanolic acid **(1)** (105.6 mg; 0.2316 mmol) in DCM was added PDC (1.31 g; 3.474 mmol) and the solution stirred at 25°C under N_2 for 12h. The solution was filtered and the residue chromatographed with EtOAc:hexane to afford the white crystalline *product* (68.4 mg; 65%). M.p. 109 - 111°C (from

DCM:hexane).

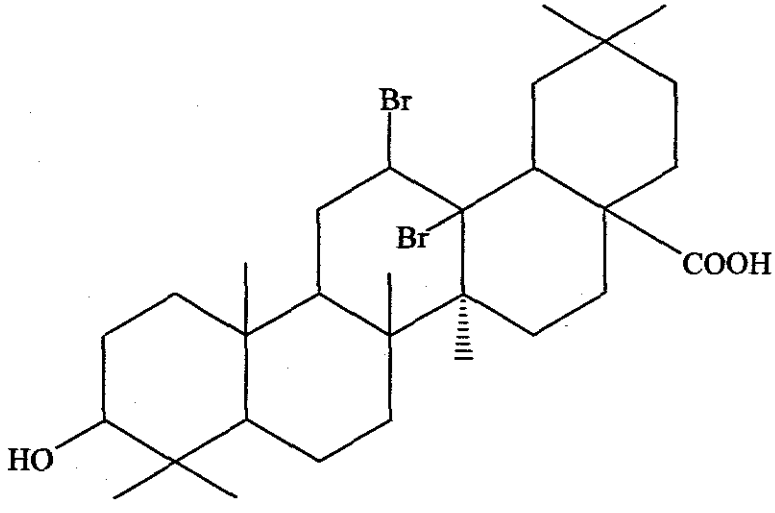
IR (cm^{-1}): ν_{max} 3388 (OH), 1718 and 1693 (C=O for ketone and carboxylic acid);

^1H NMR: δ ($CDCl_3$) 0.81, 0.90, 0.93, 1.03, 1.04, 1.08, 1.14 (7x3H, s, CH_3), 2.82 (1H, dd, J 13.6 and 5.3 Hz, H-18), 5.30 (1H, t, J 3.6 Hz, H-12);

^{13}C-NMR : 15.4, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 122.6, 143.8, 182.4, 217.6;

Found M^+: 468.32268, calc for $C_{30}H_{44}O_4$ 468.32396.

12, 13 dibromo oleanolic acid (11)



To a solution of oleanolic acid **(1)** (102 mg; 0.224 mmol) in DCM (10 ml) was added bromine water (10 ml) and the resultant mixture was stirred for 30 min. The organic phase was separated and the residue obtained upon workup was chromatographed with EtOAc:hexane (1:4) to afford the *product* (71.7 mg, 52

%). M.p. 243 – 245°C (from DCM:hexane).

IR (cm^{-1}): ν_{max} 3406 (OH), 1767 (C=O).

^1H NMR: δ ($CDCl_3$) 0.78, 0.89, 0.90, 1.0, 1.22, 1.44, 1.52 (7 x 3H, s, CH_3), 3.26 (1H, dd, J 11.0 and 5.4 Hz, H-3), 4.30 (1H, dd, J 3.6 and 2.2, H-12);

^{13}C-NMR : 15.4, 17.0, 18.5, 23.2, 23.5, 23.8, 26.1, 27.4, 27.9, 28.2, 30.8, 32.5, 32.8, 33.2, 34.0, 37.2, 37.8, 38.6, 39.4, 41.5, 41.8, 46.0, 46.9, 47.8, 51.6, 55.4, 78.8, 178.9, 217.5;

Found M-1^+: 613.1 for $C_{30}H_{47}O_3Br_2$.

CHAPTER 6

ANTIMICROBIAL PROCEDURES AND EVALUATIONS

Laboratory procedures are commonly used to test compounds for antibacterial or antifungal activity. The sensitivity of this *in vitro* assay is dependent on qualities such as the solubility of the antibiotic, the antibiotic's initial concentration, the diffusion of the drug through the medium used as well as the procedure used to determine the susceptibility of the pathogen. In our case, qualitative tests were conducted to determine whether OA or any of the derivatives synthesised had the ability to inhibit the growth of bacteria or fungi of importance to humans.

The minimum inhibitory concentration (MIC) is the least amount of antimicrobial that will inhibit visible growth of an organism after overnight incubation. The minimum bactericidal concentration (MBC) is the amount of agent that will prevent growth after subculture of the organism to antibiotic-free medium [42].

The compounds were tested against three Gram-positive and three Gram-negative organisms. The difference between these two types of bacteria is determined by the physical nature of their cell walls[43]. If the cell wall of Gram-positive bacteria is removed it becomes Gram-negative. The peptidoglycan

layer is not stained, but acts as a permeability barrier to prevent the loss of stain. During the staining procedure the bacteria are first stained with crystal violet followed by iodine to promote dye retention. When bacteria are decolourised with ethanol, Gram-positive bacteria remain purple but the colour is removed from Gram-negative bacteria. Further staining with safranin causes Gram-negative bacteria to become counter stained, giving them a red colour. The Gram-positive cell wall has a relatively thick murein layer that traps the primary stain. Gram-positive bacteria are more susceptible than Gram-negative bacteria to the antibacterial actions of penicillin, acids, iodine, basic dyes, detergents and lysozyme, and less susceptible to alkalies, azide, tellurite, proteolytic enzymes, and plasmolysis in solutes of high osmotic pressure.[44]

The organisms used for testing purposes were Gram-positive *Staphylococcus aureus, Bacillus subtilis* and the fungus *Candida albicans*. Gram-negative organisms were *Pseudomonas aeruginosa, Proteus vulgaris* and *Eschericia coli.* These test organisms were obtained from the South African Bureau of Standards (SABS) in Pretoria for experimental work.

Staphylococcus aureus is a cluster-forming Gram-positive cocci[44]. The organisms are non-sporing, non-motile and usually non-capsulate. *Staph. aureus*, which is present in the nose and on the skin of healthy people, is an opportunistic pathogen, as it causes infection at sites of lowered host resistance, such as, damaged skin or mucous membranes. *Staph. aureus* is sensitive to many antimicrobial agents, especially penicillins.

Bacillus subtilis has been implicated in causing food poisoning. They may also

be found in wounds and tissues of burned patients.

Pseudomonas aeruginosa is a Gram-negative bacillus, motile using one or two polar flagella. The organism grows readily over a wide temperature range and emits a sweet grape-like odour. *Ps. aeruginosa* infections are usually mild and shallow such as varicose ulcers, but can become more severe such as urinary tract infection, bed sores and eye infections.

Proteus vulgaris has the ability to *swarm* on solid media : the bacterial growth spreads from the edge of the colony and eventually covers the whole surface of the medium. The species has the ability to oxidatively de-aminate amino acids. Strains are resistant to penicillins, but intrinsically sensitive to aminoglycosides.

Strains of the Gram-negative 'coliform' bacteria, *Eschericia coli*, predominate among the aerobic parasitic flora present in the gut of man and animals. Certain strains are pathogens in man and animals and cause both septic infection and diarrhoea.

Yeasts, such as *Candida albicans,* affect the skin, nail and mucous membranes of the mouth and vagina; these are commonly known as *thrush*. Superficial *Candida* infections respond well to treatment with amphotericin B.

Three different methods were used in the determinations for antimicrobial activity of each of the compounds synthesised.

1. Kirby-Bauer Disk Diffusion Technique

The disk diffusion technique used to measure inhibition by the compounds was the Kirby-Bauer method. This agar diffusion technique is used to determine bacterial susceptibility to antimicrobial agents[45]. The size of the zone of inhibition reflects the degree of susceptibility of the test strain: the larger the zone of inhibition, the more susceptible the test organism.

Experimental

Cultures of each organism were grown overnight on nutrient agar and these were used to streak freshly prepared agar plates. Three concentrations of each compound were prepared namely 2.81 μmol, 1.4 μmol and 0.7 μmol and were dissolved in acetone : DCM (1:1) as the solvent. This was chosen as the result of the low solubility and hydrophobic properties of OA and its derivatives. The solutions were injected onto filter paper disks (740-E, Schleider & Schuell, 9mm), making sure that the excess solvent had been removed. Three disks were placed onto the nutrient agar plate and were incubated at 37°C for approximately 18 hours. Blank filter paper disks which had been saturated with the same volume of solvent were prepared. Chloromycetin (Chloramphenicol) and Ampicillin (Amphotericin) were used as standards.

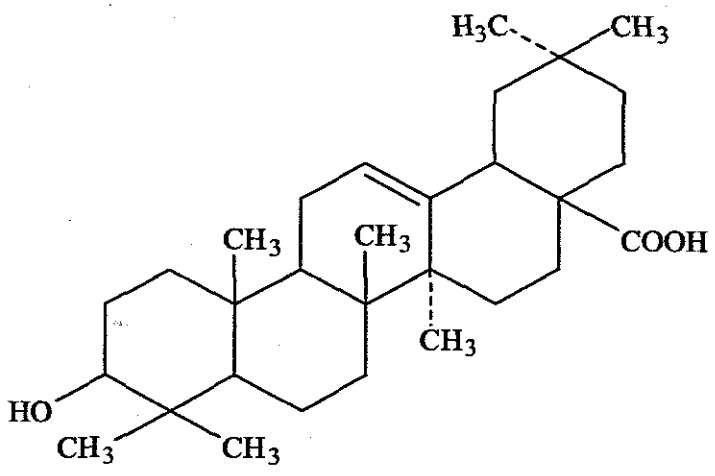
Diffusion of the antibiotic into the agar established a concentration gradient. A clear zone (zone of inhibition) around the antibiotic disk indicated inhibition of microbial growth. The diameter of the zone of inhibition reflects the solubility of the particular antibiotic - that is, the concentration gradient established by diffusion of the antibiotic into the agar – and the sensitivity of the given micro

organism to the specific antibiotic.

Evaluations

Tables 6.1 to 6.11 show the inhibitory activity of the eleven compounds tested.

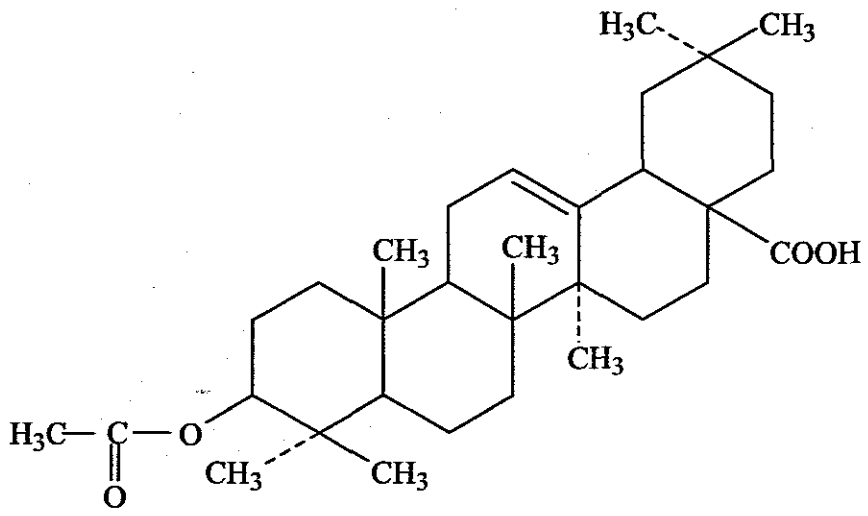
Table 6.1. Inhibitory activity of oleanolic acid (1) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.80 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.80 1.4 0.7	0 0 0
Candida albicans fungus	2.80 1.4 0.7	11 10.8 0

Gram Negative Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.80 1.4 0.7	0 11 10
Proteus vulgaris SATCC Pre 1	2.80 1.4 0.7	11 11 0
Eschericia coli SATCC Esc 25	2.80 1.4 0.7	11 0 0

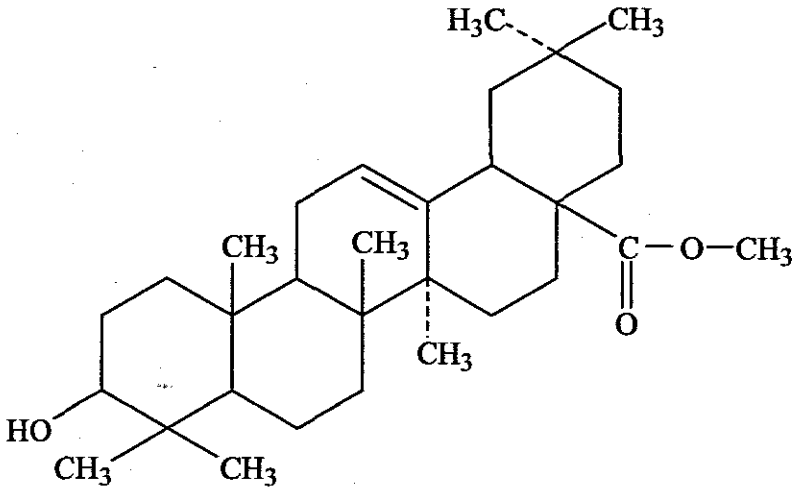
Table 6.2. Inhibitory activity of 3-O-acetyloleanolic acid (2) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilus SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	11 10.8 0

Gram Negative Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 11.3 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	10.8 12.5 11
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 11 10.8

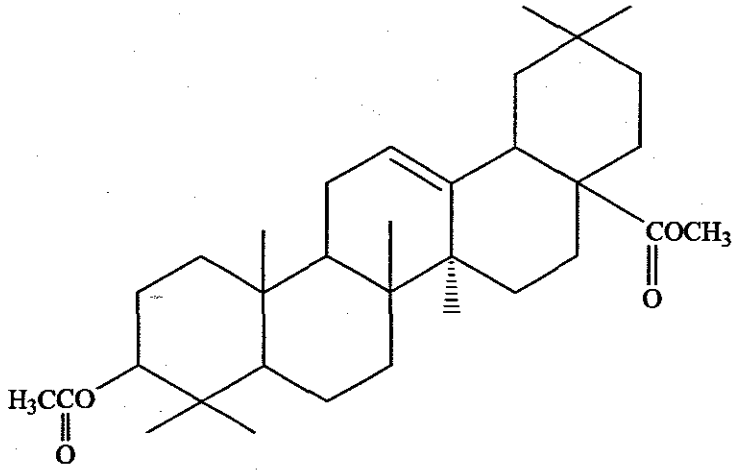
Table 6.3. Inhibitory activity of methyl oleanolate (3) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus Subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

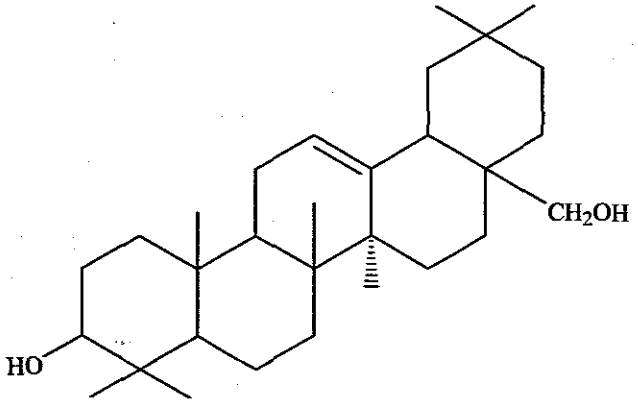
Table 6.4. Inhibitory activity of diester (4) against test organisms as measured by zones of inhibition (mm)



Gram Positive Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

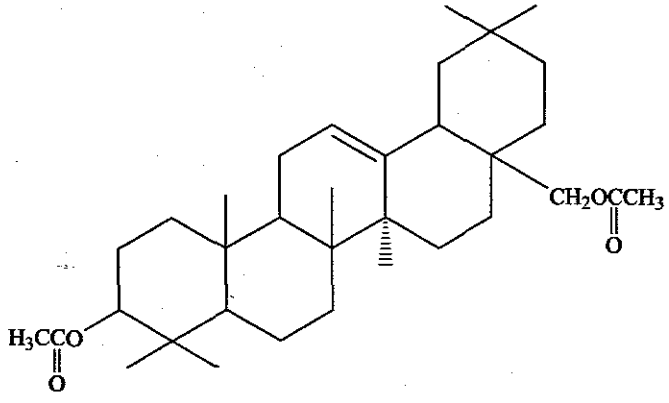
Table 6.5. Inhibitory activity of diol (5) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

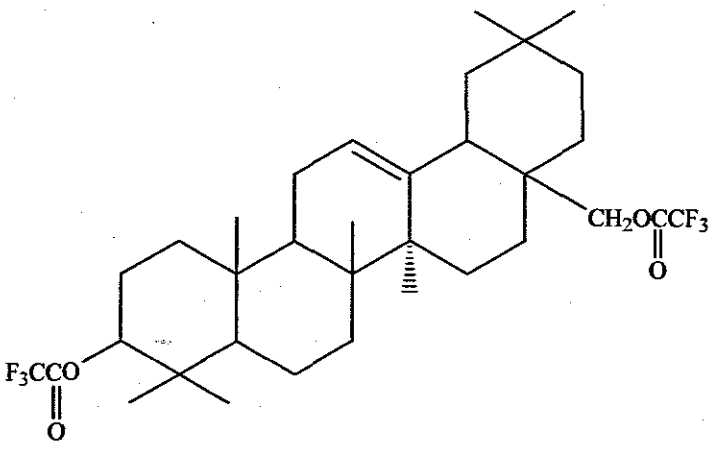
Table 6.6. Inhibitory activity of 3-acetoxy-17- acetoxymethyl olean-12-ene (6) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

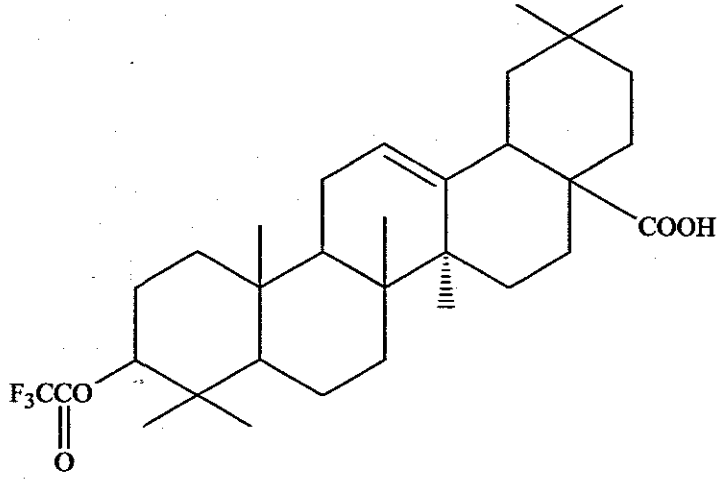
Table 6.7. Inhibitory activity of bis-trifluoroacetoxy oleanolic acid (7) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

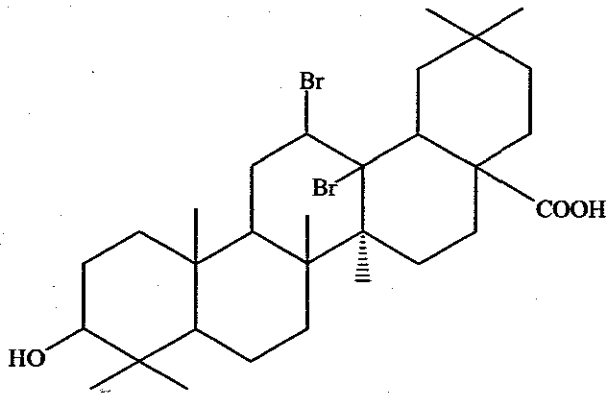
Table 6.8. Inhibitory Activity of trifluoroacetoxy oleanolic acid (9) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

Table 6.9. Inhibitory activity of 12, 13 dibromo oleanolic acid (11) against test organisms as measured by zones of inhibition (in mm)



Gram Positive Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	0 0 0
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	0 0 0
Candida albicans fungus	2.81 1.4 0.7	0 0 0

Gram Negative Organism and SABS culture number	Dose in µmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	0 0 0
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	0 0 0
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	0 0 0

Table 6.10. Inhibitory activity of standard Chloromycetin (Chloramphenicol) against test organisms as measured by zones of inhibition (in mm)

Gram Positive Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	27 29 29
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	33 36 31
Candida albicans fungus	2.81 1.4 0.7	25 25 26

Gram Negative Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	18 24 22
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	23 27 28
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	25 25 26

Table 6.11. Inhibitory activity of standard Ampicillin against test organisms as measured by zones of inhibition (in mm)

Gram Positive Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Staphylococcus aureus SATCC Sta 53	2.81 1.4 0.7	42 41 46
Bacillus subtilis SATCC Bac 96	2.81 1.4 0.7	23 23 27
Candida albicans fungus	2.81 1.4 0.7	15 15 17

Gram Negative Organism and SABS culture number	Dose in μmol	Zone of inhibition in mm
Pseudomonas aeruginosa SATCC Pse 2	2.81 1.4 0.7	- 16 14
Proteus vulgaris SATCC Pre 1	2.81 1.4 0.7	25 21 27
Eschericia coli SATCC Esc 25	2.81 1.4 0.7	18 20 23

Discussion

The results measured for each concentration were averaged for triplicate results. In the case of the first four compounds the antimicrobial test had been repeated a number of times. The results for oleanolic acid and acetoxy oleanolic acid had shown some activity only once during the repeated testing. To verify these results the concentration of the solution of compound was increased to 6 μmol. The results from this further testing showed a lack of any inhibition by the compound against the bacteria or fungal organisms.

In a study by F.Hichri et al. oleanolic acid had been isolated from fruit barks of *Periploca laevigata* and acetoxy oleanolic acid was synthesised[19]. These two compounds showed a moderate activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* bacteria. We were unable to show any level of activity against the bacteria or fungus using the Kirby-Bauer disk diffusion method. Even though activity was shown in Tables 6.1 and 6.2 for **(1)** and **(2),** this was not repeated when the concentrations were increased to 6 μmol of the compound.

2. Broth Dilution Method

The second method for antimicrobial activity involved preparation of the organisms in nutrient broth. A decimal dilution method was used to prepare solutions in an emulsified solvent[42]. To overcome the hydrophobicity of the compounds Tween 80 was added to the sterile physiological water at a concentration of 10% (v/v). To 1 ml of the compound solution 9 ml of broth prepared with the bacteria 24 hours previously and incubated at 37°C was added. A blank and a standard were also analysed.

Experimental

After 18 hours incubation, the amount of turbidity was measured against the blank. A decrease in turbidity indicated an inhibitory affect of the compound over the organism. A subculture was prepared by streaking an agar plate with a loopful from the tube. After incubation at 37°C for 18 hours the plates were

examined for growth. The tube containing the lowest concentration of antibiotic that fails to yield growth on the subculture plate contains the MBC of antibiotic for the test strain.

The products were tested against the same organisms as in the Kirby-Bauer method. The solutions were made to the following concentrations: 128; 64; 32; 16; 8; 4; 2; 1; 0.5 and 0.25 mg/l. The organisms were grown in broth solution. One ml of the solution (acetone : DCM (1:1)) plus 9 ml of broth solution were incubated for 18 hours and inspected for growth inhibition.

Evaluation

The results for the broth dilution method are shown in Table 6.12.

Table 6.12. Results showing activity / inhibition across the highest and lowest concentrations of the compounds

Compound	Organism	Highest concentration (128mg/l)	Lowest concentration (0.25mg/l)
Oleanolic acid **(1)**	*Staph. aureus*	+	+
	B. subtilis	-	-
	C. albicans	+	+
	Ps. aeruginosa	+	+
	P. vulgaris	+	-
	E. coli	-	-
3-O-Acetyloleanolic acid **(2)**	*Staph. aureus*	+	-
	B. subtilis	-	-
	C. albicans	-	-
	Ps. aeruginosa	+	-
	P. vulgaris	-	-
	E. coli	+	-
Methyl oleanolate **(3)**	*Staph. aureus*	+	-
	B. subtilus	-	-
	C. albicans	-	-
	Ps. aeruginosa	+	-
	P. vulgaris	-	-
	E. coli	-	-
Diester **(4)**	*Staph. aureus*	+	+
	B. subtilus	-	-
	C. albicans	+	-
	Ps. aeruginosa	+	+
	P. vulgaris	-	-
	E. coli	-	-
Standard (Chloromycetin)	*Staph. aureus*	+	±
	B. subtilis	-	-
	C. albicans	+	+
	Ps. aeruginosa	+	+
	P. vulgaris	+	+
	E. coli	-	-

Key : + implies activity ; - implies no activity; ± implies possible activity

The activity or inhibition of the compound was difficult to measure due to the

lack of miscibility of the compound solution with the aqueous broth solution. Activity might have been shown had the compound reached the bacterial cells. However, the denser compound solution remained on the bottom of the tube.

DMSO solvent was also attempted for the compound. The strongest concentration (128 mg/l) and the weakest concentration (0.25 mg/l) were mixed with the broth (2 ml:8 ml) and the turbidity after 18 hours incubation at 37°C was determined for antibacterial activity. These are shown in Table 6.13.

Table 6.13. Results showing activity / inhibition against highest and lowest concentrations in DMSO

Compound	Organism	High conc (128 mg/l)	Low conc (0.25 mg/l)
Oleanolic acid **(1)**	*Staph. aureus*	-	-
	B. subtilis	-	+
	C. albicans	-	-
	Ps. aeruginosa	-	-
	E. coli	-	-
3-O-Acetyloleanolic acid **(2)**	*Staph. aureus*	-	-
	B. subtilis	-	+
	C. albicans	-	-
	Ps. aeruginosa	-	+
	E. coli	-	+
Diester **(4)**	*Staph. aureus*	-	-
	B. subtilus	+	-
	C. albicans	-	-
	Ps. aeruginosa	-	+
	E. coli	-	+
Standard (Chloromycetin)	*Staph. aureus*	-	-
	B. subtilis	+	+
	C. albicans	+	+
	Ps. aeruginosa	+	+
	E. coli	+	+

Discussion

The explanation of the results obtained is possibly again linked to the solubility of the compound in the broth. Being an aqueous mixture, the oleanolic acid and its derivatives will precipitate in this medium, as they are all hydrophobic. This explains the reason why precipitate or turbidity was picked up by the high concentration of the compound in DMSO whereas the low concentration appeared clear due to the higher solubility of a low concentration of compound.

Due to the properties of the oleanolic acid and its derivatives it was felt that the broth solvent should be changed to an emulsified aqueous solution. An emulsifier in the form of 10% Tween was added to the broth for incubating the bacteria. This method of sample preparation had been used by F.Hichri for non water-soluble compounds[19]. The compound made up in 10% Tween solution, was diluted decimally to concentrations of 2000, 1000, 100, 10 and 1 $\mu g.ml^{-1}$. These were diluted 1:9 with the nutrient broth before incubation. The solution after 18 hours incubation at 37°C was streaked on agar plates. These were incubated for another 18 hours and the numbers of bacteria on each plate were counted.

A number of trials were carried out using this method of broth containing Tween, but, together with the extremely tedious method of counting all the plates, the results did not agree very well, and we were still in a situation without any definite answers regarding the activity of each of these compounds.

3. Tetrazolium-based colorimetric chemosensitivity test

The third method was based on the assay established by Tim Mosmann in 1983[46] where cellular growth was studied using the formation of a quantitative colorimetric method using a tetrazolium salt. The method can be used to measure surviving and / or proliferating bacterial cells. Dead cells are not detected and the signal generated is dependent on the degree of activation of the cells. The results can be measured on a spectrophotometric microplate reader which results in a rapid and precise method.

Tetrazolium salts have been utilised to quantitate cellular reductive capacity. These salts accept electrons from living cells that result in their reduction to a coloured formazan product[47]. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population. The properties are consistent with the cleavage of the tetrazolium salt only by active mitochondria.

Two different tetrazolium salts , the hydrogen acceptor 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, thiazolyl blue) and 2,3,5 triphenyl tetrazolium chloride (TTC, tetrazolium red) are available for measuring antimicrobial activity. Where bacterial growth had occurred, the suspension changed to blue with MTT and to red with TTC within 10 – 60 minutes[48]. Mosmann[46] had found MTT the most promising reagent as it is a pale yellow

substrate that produces a dark blue formazan product when incubated with live cells. The MTT formazan reaction product is only partially soluble in the medium, so isopropanol was used to dissolve the formazan for measurement of optical density. To minimise interference from the phenol red, it was converted to the fully acidic yellow form at the end of the assay.

The advantages of the colorimetric assay are the speed with which samples can be processed, the lack of interference by the substrate and the medium and the stability of the colour for a few hours at room temperature. Rapid qualitative results are obtained by reading the formazan spectrophotometrically at 540 nm[49].

The hydrophobic nature of the compounds and lack of miscibility with any solvents used caused us to look further for a better method based on the tetrazolium salt microplate method above. This procedure was suggested by Eloff [50], where the compound being tested for antimicrobial activity was dried onto a t.l.c. plate. This was sprayed with actively growing bacterial cells and incubated. The plates were then sprayed with a tetrazolium salt solution and a clear zone on the plate indicated inhibition of growth.

Experimental

The compound in acetone : DCM (1 : 1) solution was pipetted onto the t.l.c. plate in amounts of 0.7 and 2.8 μmol. The plate was dipped into a trough of the actively growing bacteria broth. This was incubated at 37°C in a sealed container with a high relative humidity for 18h then dipped in 0.25% MTT

solution. Following incubation under the same conditions for 4h, the plate was dipped in acid-isopropanol for 1h. The plates were then evaluated and photographed. Clear or yellow zones on the t.l.c. plate indicated inhibition of bacterial growth, while blue indicated no inhibition of growth.

Chloromycetin was used as the standard except for Candida albicans, where the more appropriate amphotericin was used. For each compound, triplicate amounts were pipetted on the t.l.c. plate. A solvent blank was applied in triplicate at the top of each plate. The diffusion of the standard on the plate was good, causing it to interfere with the activity of the compounds, hence the standard was applied to its own plate.

Evaluation

The best set of qualitative results for the antimicrobial testing was achieved by this method of t.l.c. application with MTT. Inhibition or activity against the bacteria was identified by this procedure. Photographic evaluations follow in the Figures below.

A. Staphylococcus aureus

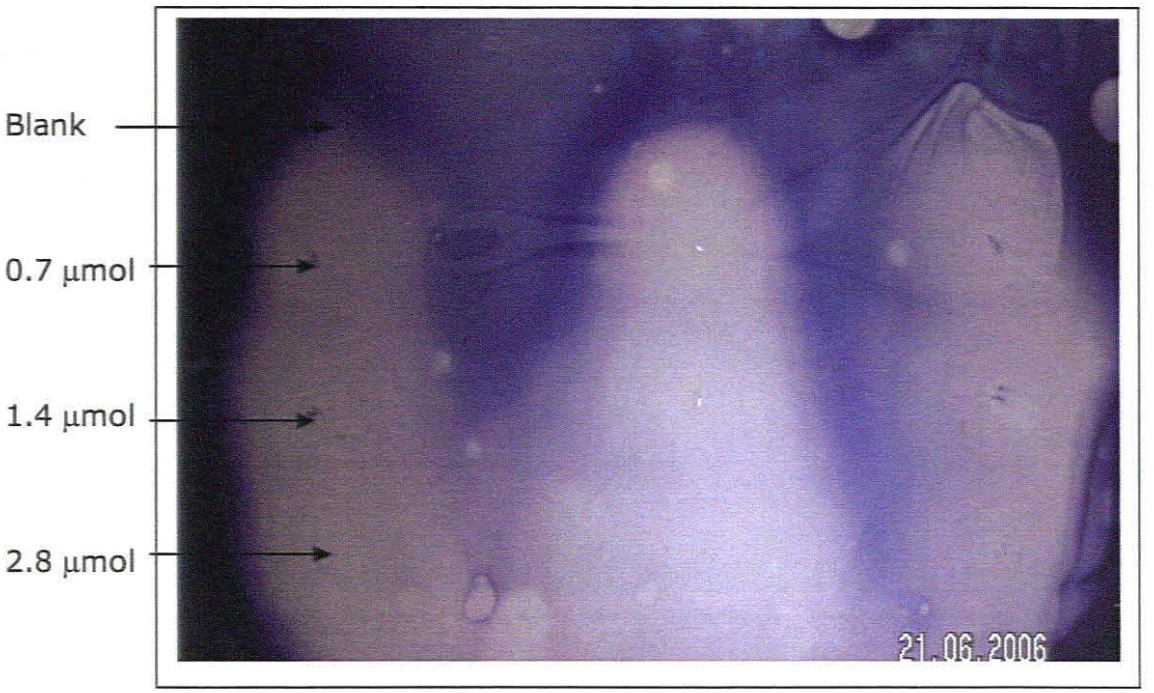


Figure A.1. Chloromycetin

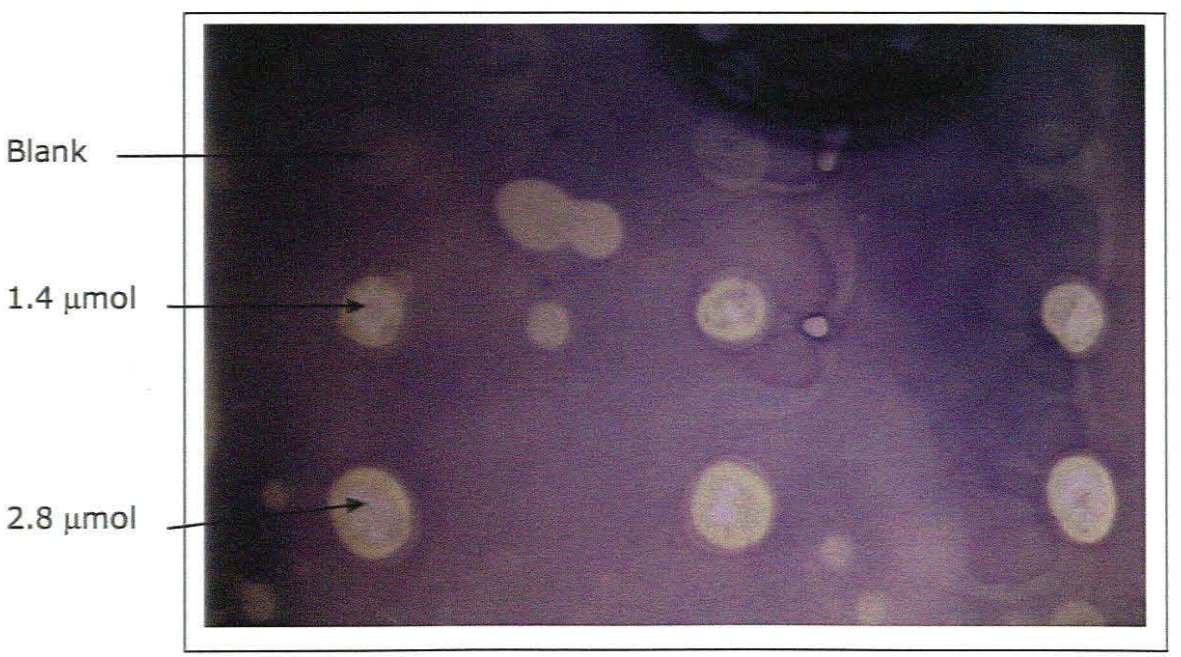


Figure A.2. Oleanolic acid (1)

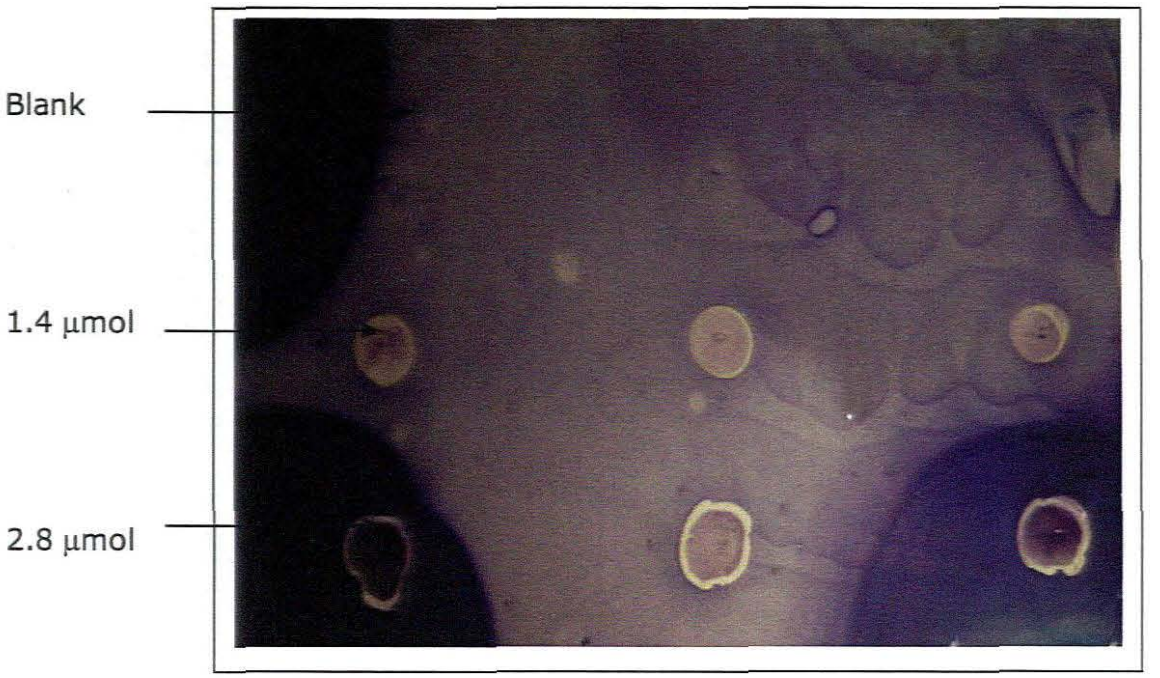


Figure A.3. 3-O-Acetyloleanolic acid (2)

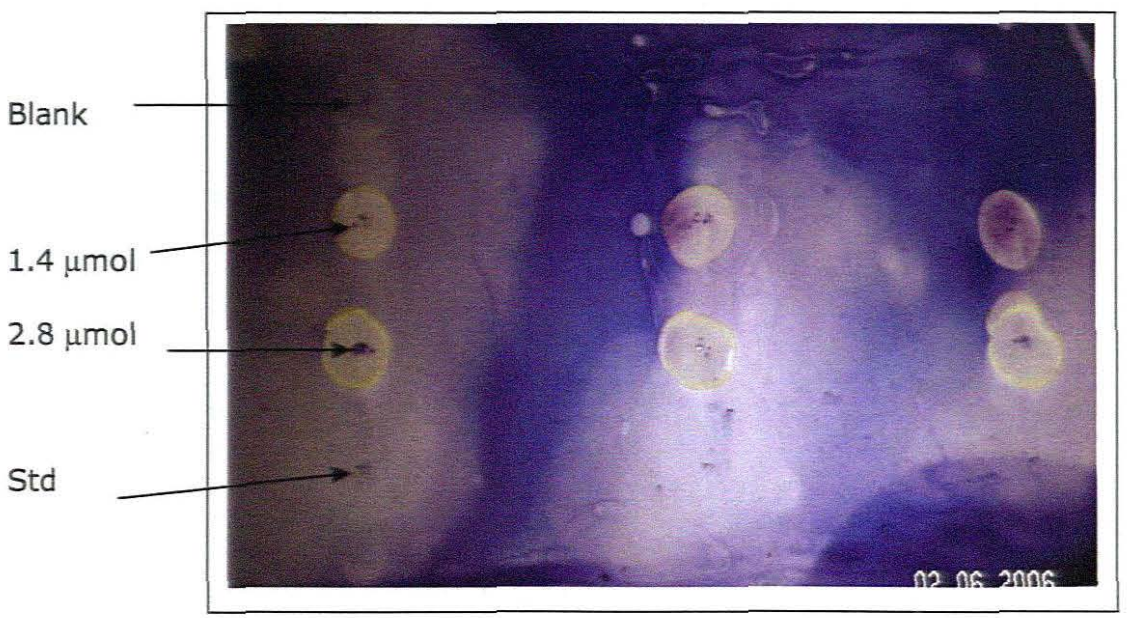


Figure A.4. 3-O-Acetyloleanolic acid (2) plus std

Figure A.5. Methyl oleanolate (3)

Figure A.6. Diester (4)

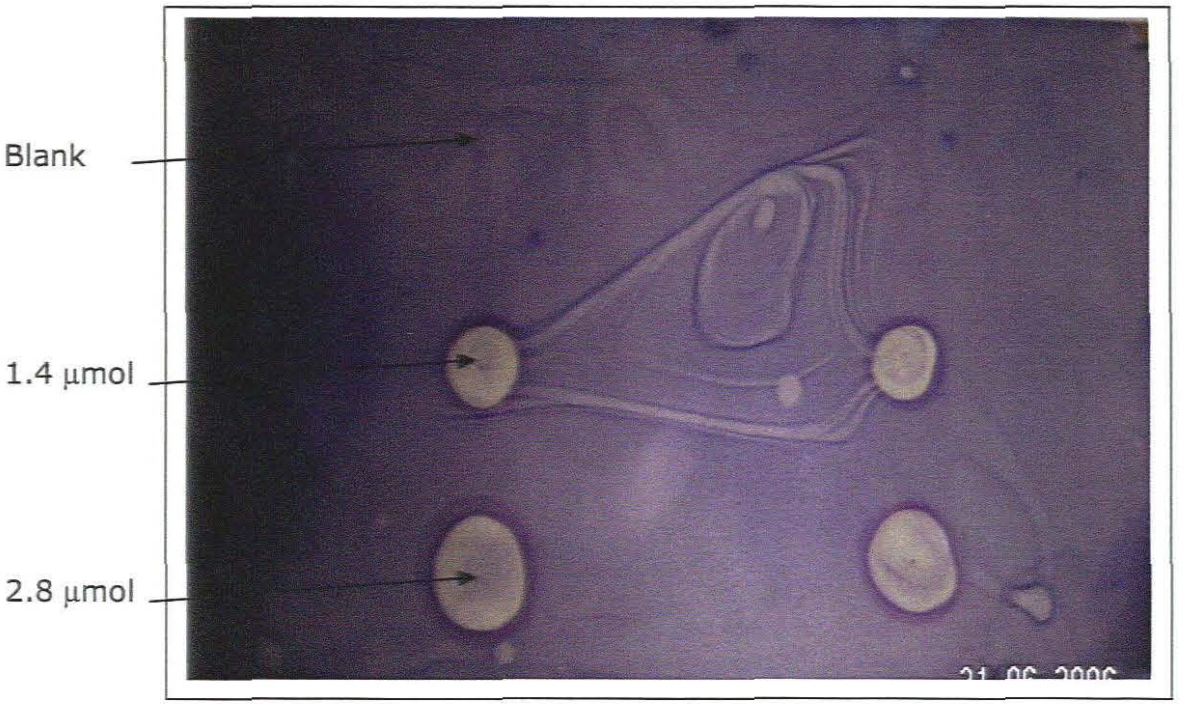


Figure A.7. Diol (5)

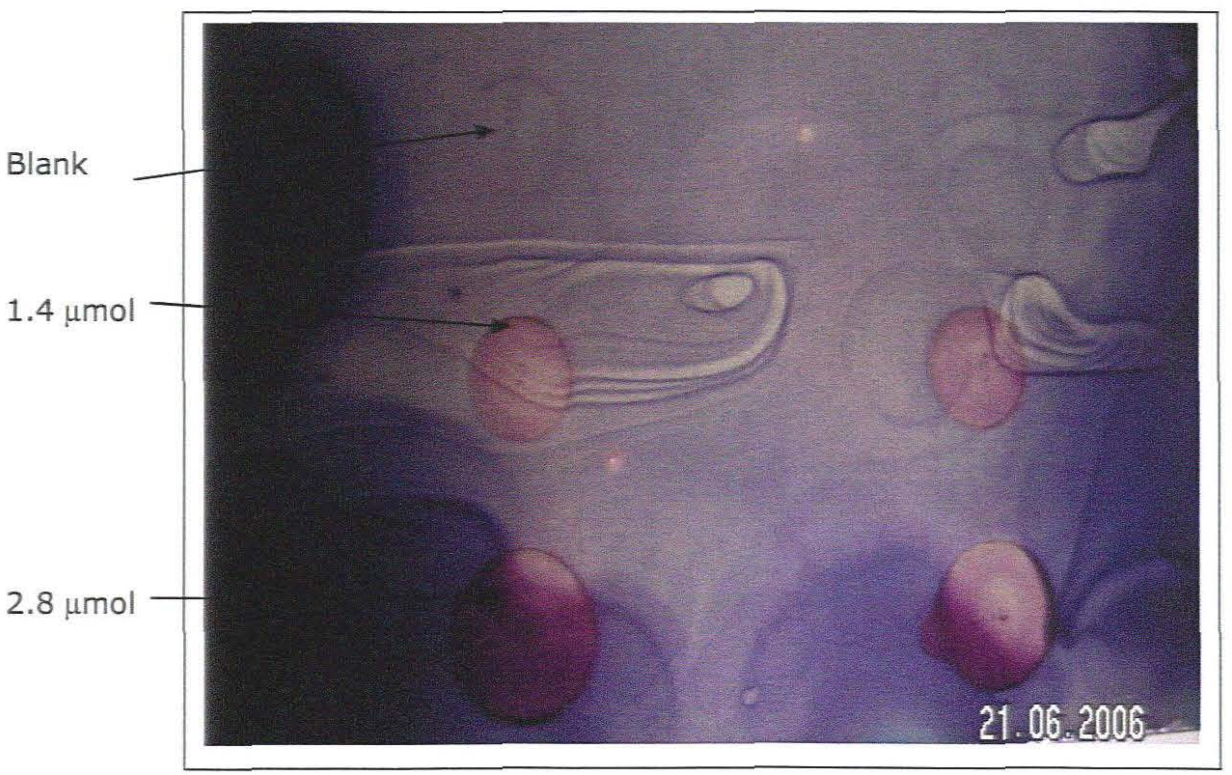


Figure A.8. Diester of diol (6)

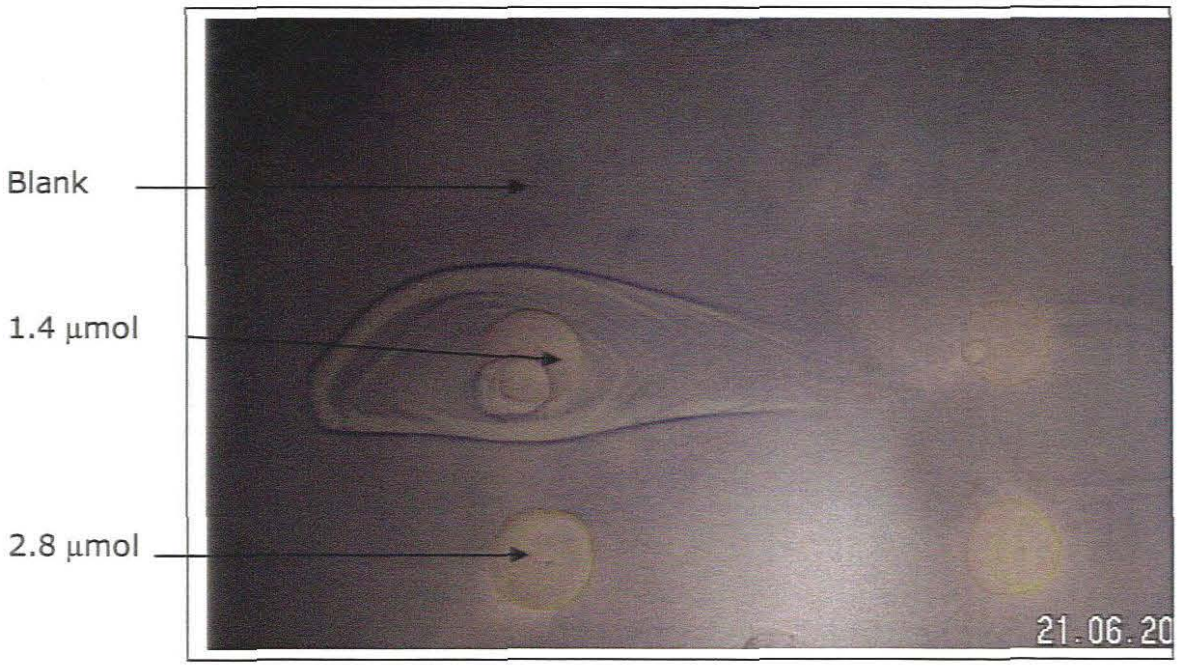


Figure A.9. Bis-trifluoracetoxy oleanolic acid (7)

Figure A.10. Trifluoro-acetoxy oleanolic acid (9)

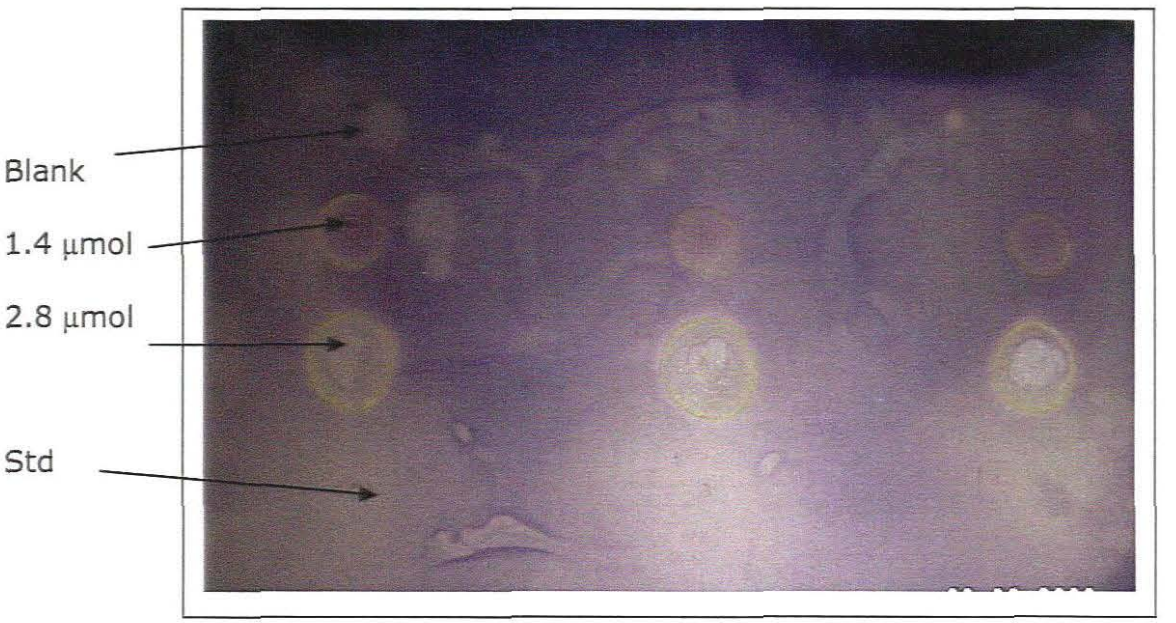


Figure A.11. Trifluoro-acetoxy oleanolic acid (9) with std

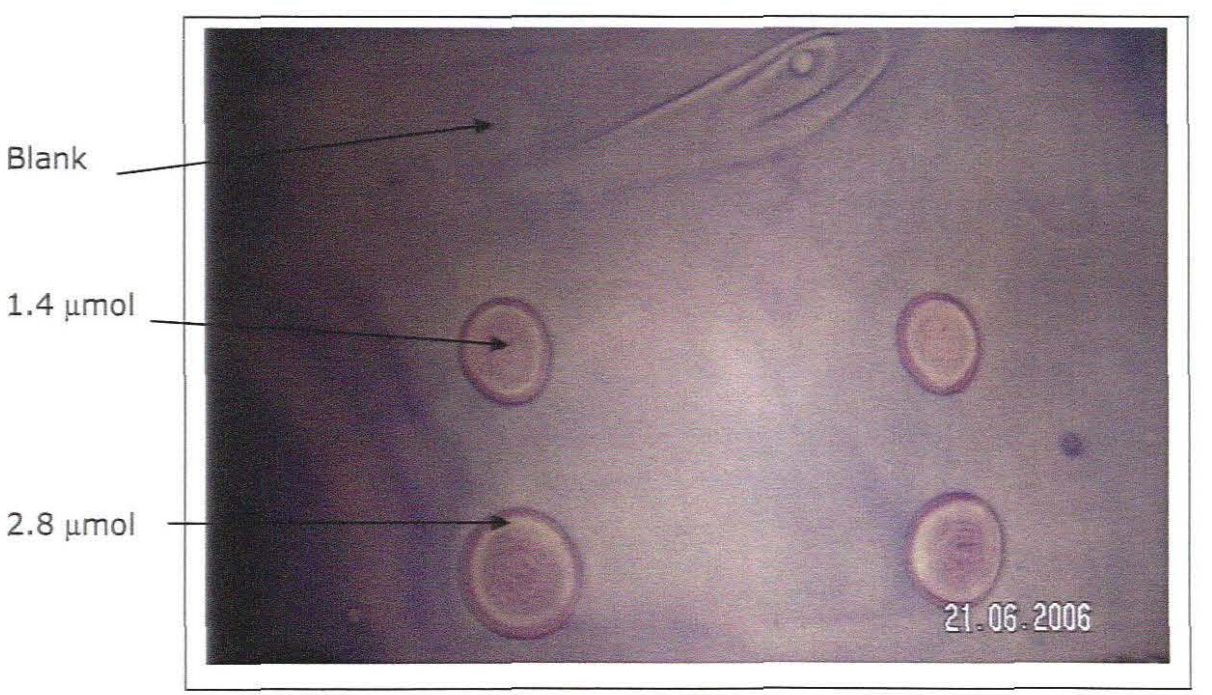


Figure A.12. 12,13-Dibromo oleanolic acid (11)

Table 6.14. Evaluation of antimicrobial activity of compounds against *Staph aureus* organisms

Figure	Compound	Inhibition 1.4 μmol	Inhibition 2.8 μmol	Discussion
A.1	Chloro	+	+	Diffusion of std across plate
A.2	(1)	+	+	Inhibition
A.3	(2)	+	+	White outer ring at highest concentration
A.4	(2)+Std	+	+	White outer ring, std inhibition
A.5	(3)	-	-	No inhibition, or diffusion and inhibition by compound (3)
A.6	(4)	-	+	Outer ring inhibition, blue in middle
A.7	(5)	+	+	Inhibition, slight blue in middle
A.8	(6)	-	-	No inhibition
A.9	(7)	-	±	Some inhibition by right hand spot
A.10	(9)	-	±	Inhibition by 2.8 μmol in centre of spots
A.11	(9)+Std	-	+	Inhibition, diffusion of std
A.12	(11)	±	+	Outer ring of inhibition

B. Bacillus subtilis

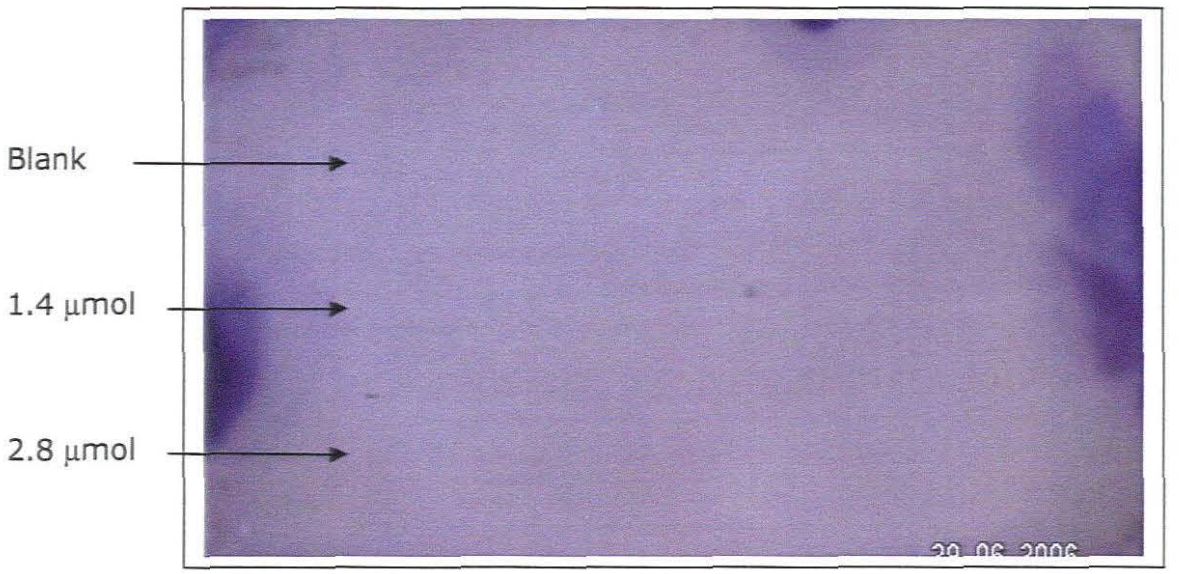


Figure B.1 : Chloromycetin

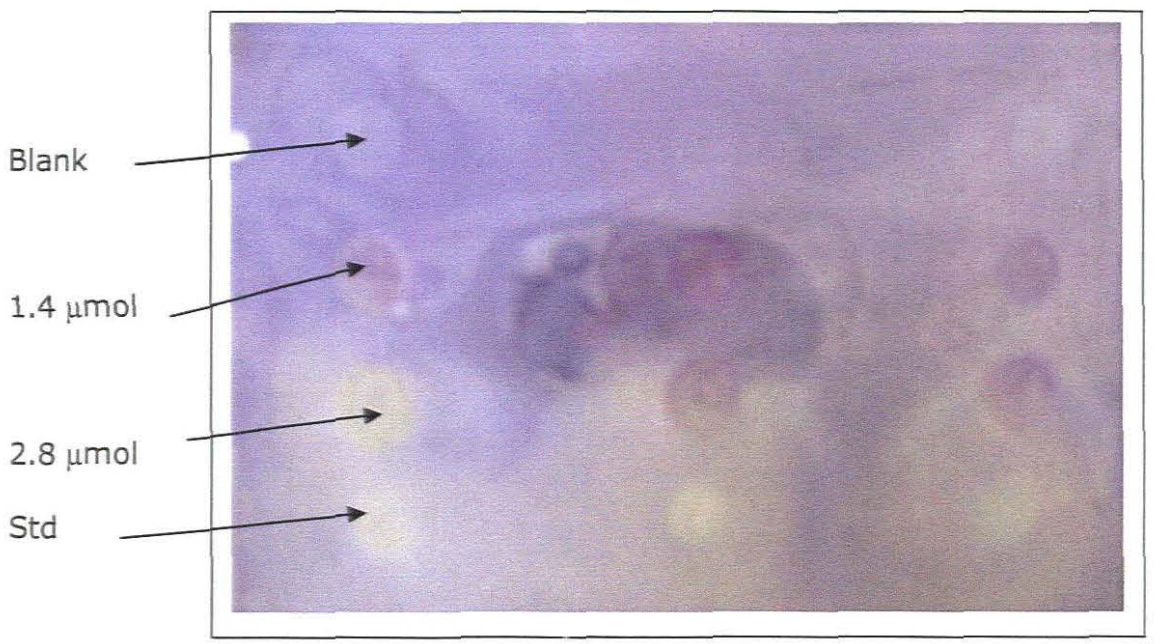


Figure B.2 : Oleanolic acid (1)plus std

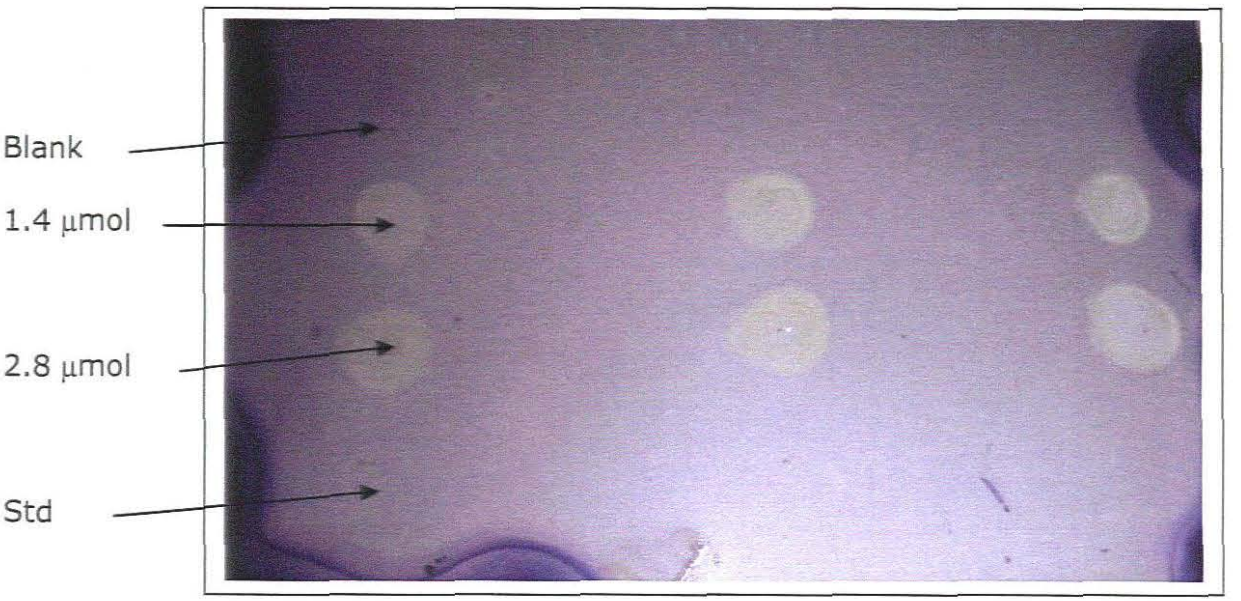


Figure B.3 : Oleanolic acid (1) plus std

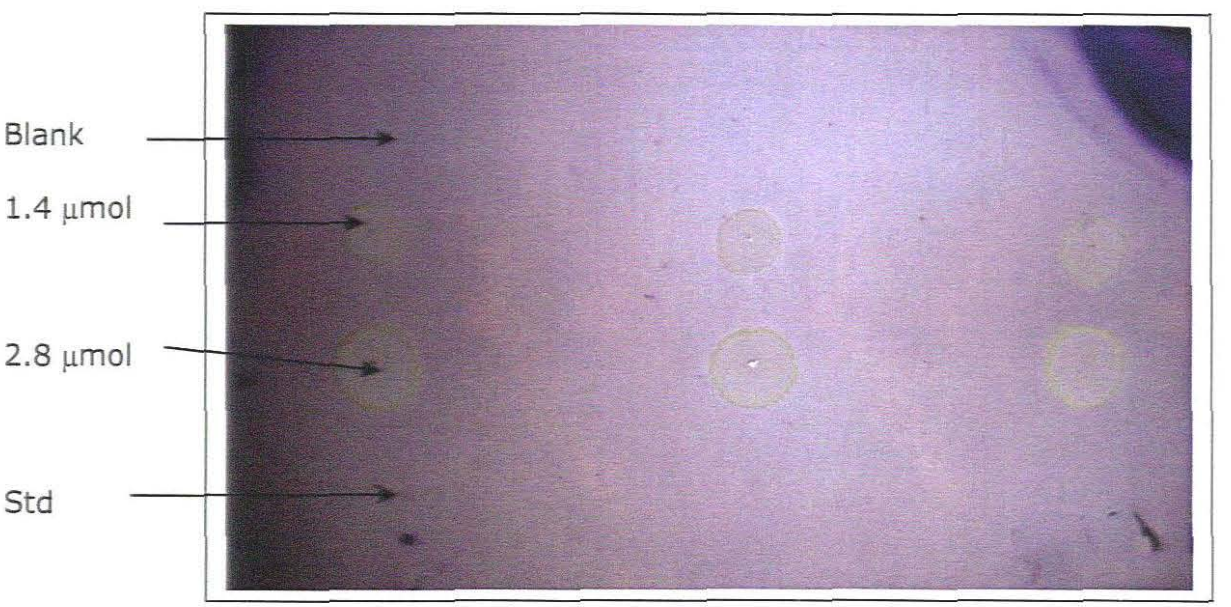


Figure B.4 : 3-O-Acetyloleanolic acid (2) plus std

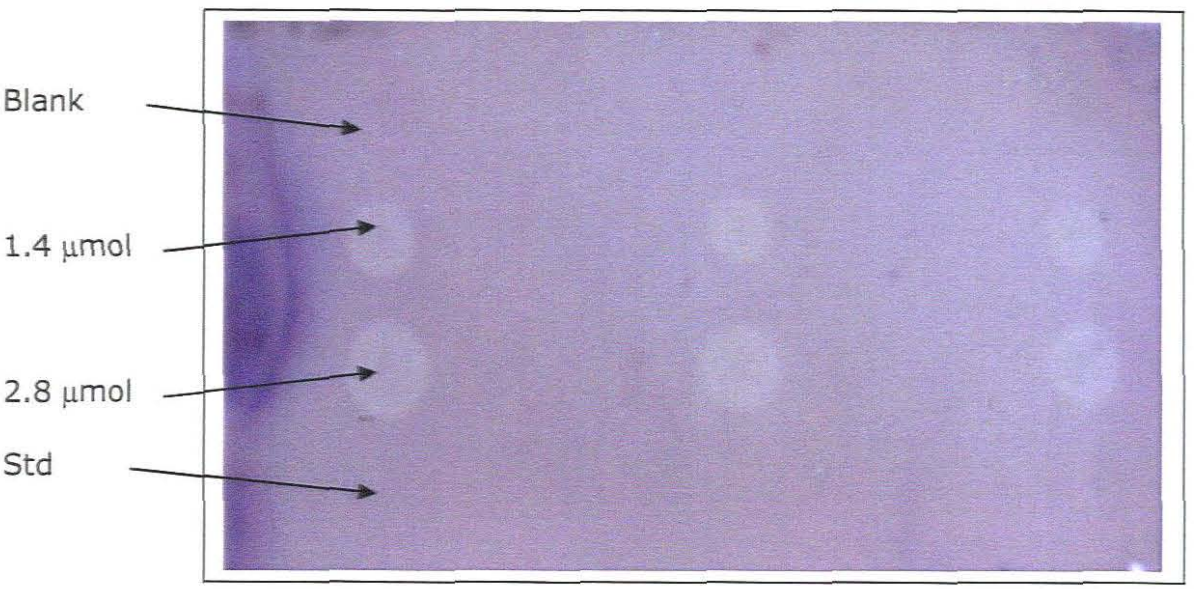


Figure B.5 : Methyl oleanolate (3) plus std

Figure B.6 : Diester (4) plus std

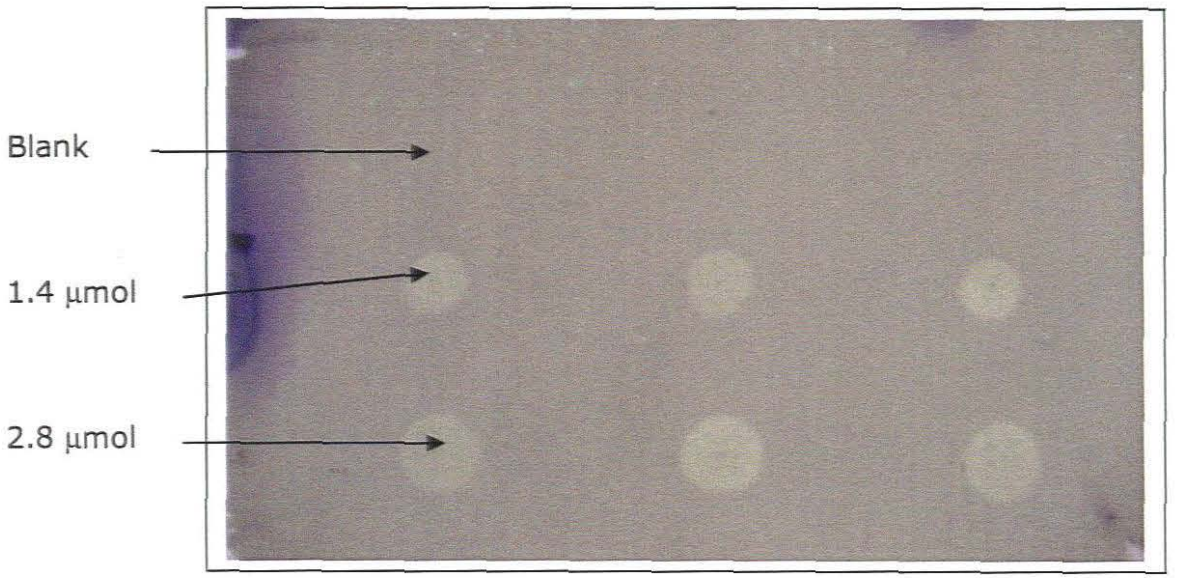


Figure B.7 : Diol (5)

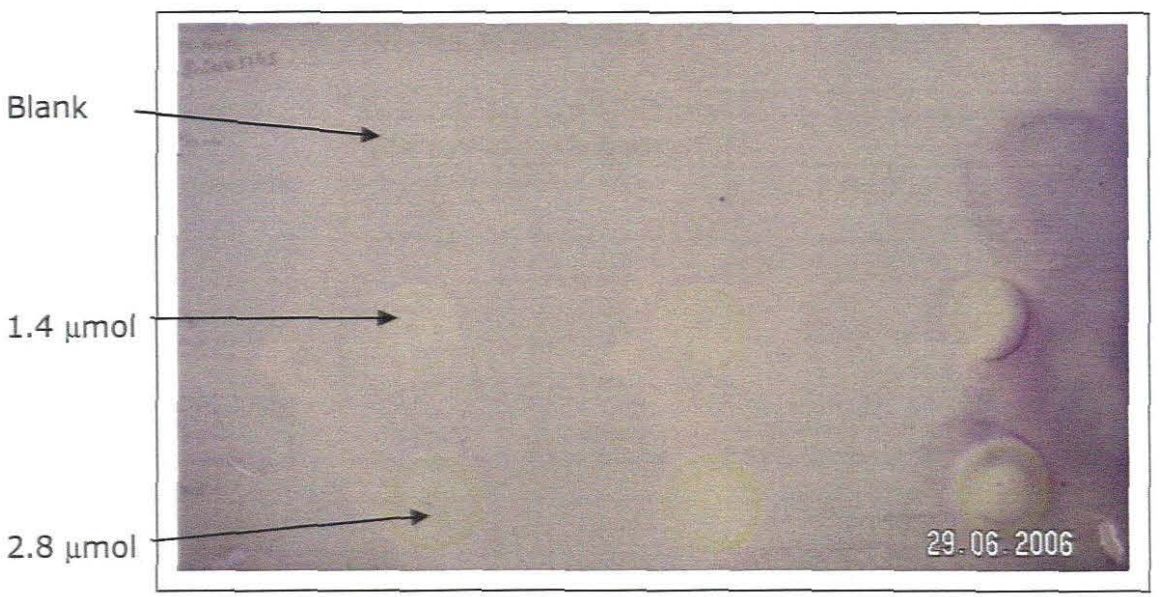


Figure B.8 : Bis-trifluoro acetoxy oleanolic acid (7)

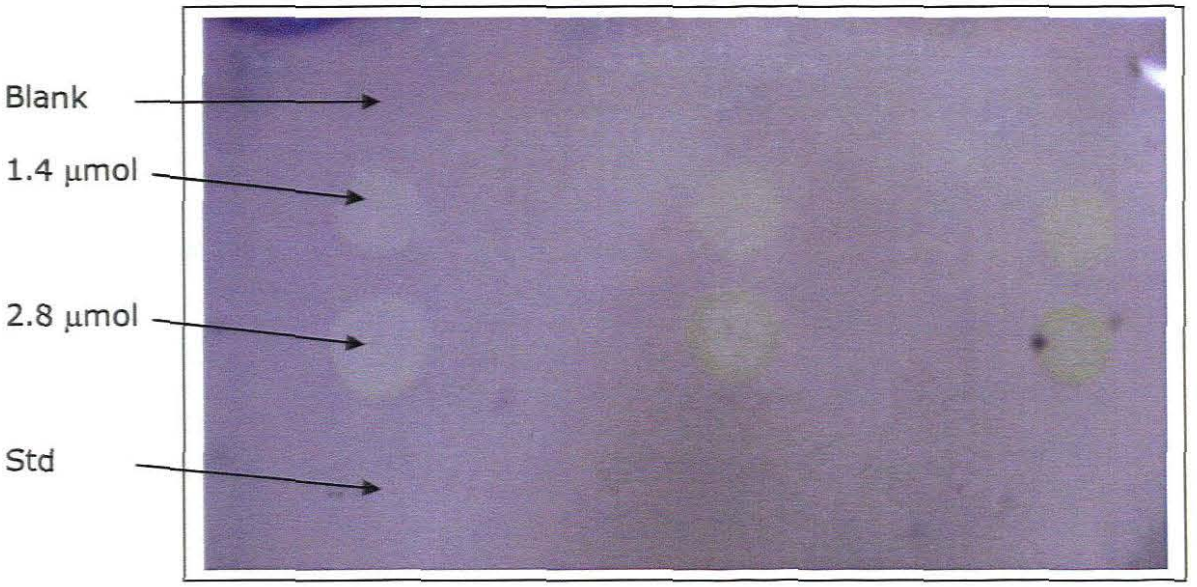


Figure B.9 : Trifluoroacetoxy oleanolic acid (9) plus std

Table 6.15. Evaluation of antimicrobial activity of compounds against *Bacillus subtilis* organisms

Figure	Compound	Inhibition 1.4 μmol	Inhibition 2.8 μmol	Discussion
B.1	Chloro	-	-	No inhibition, or diffusion on plate
B.2	(1)+Std	-	±	Low inhibition, but inhibition by std
B.3	(1)+Std	+	+	Good inhibition at both concentrations
B.4	(2)+Std	-	±	Inhibition at bottom right spot only
B.5	(3)+Std	-	-	Inhibition
B.6	(4)+Std	+	+	Inhibition by both concentrations, diffusion of std
B.7	(5)	+	+	*Inhibition*
B.8	(7)	±	±	Inhibition at edges, blue in centre
B.9	(9)+Std	±	±	Some inhibition

C. Candida albicans

Figure C.1 : Chloromycetin

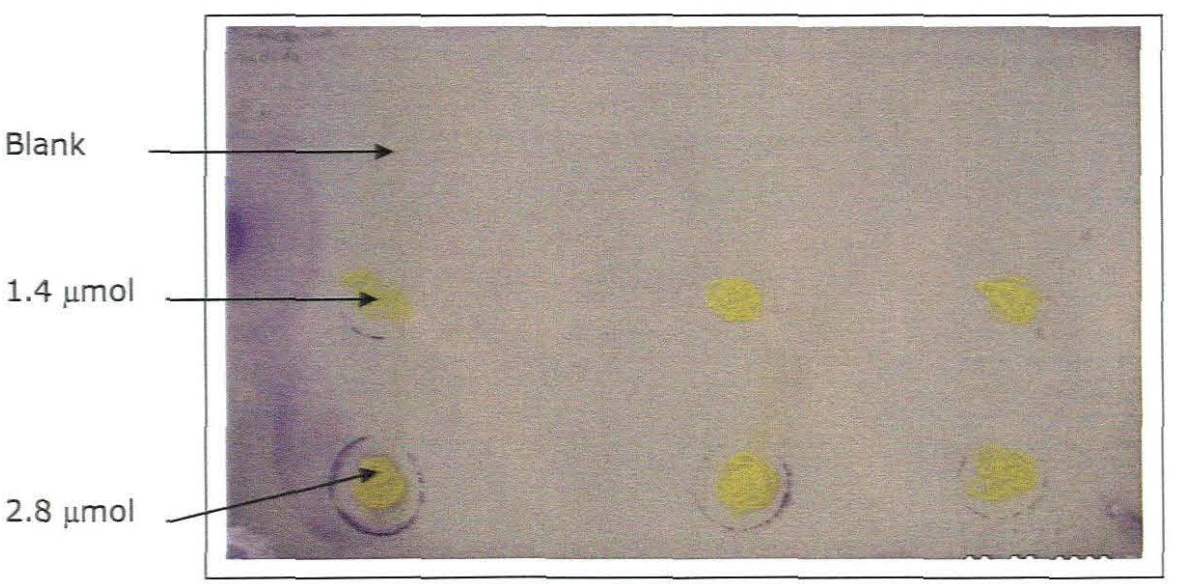


Figure C.2 : Amphotericin

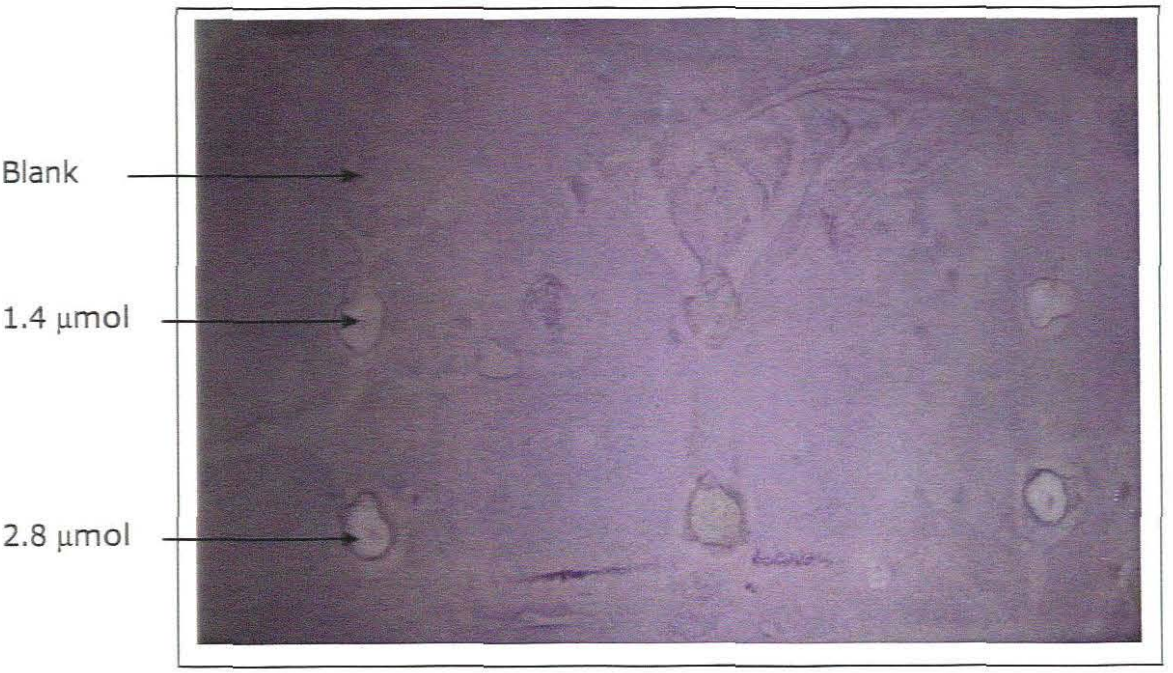


Figure C.3 : Oleanolic acid (1)

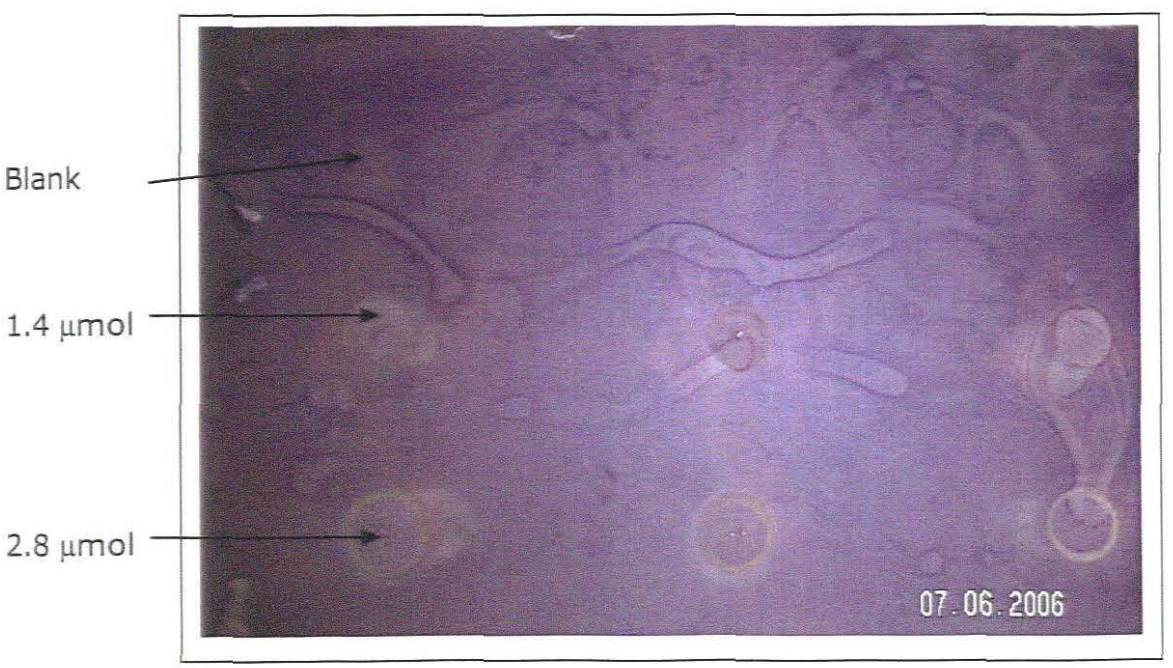


Figure C.4 : 3-O-Acetyloleanolic acid (2)

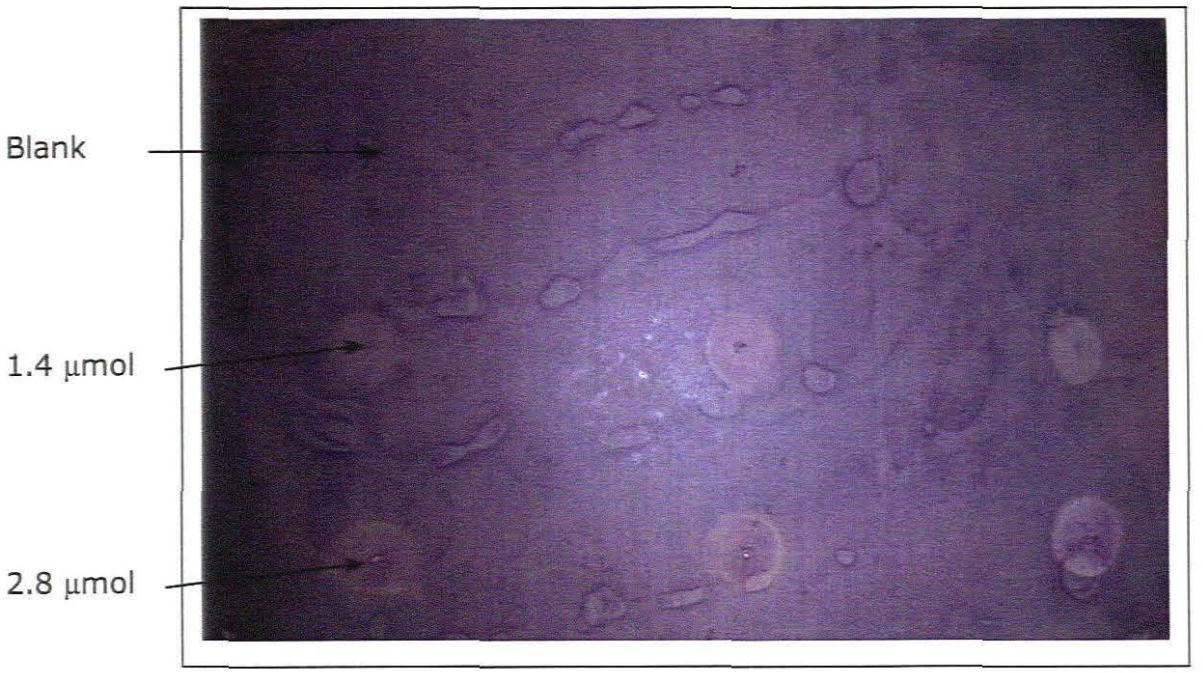


Figure C.5 : Methyl oleanolate (3)

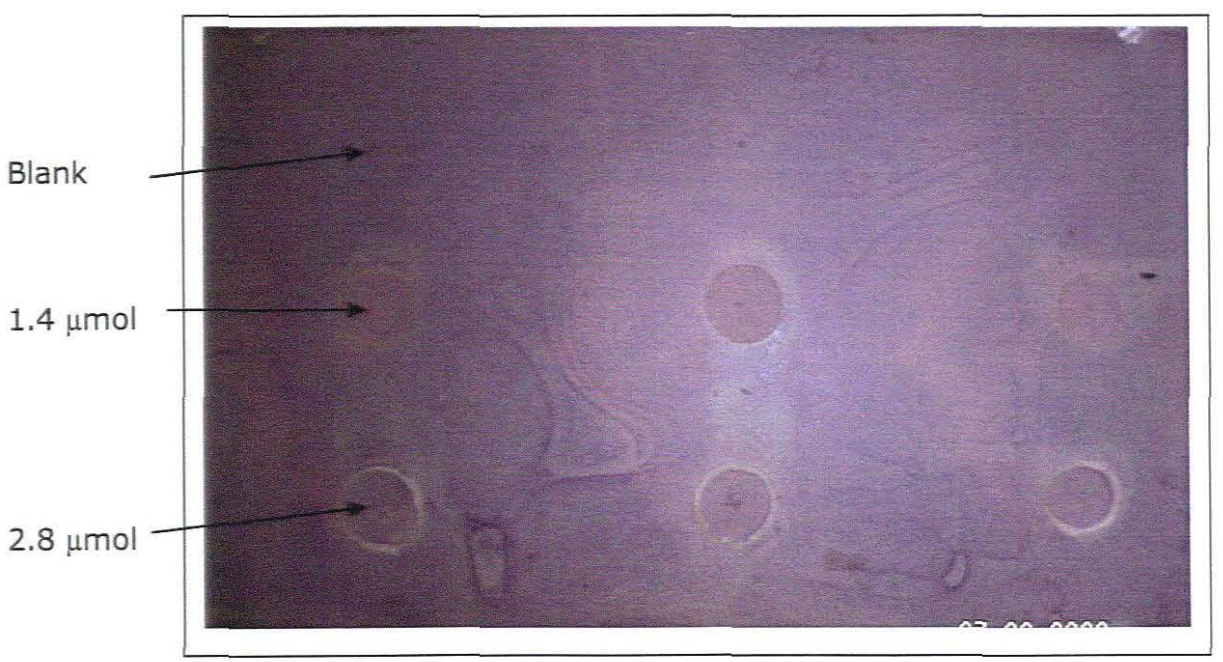


Figure C.6 : Diester (4)

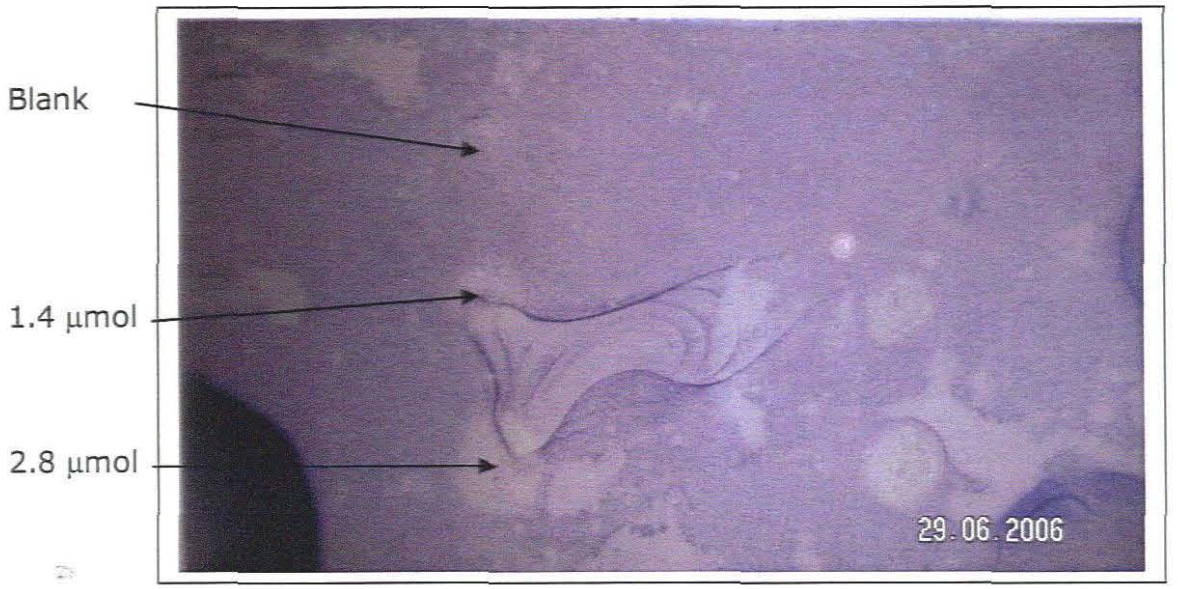


Figure C.7 : Diol (5)

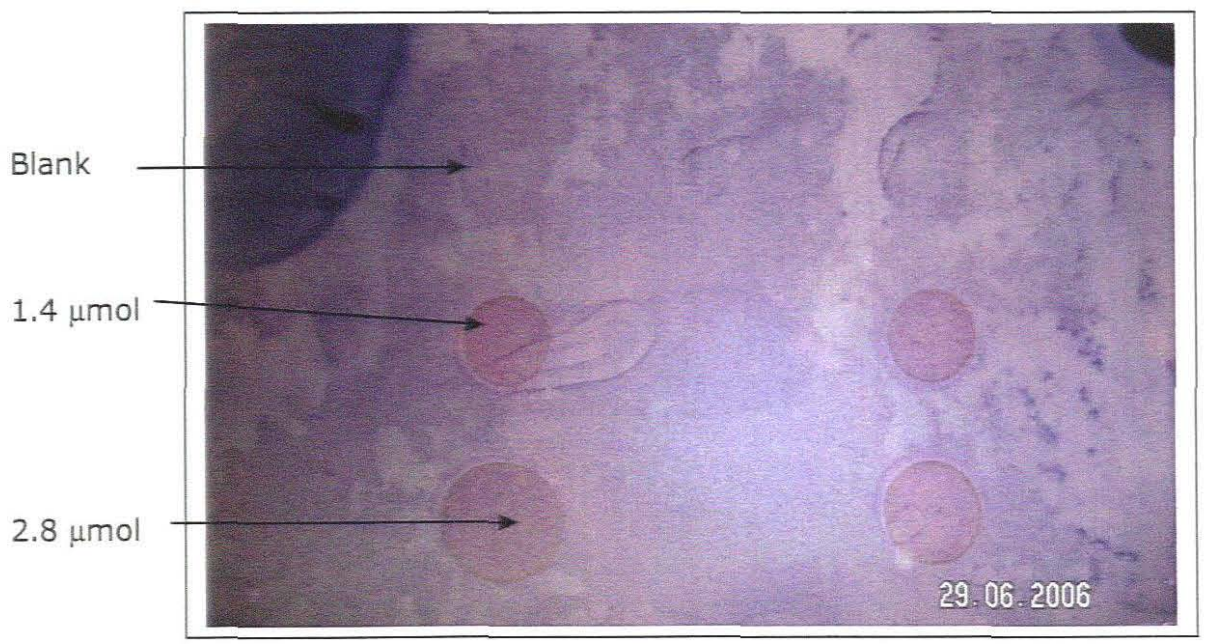


Figure C.8 : Diester of diol (6)

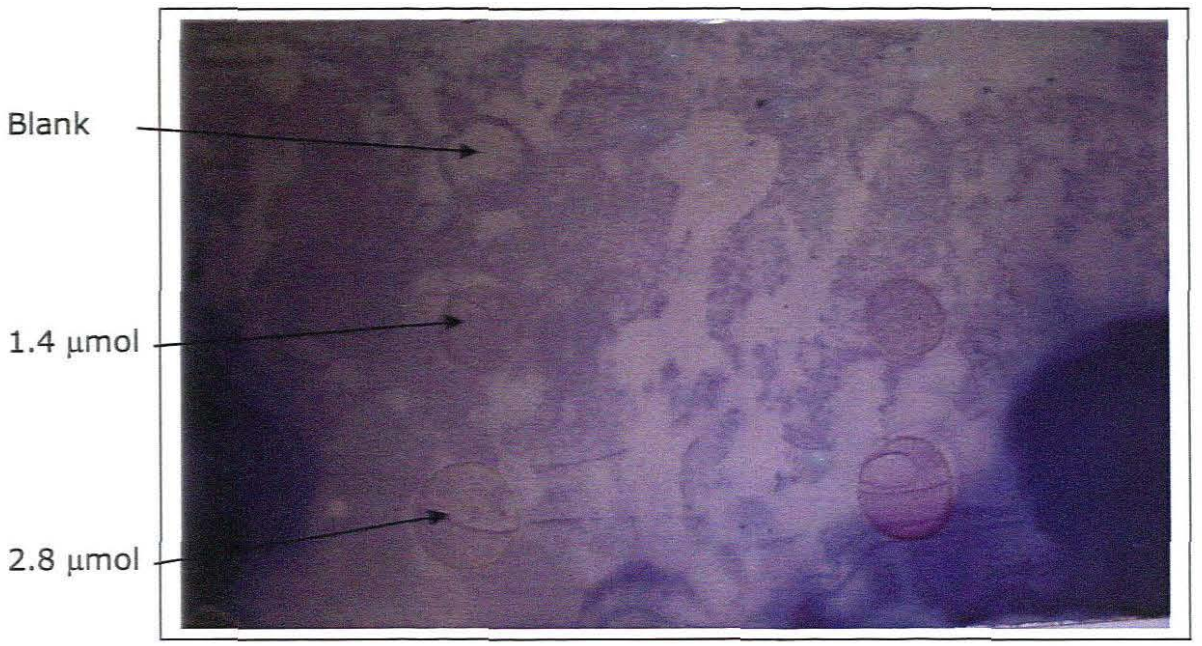


Figure C.9 : Bis-trifluoroacetoxy oleanolic acid (7)

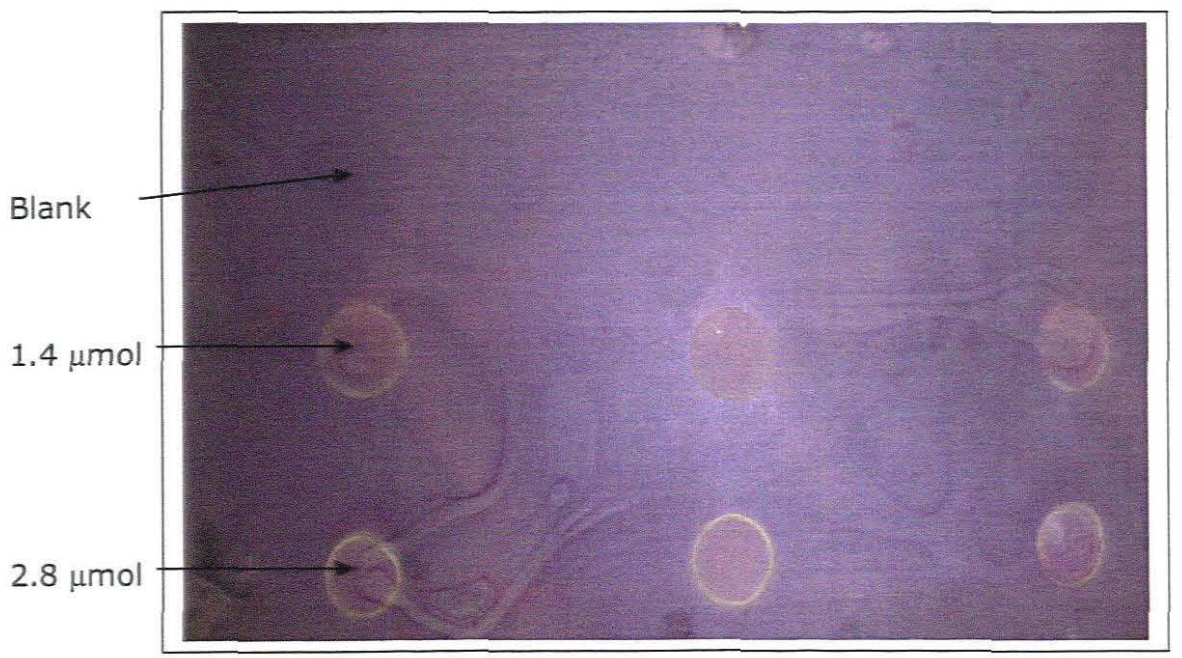


Figure C.10 : Trifluoroacetoxy oleanolic acid (9)

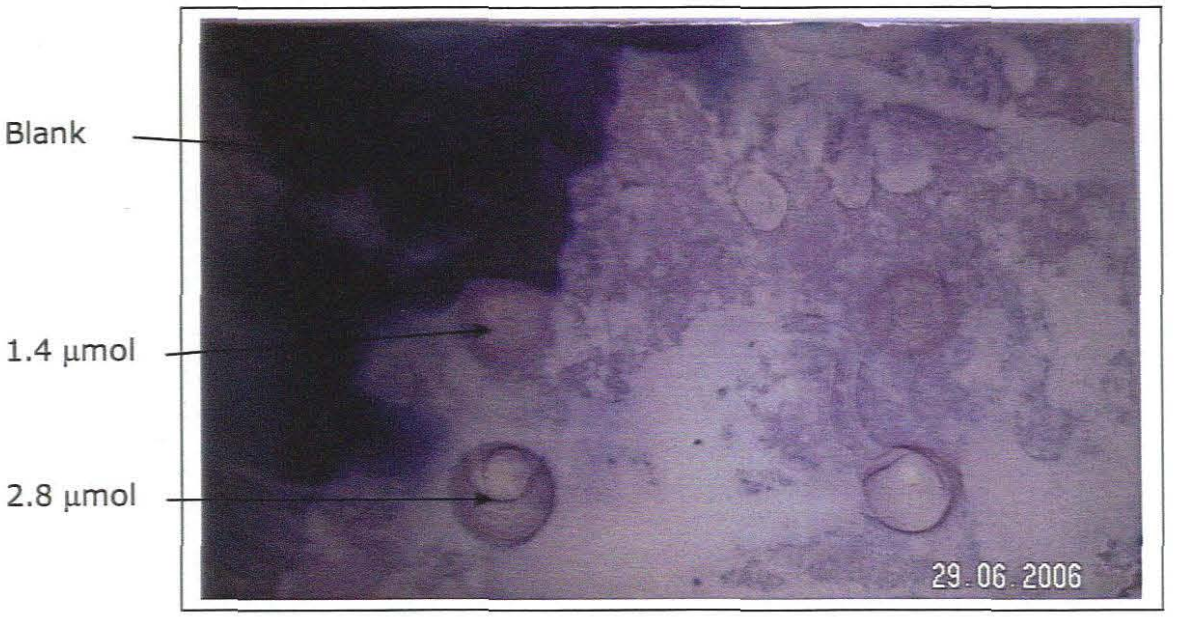


Figure C.11 : 12,13-Dibromo oleanolic acid (11)

Table 6.16. Evaluation of antimicrobial activity of compounds against *Candida albicans* organisms

Figure	Compound	Inhibition 1.4 µmol	Inhibition 2.8 µmol	Discussion
C.1	Chloro	±	±	Blue rings around possible inhibition
C.2	Ampho	-	±	Possible inhibition, blue outer ring
C.3	(1)	+	+	Inhibition
C.4	(2)	-	±	Outer ring of inhibition, blue centre
C.5	(3)	-	±	Outer ring of inhibition
C.6	(4)	-	±	Thin outer ring of inhibition by 2.8 µmol, blue centre
C.7	(5)	+	+	Inhibition
C.8	(6)	-	-	No inhibition
C.9	(7)	-	-	No inhibition
C.10	(9)	-	±	Outer ring of inhibition, blue centre
C.11	(11)	-	-	No inhibition

D. Pseudomonas aeruginosa

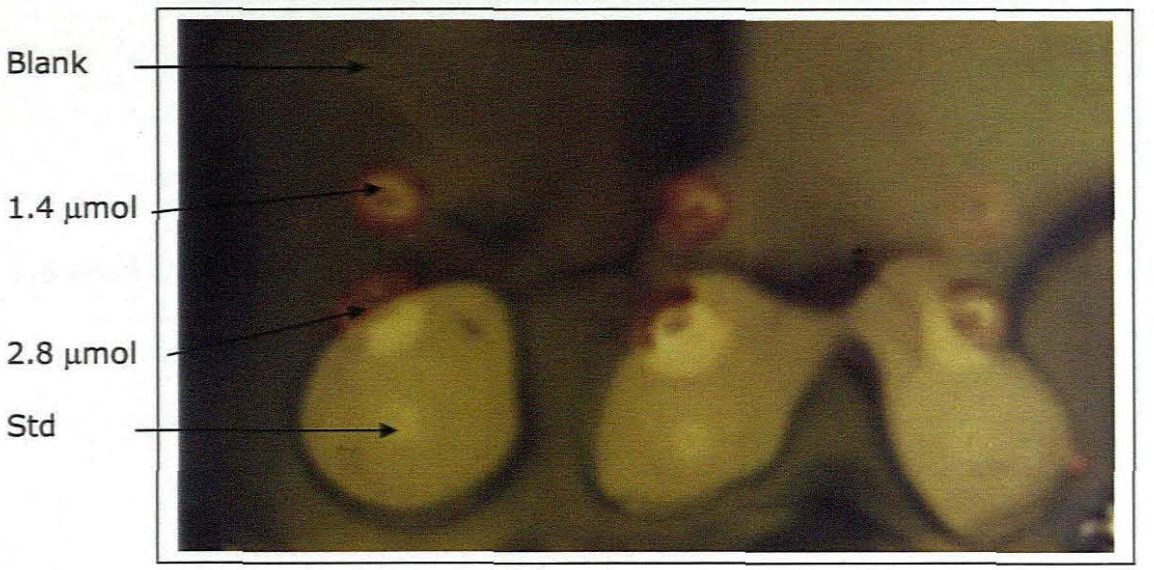


Figure D.1 : Oleanolic acid (1) plus std

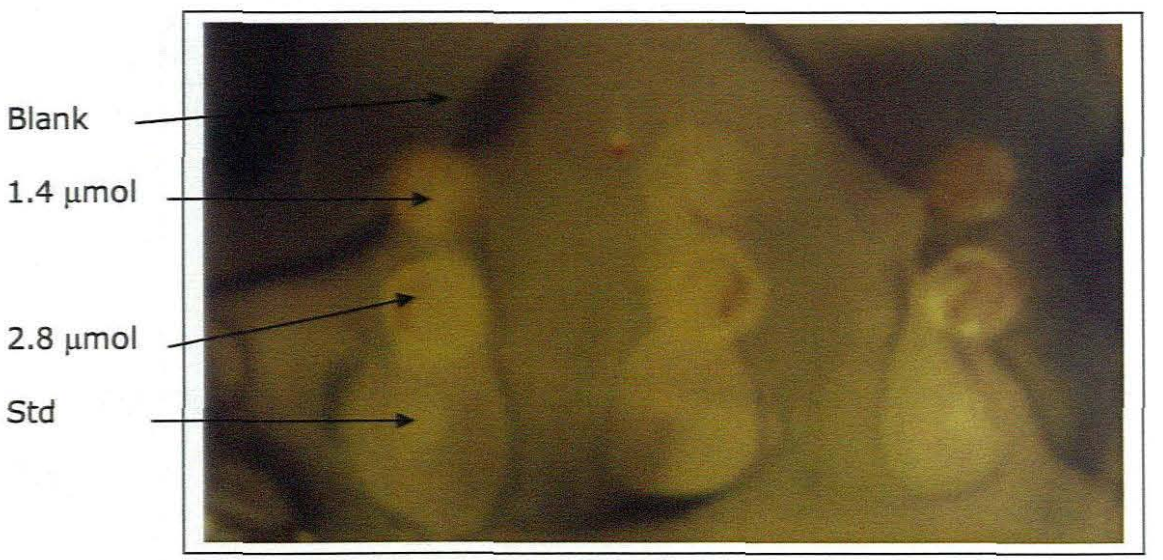


Figure D.2 : 3-O-Acetyloleanolic acid (2) plus std

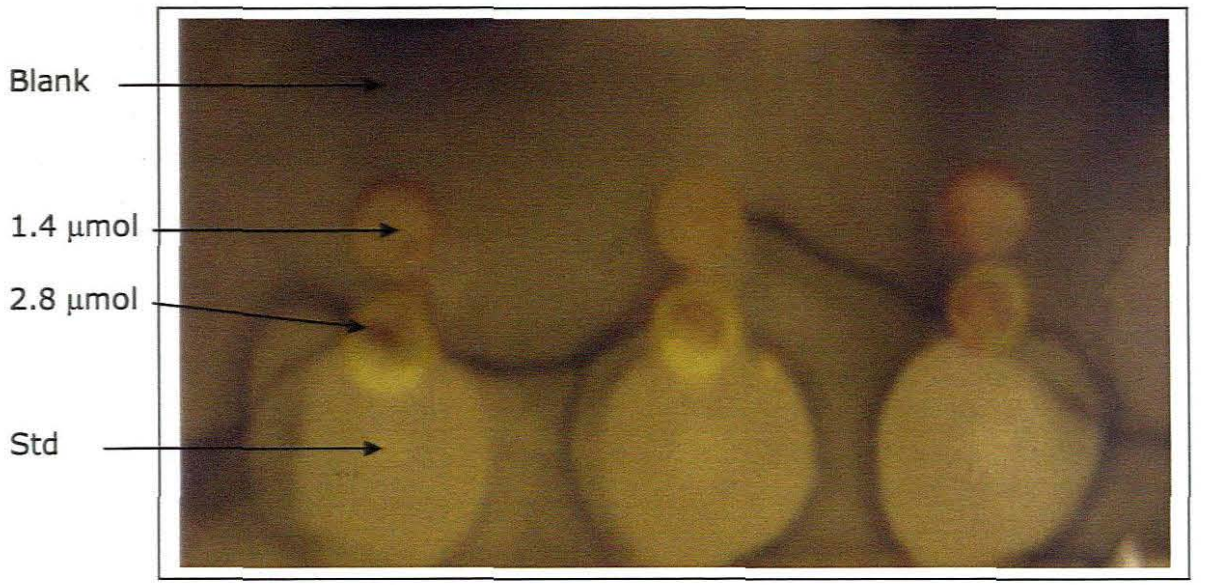


Figure D.3 : Diester (4) plus std

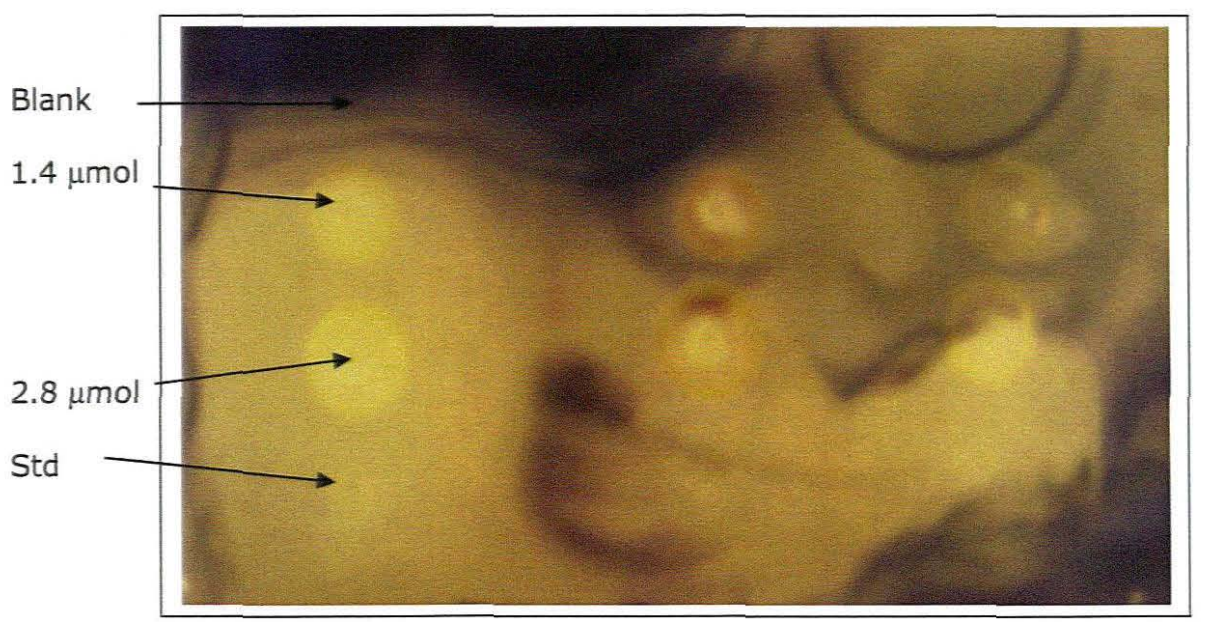


Figure D.4 : Trifluoroacetoxy oleanolic acid (9) plus std

Table 6.17. Evaluation of antimicrobial activity of compounds against *Pseudomonas aeruginosa* organisms

Figure	Compound	Inhibition 1.4 μmol	Inhibition 2.8 μmol	Discussion
D.1	(1)+Std	±	±	Inhibition at middle of spots, diffused inhibition by std
D.2	(2)+Std	-	±	Low inhibition at edges of spots, blue in middle, diffused inhibition by std
D.3	(4)+Std	-	±	Inhibition at edges of spots, diffused inhibition by std
D.4	(9)+Std	±	±	Inhibition by compound apparent, diffused inhibition by std

Antimicrobial activity results for the compounds tested against *Pseudomonas aeruginosa* are limited as the organism died during the experimental time. We were unable to replace it timeously. Testing was performed with the standard spotted at the bottom of each plate hence diffusion caused interference between the compound and the standard in many cases.

E. Proteus vulgaris

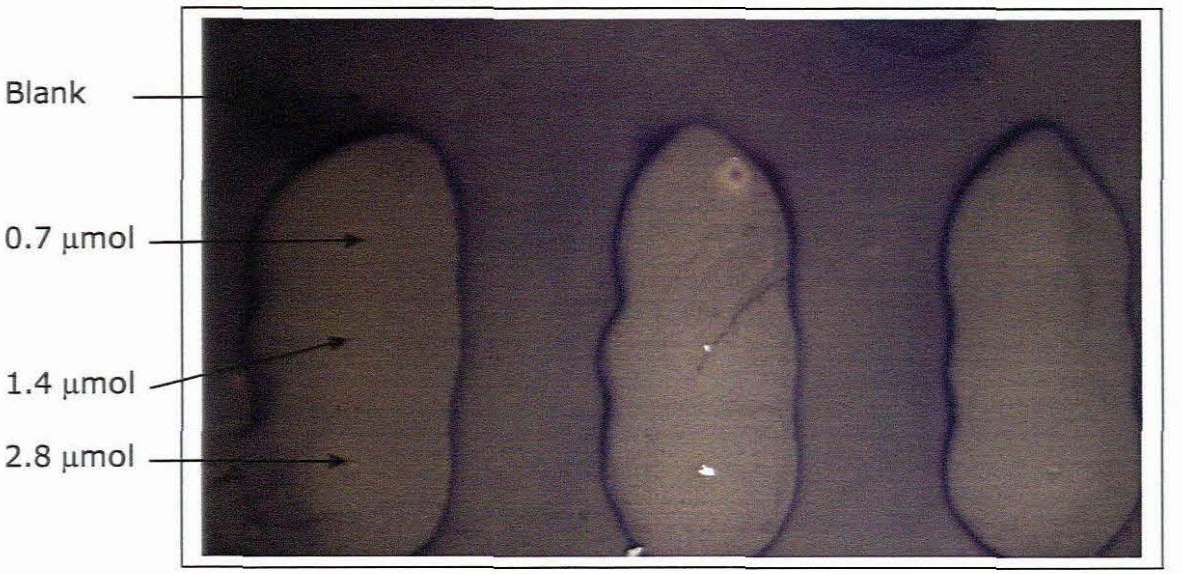


Figure E.1 : Chloromycetin

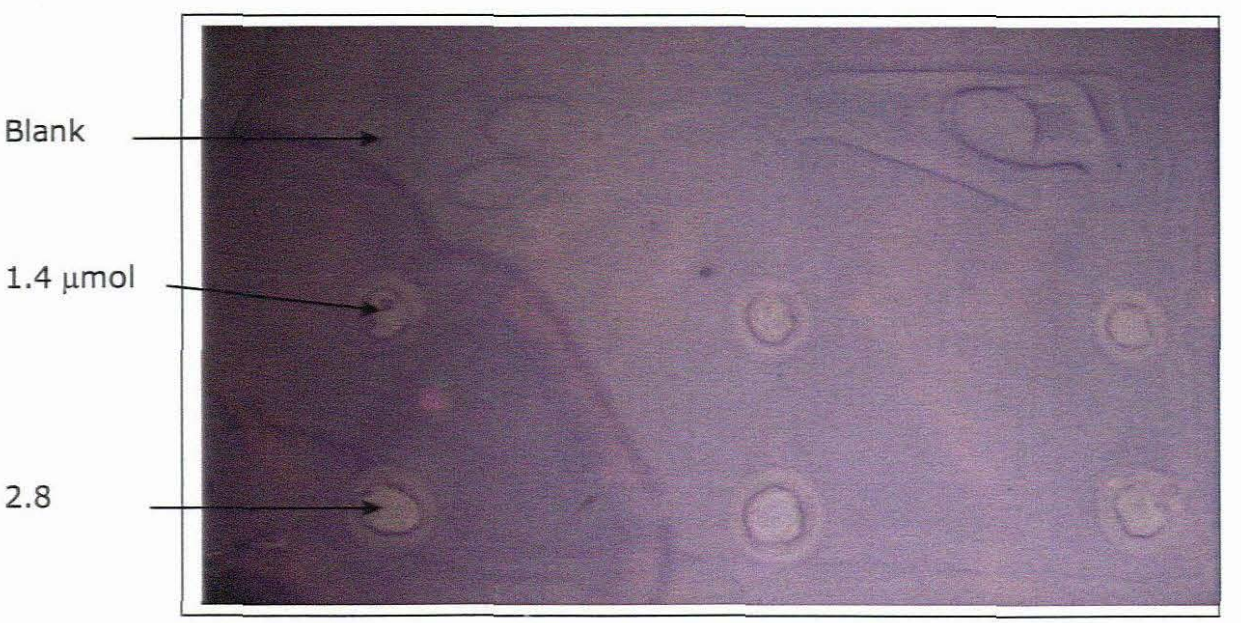


Figure E.2 : Oleanolic acid (1)

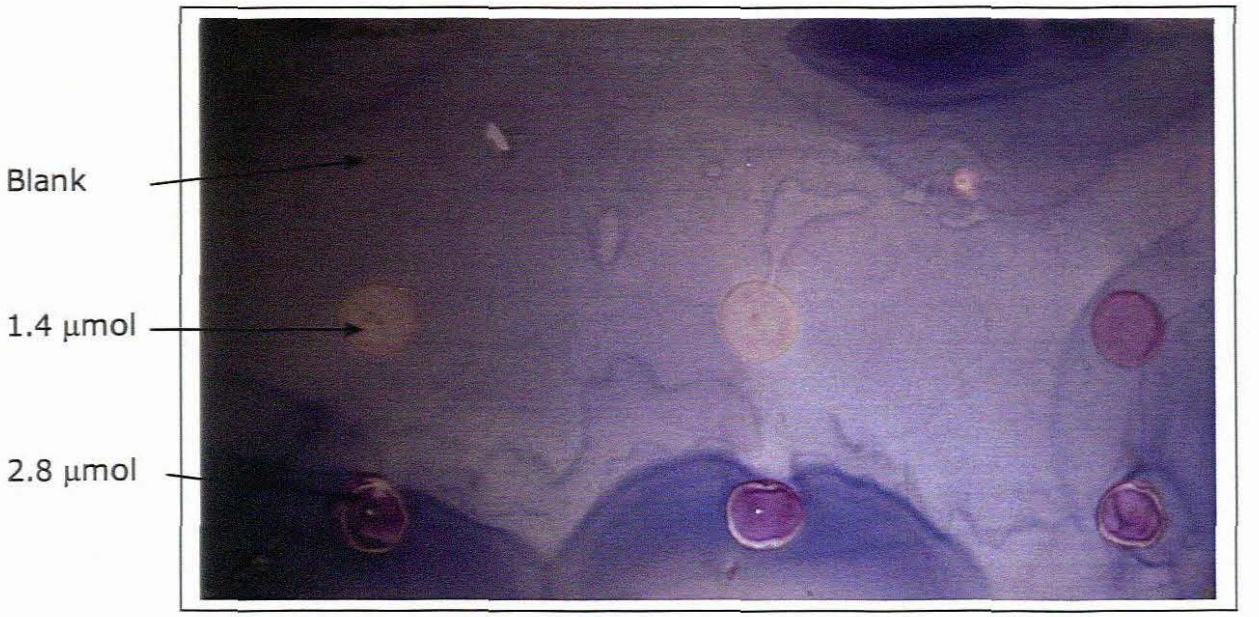


Figure E.3 : 3-O-Acetyloleanolic acid (2)

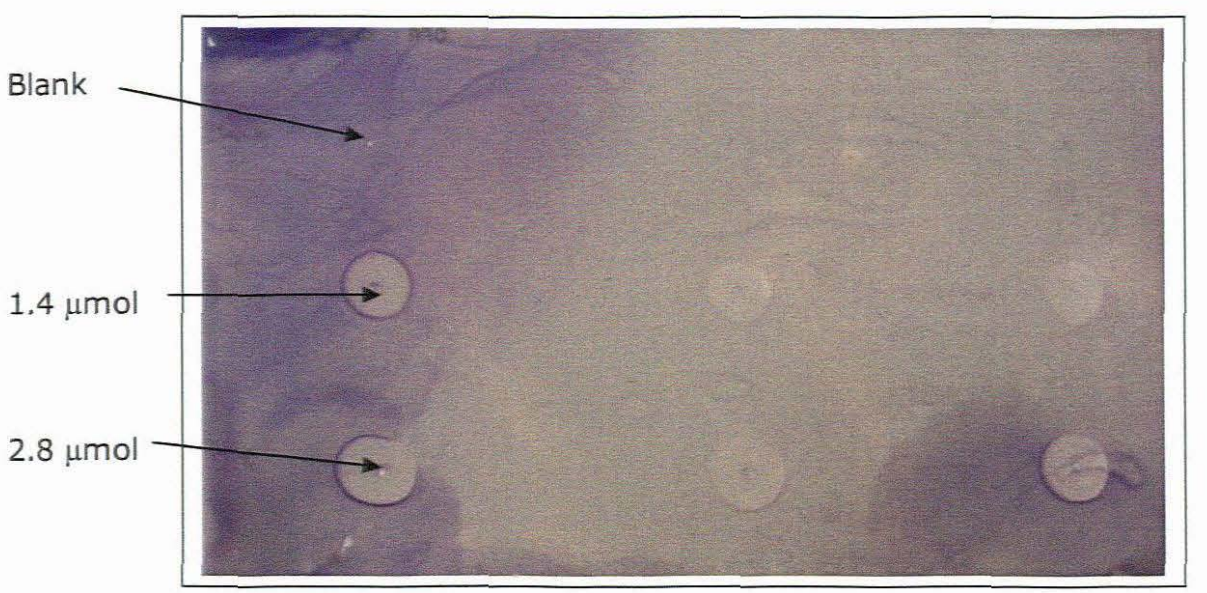


Figure E.4 : Methyl oleanolate (3)

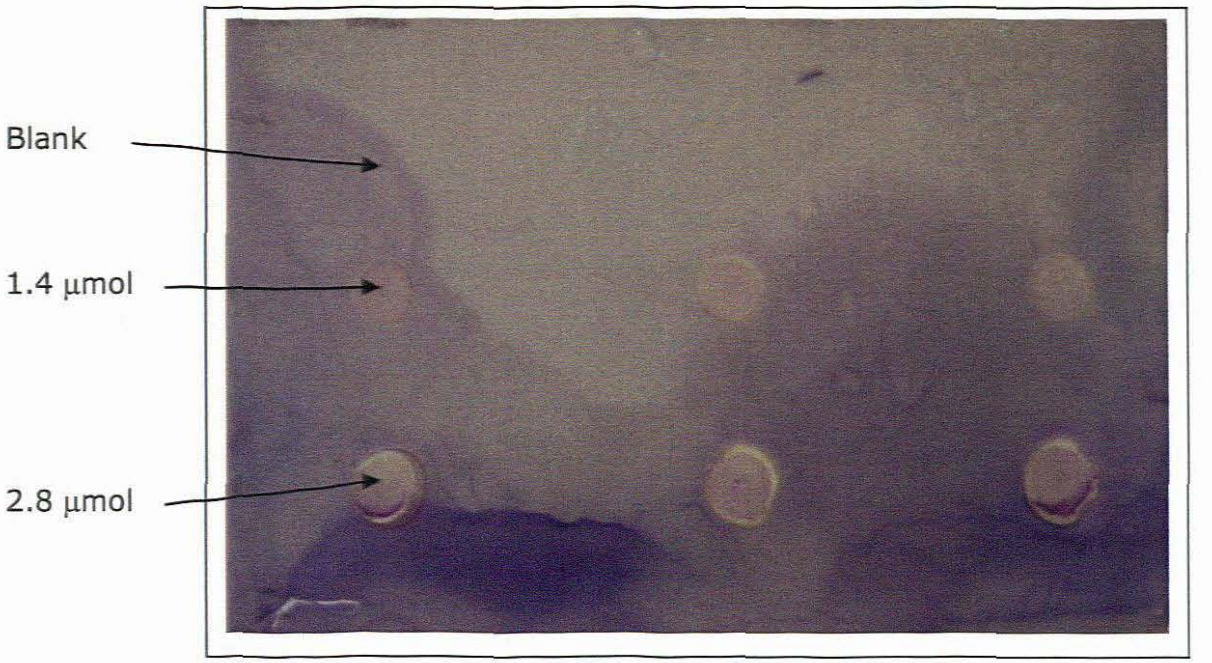


Figure E.5 : Diester (4)

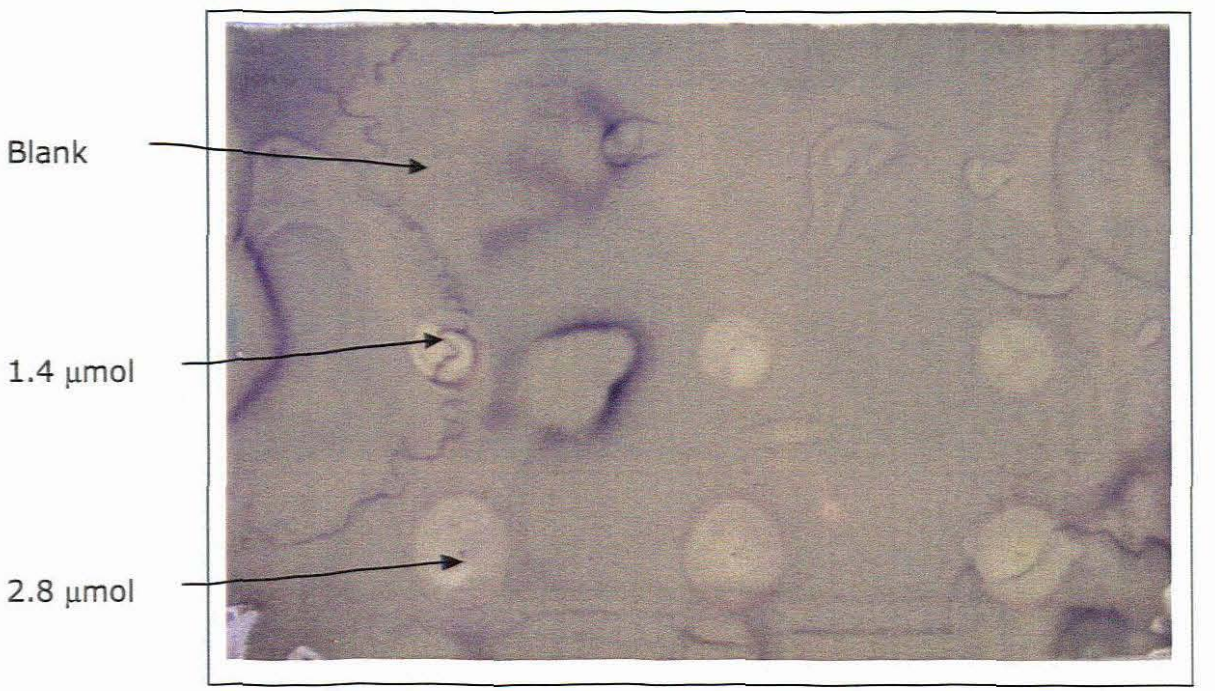


Figure E.6 : Diol (5)

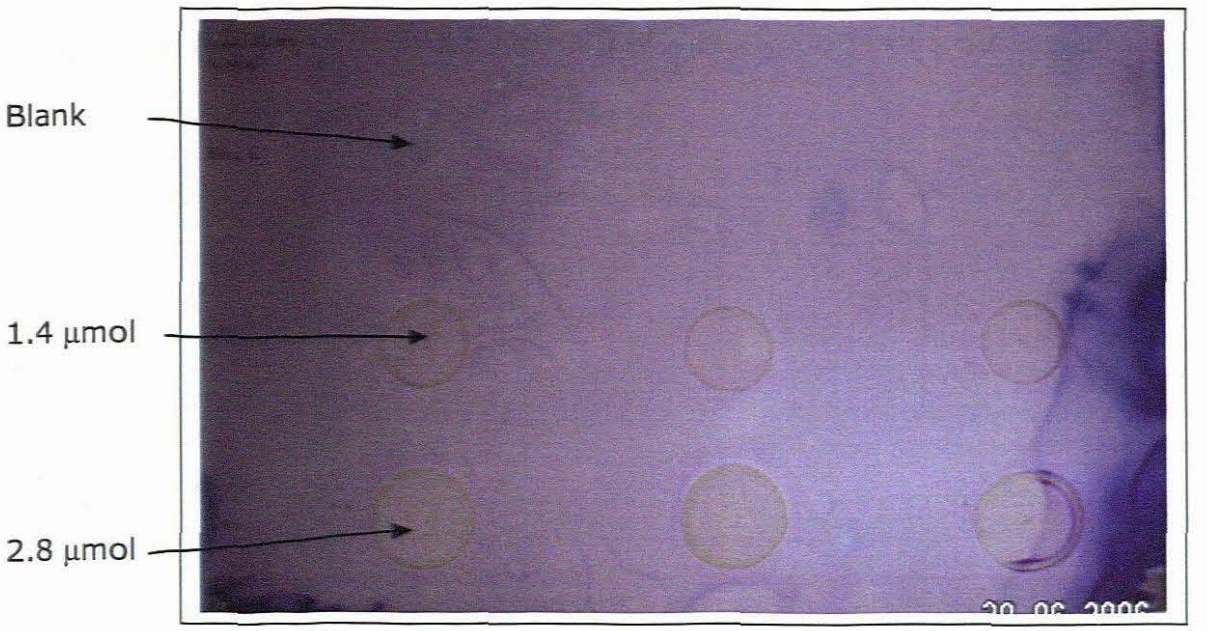


Figure E.7 : Bis-trifluoroacetoxy oleanolic acid (7)

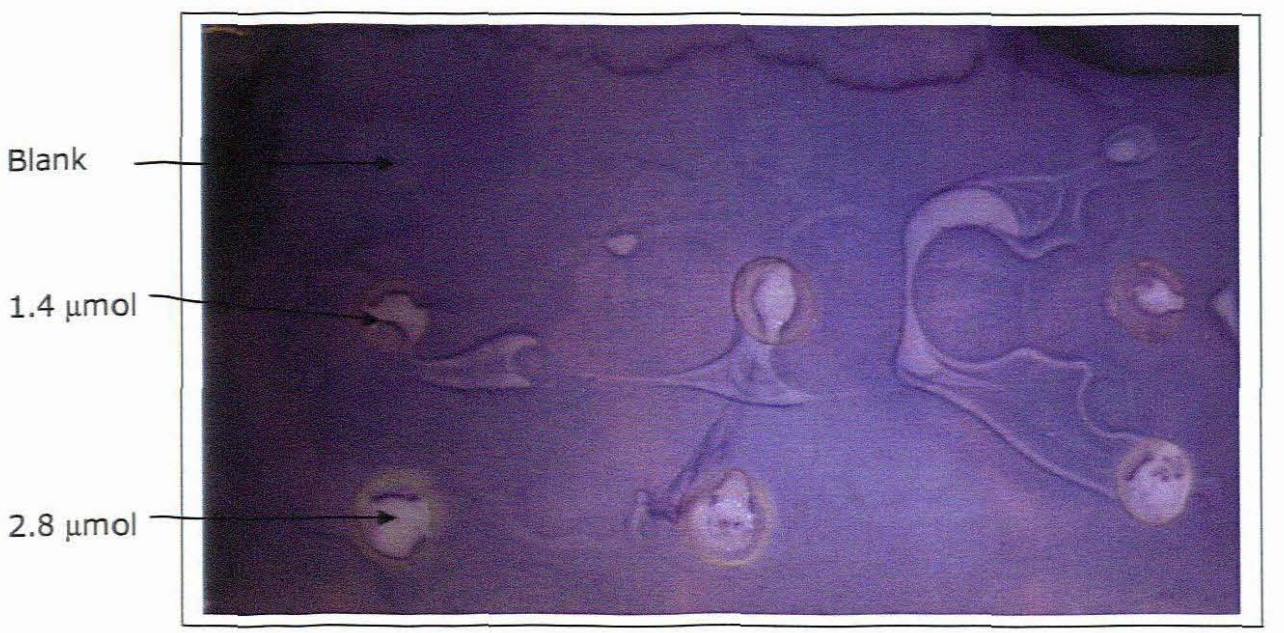


Figure E.8 : Trifluoroacetoxy oleanolic acid (9)

Table 6.18. Evaluation of antimicrobial activity of compounds against *Proteus vulgaris* organisms

Figure	Compound	Inhibition 1.4 μmol	Inhibition 2.8 μmol	Discussion
E.1	Chloro	+	+	Diffused inhibition, diffusion of area of inhibition
E.2	(1)	+	+	Inhibition, blue ring in middle of each spot
E.3	(2)	+	±	Inhibition, outer yellow circle for 2.8 μmol spots, with blue centre
E.4	(3)	+	+	Inhibition
E.5	(4)	-	+	Inhibition at edges, blue centres
E.6	(5)	±	±	Inhibition
E.7	(7)	-	-	No inhibition
E.8	(9)	+	+	Inhibition in middle of each spot

F. Eschericia coli

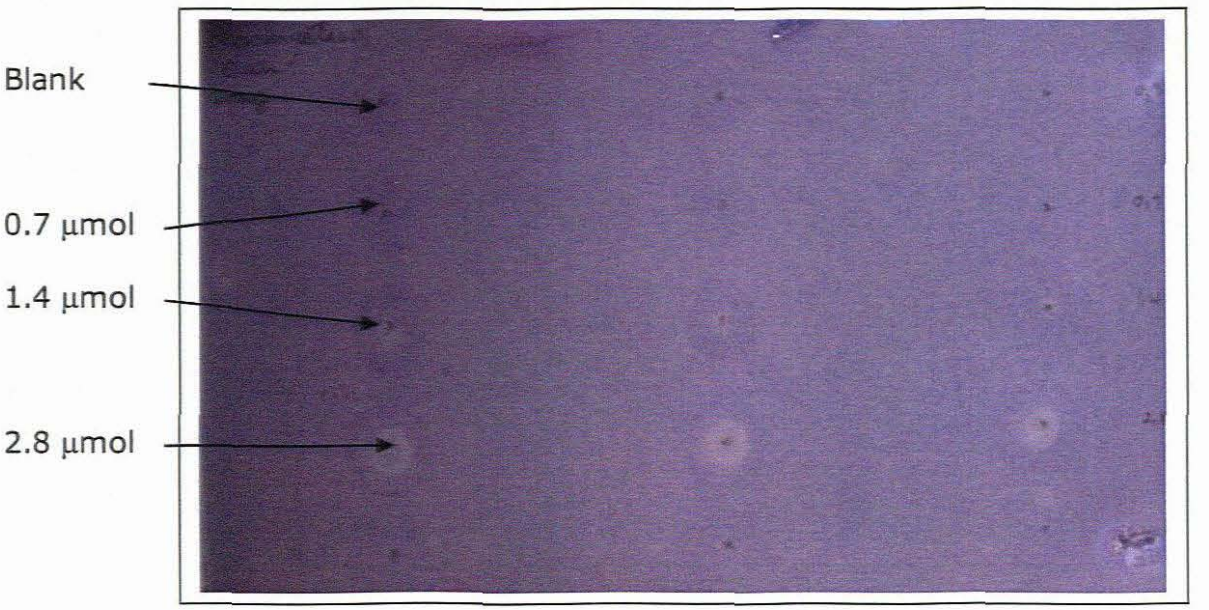


Figure F.1 : Chloromycetin (isopropanol)

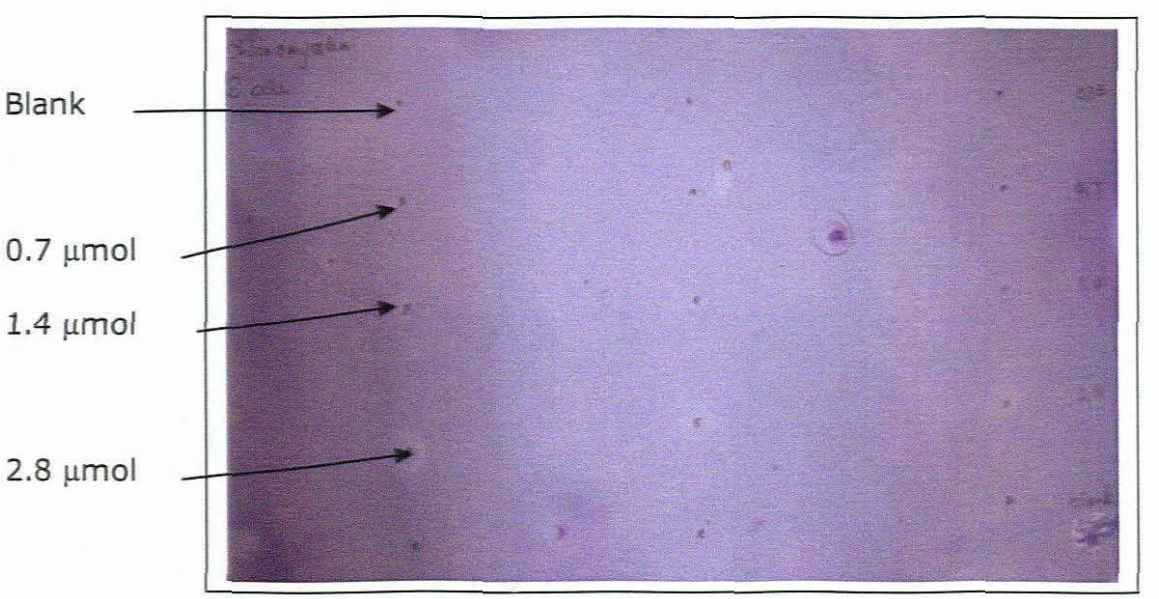


Figure F.2 : Chloromycetin (no isopropanol)

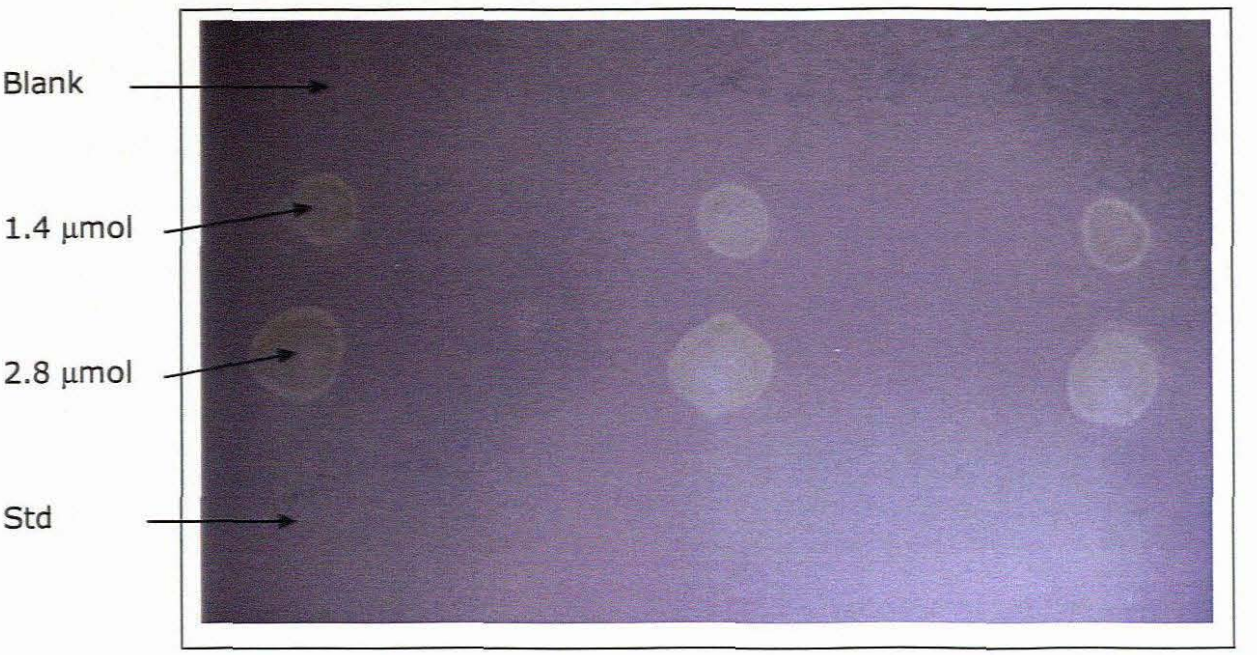


Figure F.3 : Oleanolic acid (1) plus std

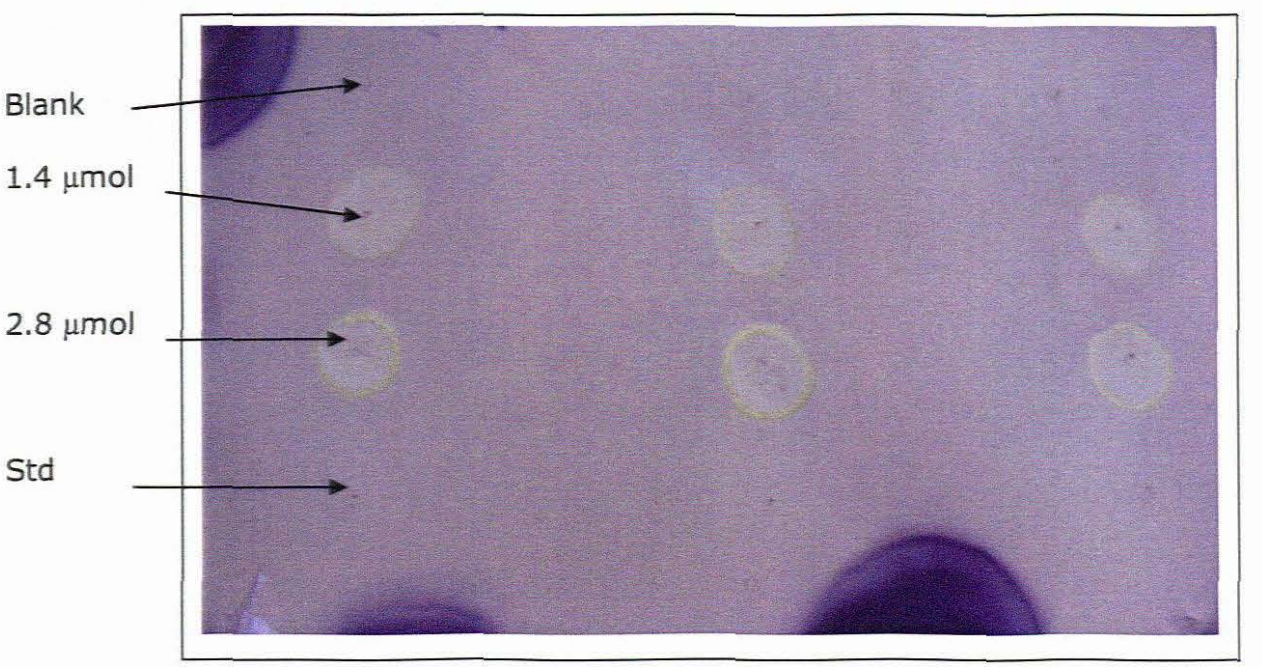


Figure F.4 : 3-O-Acetyloleanolic acid (2) plus std

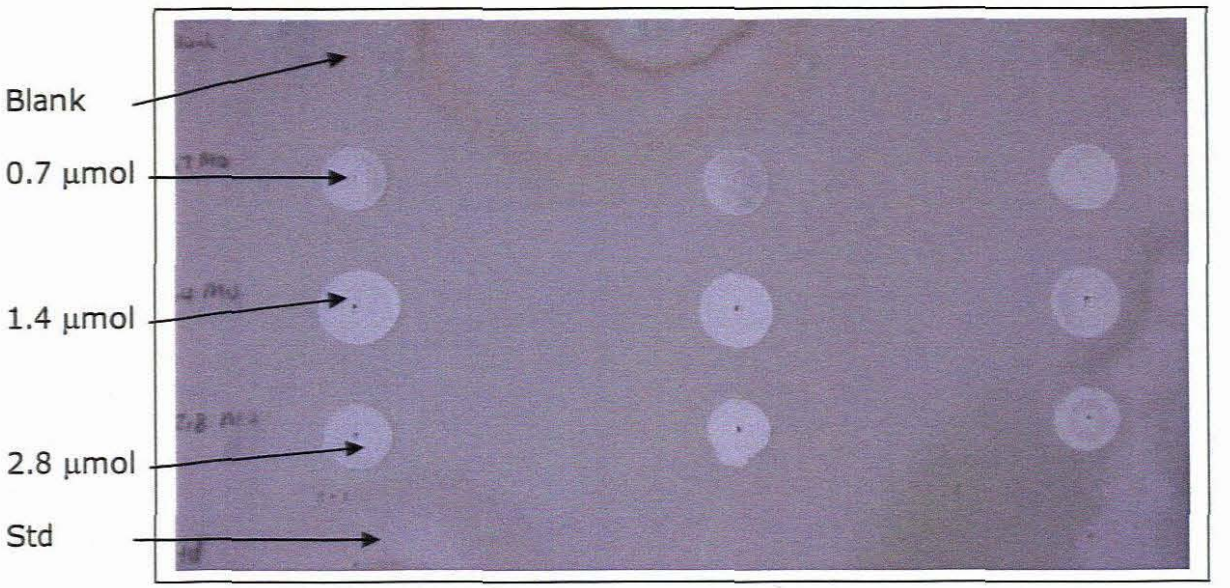


Figure F.5 : Methyl oleanolate (3) plus std

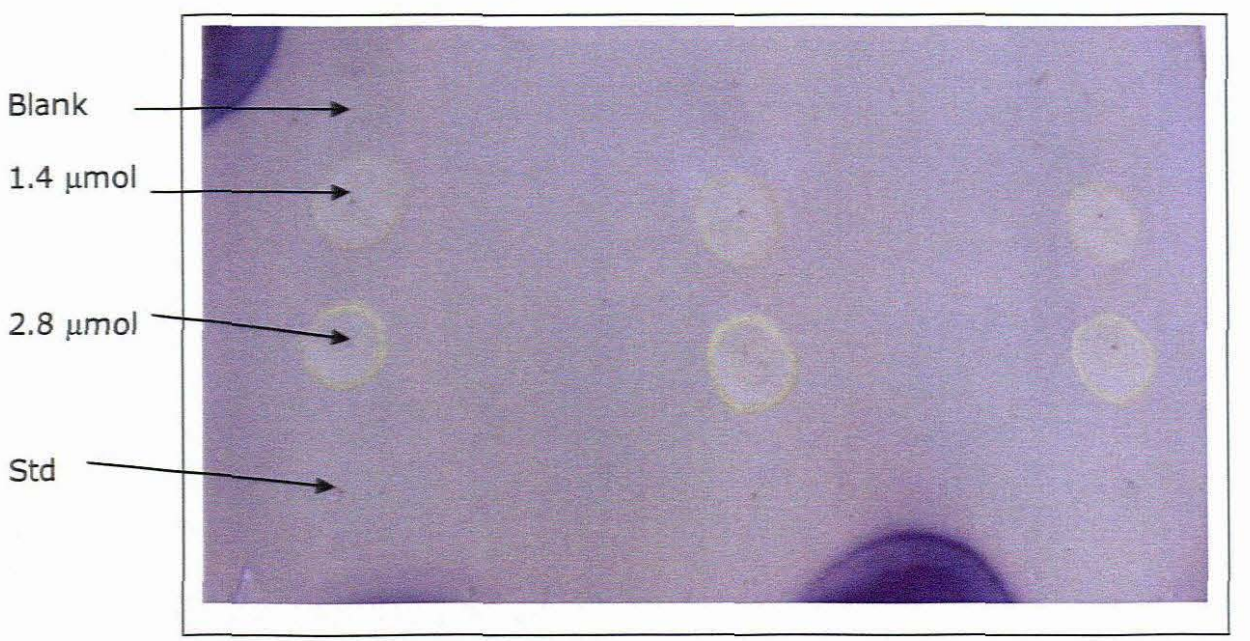


Figure F.6 : Methyl oleanolate (3) (isopropanol) plus std

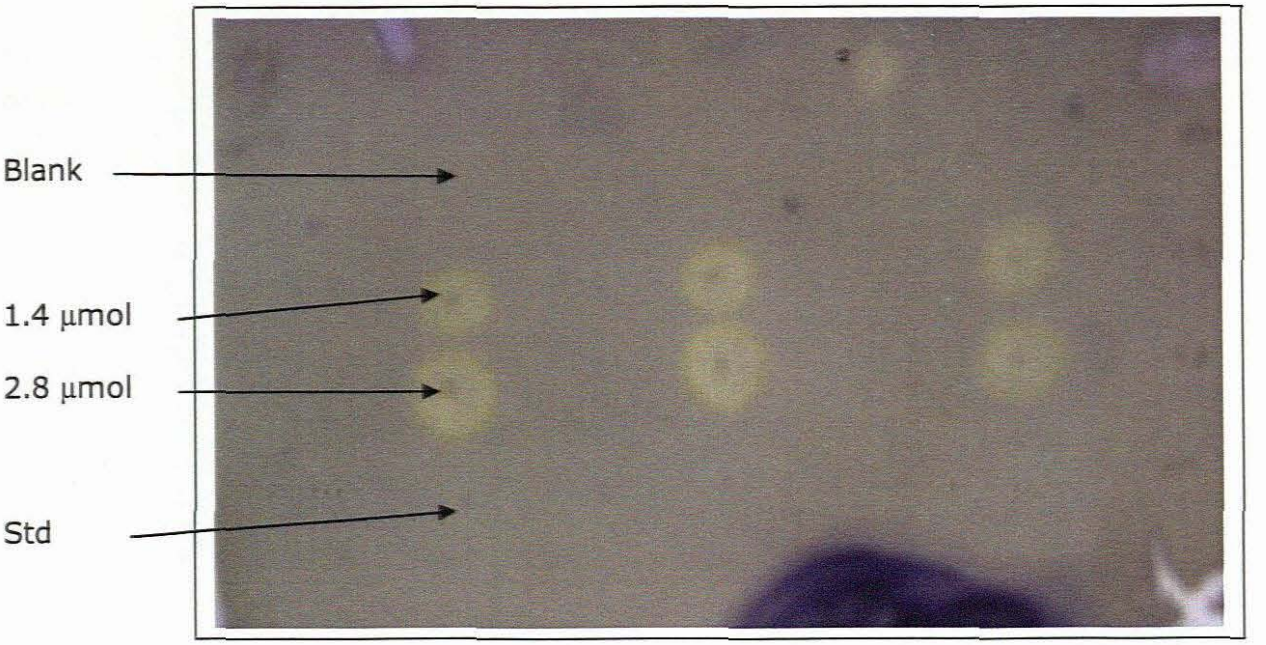


Figure F.7 : Diester (4) plus std

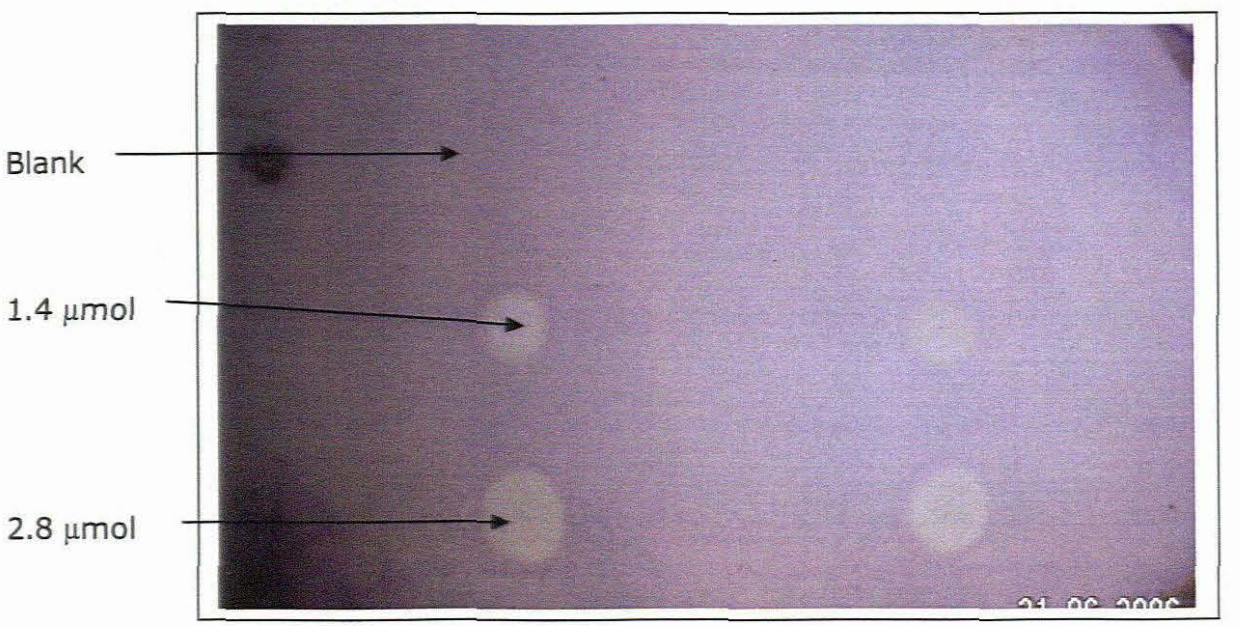


Figure F.8 : Diol (5)

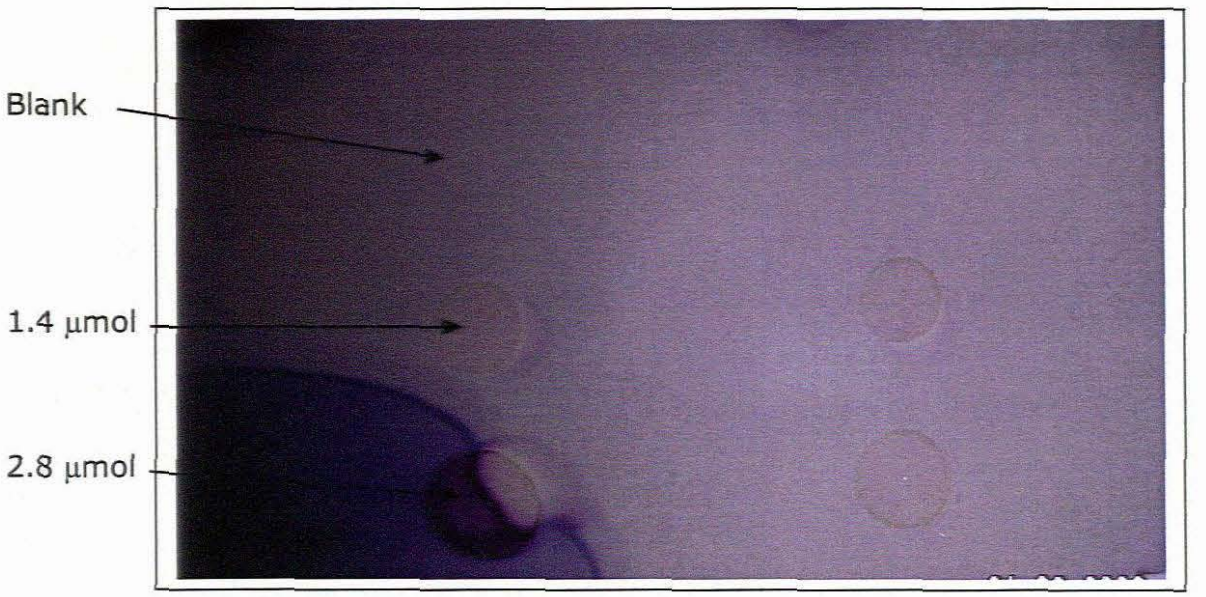


Figure F.9 : Diester of Diol (6)

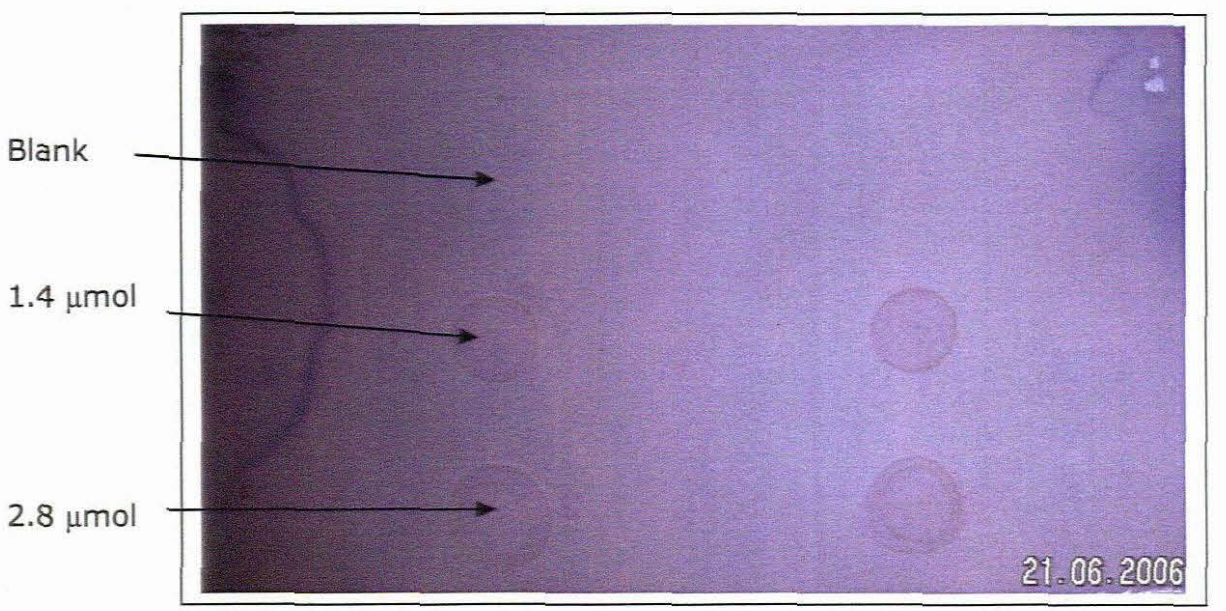


Figure F.10 : Bis-trifluoro acetoxy oleanolic acid (7)

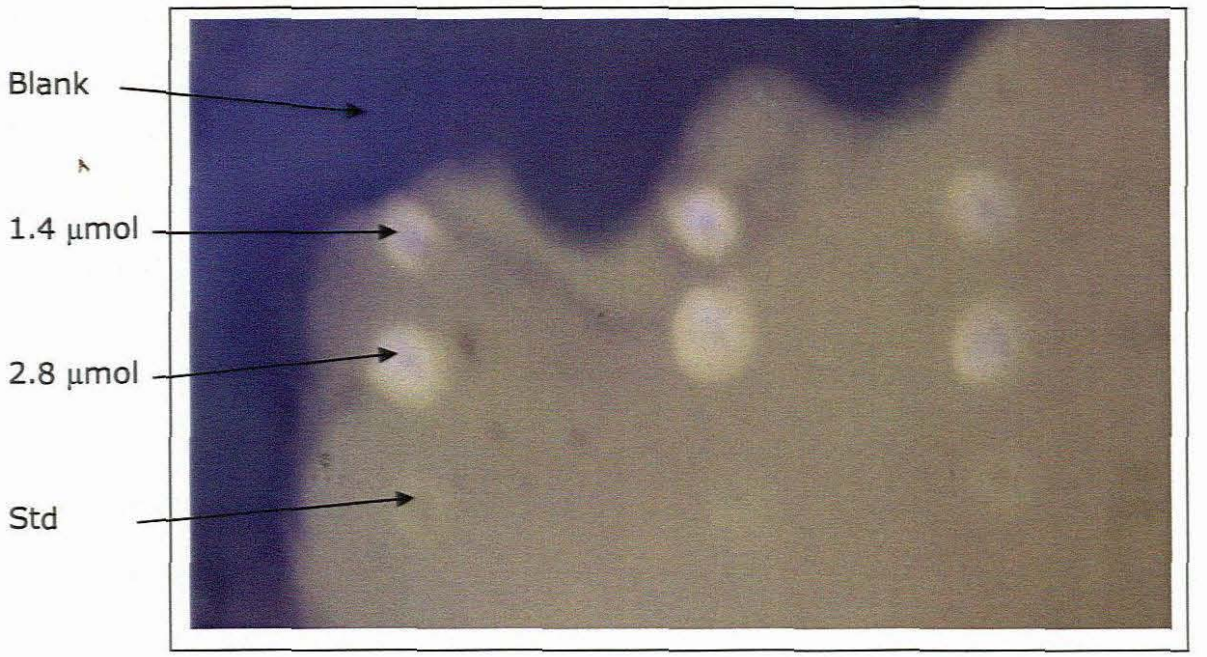


Figure F.11 : Trifluoracetoxy oleanolic acid (9) plus std

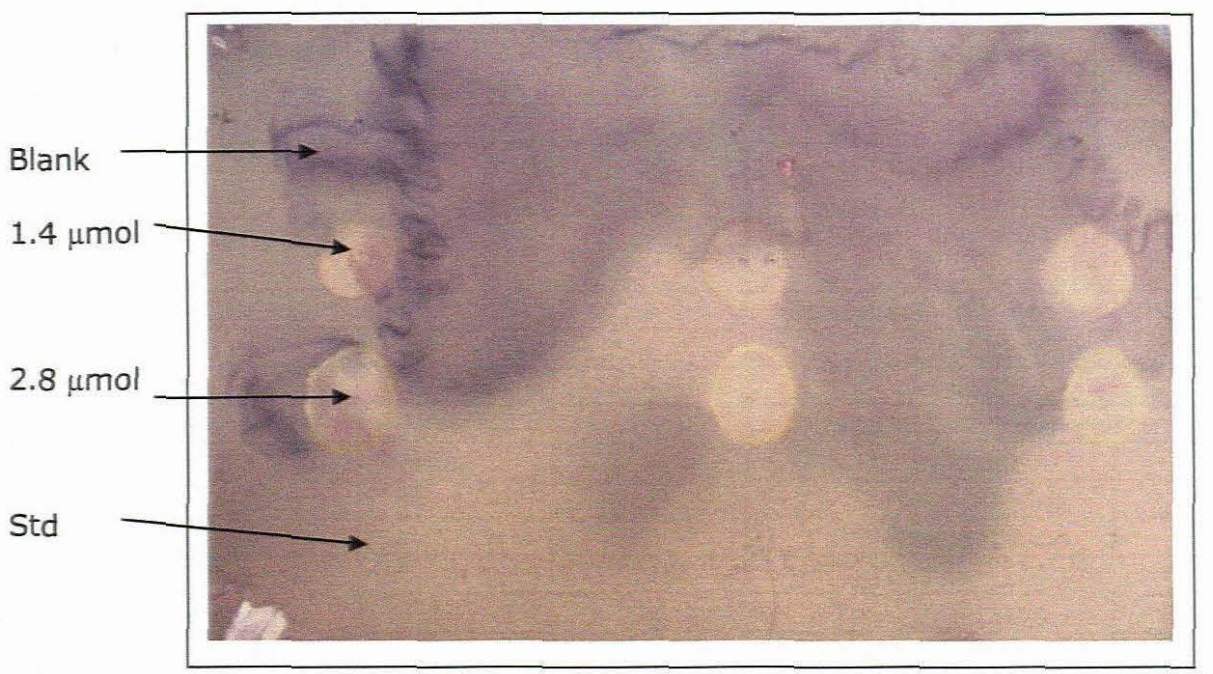


Figure F.12 : Trifluoroacetoxy oleanolic acid (9) plus std

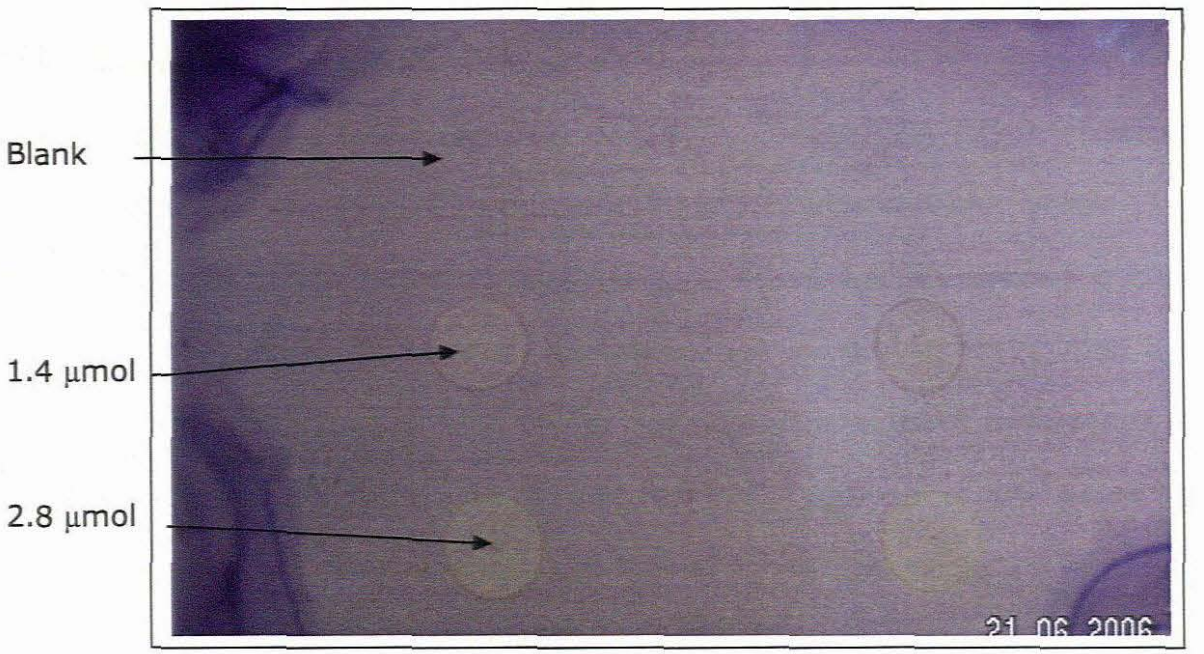


Figure E.13 : 12,13 Dibromo oleanolic acid (11)

Table 6.19. Evaluation of antimicrobial activity of compounds against *Eschericia coli* organisms

Figure	Compound	Inhibition 1.4 µmol	Inhibition 2.8 µmol	Discussion
F.1	*Chloro+Iso*	-	+	*Inhibition (with isopropanol)*
F.2	Chloro	-	+	Inhibition poor (no isopropanol)
F.3	(1)+Std	+	+	Inhibition, slightly blue inner ring
F.4	(2)+Std	+	+	Inhibition, blue inner ring
F.5	(3)+Std	+	+	Inhibition
F.6	(3)+Std+Iso	+	+	Inhibition
F.7	(4)+Std	+	+	Inhibition, slightly yellow
F.8	(5)	+	+	Inhibition
F.9	(6)	-	-	No inhibition
F.10	(7)	-	-	No inhibition
F.11	(9)+Std	+	+	Inhibition by (10) and std
F.12	(9)+Std	+	+	Inhibition
F.13	(11)	-	±	Weak outer ring of inhibition

Discussion of antibacterial results

Inhibitory activity against *Staphylococcus aureus* was shown by Oleanolic acid **(1)**, the diol **(5)** and trifluoroacetoxy OA **(9).** 3-O-Acetyl OA **(2)**, the diester **(4)** and dibromo OA **(11)** showed some inhibition with an outer yellow ring which appeared on the outer edge of the spot. There was an amount of blue staining in the middle of the spot. An explanation for this is, firstly, that the concentration of the compound is at the highest on the outer circumference of the spot; hence the compound is able to inhibit growth of the bacteria. Secondly, the Gram-positive bacterium has a strong peptidoglycan wall that could prevent the activity of the compound in inhibiting its growth at the centre of the spot. Methyl oleanolate **(3)**, the derivatives of diol viz. diester of diol **(6)** and bis-trifluoroacetoxy OA **(7)** did not show any inhibition.

In the case of *Bacillus subtilis*, there were cases where the evaluations were difficult to make, as the spots were not always clearly defined. There appears to be definite inhibition of the bacteria by **(1)** and **(5)**. The compounds have shown well-defined yellow spots without any growth of bacteria. **(2)** has shown some inconsistent inhibition. **(4)**, **(9)** and **(7)** have inhibited growth of the bacteria, but there are blue central patches on each spot as in *Staphylococcus aureus*. **(3)** showed no activity against *Bacillus subtilis*.

The activity of the compounds against *Pseudomonas aeruginosa* was evaluated once only with the standard applied at the bottom of each plate. There is the appearance of a yellow outer ring with each spot, but the standard had diffused, making it difficult to decisively analyse each plate.

The plates that had been dipped in *Proteus vulgaris* organisms were evaluated more easily. **(3)** showed good inhibition, **(1)**, **(5)** and **(10)** showed inhibition, with a circle of blue in the middle of each spot. **(2)** also had a yellow outer ring but the middle of each spot was also blue, and **(9)** showed no inhibition.

All compounds tested for antimicrobial activity except **(4)**, **(6)** and **(7)** showed inhibition against *Eschericia coli*. In the case of **(11)**, it was present only at the amount of 2.8 μmol, hence the MIC would be for that amount of compound. The others, i.e. **(1)**, **(2)**, **(3)**, **(5)** and **(9)** demonstrated inhibitory activity at both 1.4 and at 2.8 μmol, giving a lower MIC value for each of these compounds.

The last organism the compounds were tested against was the yeast *Candida albicans*. The standard, chloromycetin, indicated a white circle surrounded by a thin blue boundary which showed apparent inhibition by the antibiotic; amphotericin's activity presented as a yellow circle in the middle of the spot, surrounded around the edge by a thin blue circle. Of the study compounds, inhibition was demonstrated by **(5)** only. In **(2)**, **(3)**, **(4)** and **(9)** inhibition around the outer edges of the spot with blue inside the spot, was shown. A white centre of the spot with a blue ring near the edge was given by **(1)**. Inhibitory activity is indicated by this observation. No inhibition by **(6)**, **(7)** and **(11)** were shown.

CONCLUSION

Oleanolic acid was successfully isolated from the dried buds of *Syzigium aromaticum* with a crude yield of 1.2% and a pure yield of 0.8%.

The stereochemistry of **(1)** was determined and agreed with findings previously reported by Abd el Rahim and Carlisle[29]. This section of my study was extremely challenging as the crystals were so difficult to grow to an acceptable size for the analysis.

Derivatives **(2)** to **(11)** were successfully synthesised although yields were low in some cases.

As mentioned before, the compounds analysed for antimicrobial activity were insoluble in aqueous medium and the modified tetrazolium salt sensitivity method appeared to give the best analyses of the activity.

Compounds **(6)** showed no activity against any of the six organisms which were tested. Compounds **(7)** and **(11)** showed only a questionable amount of activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Eschericia coli*.

Evaluations demonstrated that compound **(3)** was active against the Gram-negative compounds and the fungus, *Candida albicans*. Compounds **(1)**, **(2)**, **(4)**, **(5)** and **(9)** were found to be active against both Gram-positive and Gram-

negative organisms to a varying degree. From a subjective point of view, it appears as though compound **(1)** and **(5)** are the two compounds which demonstrated the highest antimicrobial activity.

Compounds **(8)** and **(10)** were not tested for antimicrobial activity due to the lack of sufficient compound. Compound **(10)** has already been shown to be an anti-tumour agent by Huang et al.[20]; hence further research to establish a more appropriate synthetic method and investigate its antimicrobial activity is required.

It is hoped that the results obtained from this study will engender further research in this field.

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