CHAPTER 5
STRUCTURE ELUCIDATION

5.1. INTRODUCTION

In the available literature, no information about compounds isolated from any *Curtisia* species was found. *Curtisia dentata* is the only species of the genus occurring in Africa. There is only one other member of the Cornaceae family occurring in Africa i.e. *Afrocrania volkensii* (Klopper *et al.,* 2006). Furthermore, there are few reports on chemical constituents of other genera within the family Cornaceae. Betulinic acid was isolated from the bark of *Cornus florida* Hook at a yield of 2% (Robertson *et al.,* 1939). Polyphenolic compounds such as tannins (Okuda *et al.,* 1984; Lee *et al.,* 1989) and iridoid compounds (Stermitz and Krull, 1998; Tanaka *et al.,* 2001) were isolated from some members of the genus *Cornus.* Different anthocyanins are present in extracts of *Cornus alterifolia, C. controversa, C. kousa* and *C. florida* (Vareed *et al.,* 2006). Triterpenoids such as A1 barringenol, oleanic acid (Silva *et al.,* 1968), 21,23-epoxytirucalla-7,24-diene-3-one and 3β-acetoxy-21,23-epoxytirucalla-7,24-diene (Bhakuni *et al.,* 1987) are common in the genus *Cornus.* There are no literature records concerning isolation and characterization of chemical components from any *Curtisia* species. The aim of this chapter was to elucidate the structures of the isolated compounds and compare them with those isolated from other members of the Cornaceae.

5.2. MATERIALS AND METHODS

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry were used to determine the structures of isolated compounds. In some instances DEPT (distortionless enhancement by polarization transfer) NMR was used to further confirm the structures of the compounds. The spectral data was first compared with data recorded in literature to determine whether the compounds were known or novel. The comparison included a search of compounds isolated from the Cornaceae family in which *Curtisia dentata* belongs.

5.2.1. Thin layer chromatography

Isolated compounds were analyzed by TLC separation using different solvent systems to ascertain their purity and determine R_f values. The compounds (10 µg) were loaded on aluminum-backed TLC plates and developed using CEF, BEA and DE (4:1) as mobile phases. The developed plates were visualized under UV light, sprayed with vanillin-sulphuric acid and then scanned.

5.2.2. ^{13}C and ^{1}H NMR spectroscopic analysis

^{1}H and ^{13}C NMR experiments were performed on a Varian 300 MHz (University of Limpopo, Medunsa Campus) spectrometer operating at 300.0 MHz for hydrogen and 75.4 for ^{13}C, using $DMSO_{d6}$ or $CDCl_3$ as solvents. Solutions were prepared with between 18 – 30 mg of pure compounds in 0.5 ml of $CDCl_3$ for **CI** and **HI** or $DMSO_{d6}$ for **CII**, **CIII** and **CIV**.

5.2.3. Mass Spectrometry

About 1 mg of each compound was submitted for mass spectrometric analysis at the University of Johannesburg. Electron impact mass spectrometry (EIMS) was used to analyze the isolated compounds.

5.3. RESULTS AND DISCUSSION

5.3.1. Thin Layer Chromatographic Analysis of Isolated Compounds

When BEA and CEF were used as mobile phases compounds **CII** and **CIII** migrated similar distances on TLC chromatograms, as confirmed by their R_f values (Table 5.1). Equal quantities (10 µg) were loaded for each compound on TLC plates. DE (4:1) resulted in effective separation of the two compounds (Fig. 5.1). This observation suggests that these two compounds fall within the same solubility range, are related and belong to probably the same family of compounds. Compound **CI**, which seemed non-polar as it migrated a similar distance with the solvent front, may also be related to **CII** and **CIII**, since they all stained red-purple upon spraying with vanillin-sulphuric acid. It is likely that they all belong to the same family and that the differences between them may be attributed to different chemical groups on the parent structure. Compound **CIV** is the most polar of the isolated compounds since it migrated the

shortest distance with the lowest R_f value (Fig. 5.1). From comparing concentration in the crude fractions, it appears that CII is present in the highest concentration in the crude fractions.

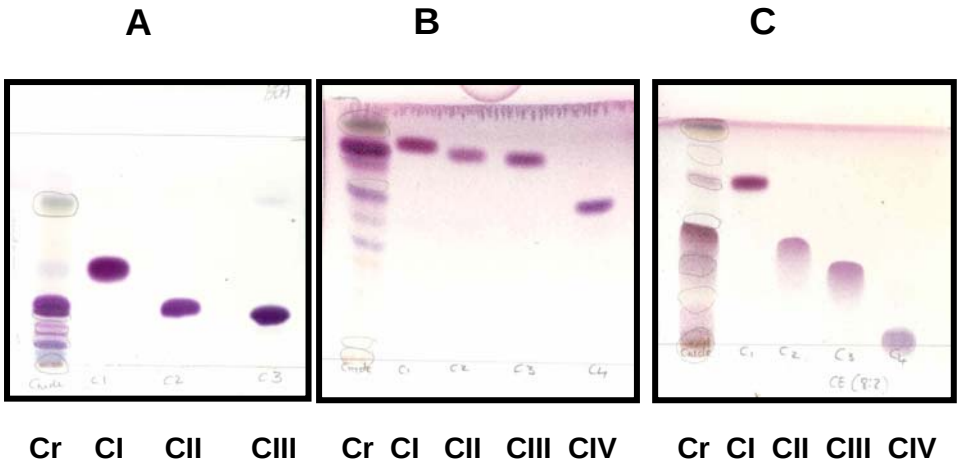


Figure 5.1. TLC analysis of the four isolated compounds (**CI, CII, CIII** and **CIV**) and dichloromethane crude fraction (Cr). BEA (A), CEF (B) and DE (4:1) (C) were used as mobile phases during development of chromatograms. TLC chromatograms were visualized under UV light and then sprayed with vanillin-sulphuric acid. Exactly10 µg of each of the isolated compounds applied

Table 5.1. R_f values of compounds isolated from *C. dentata* and quantity obtained.

Compounds	CEF	BEA	DE (4:1)	Mass (mg)
CI	0.84	0.41	0.79	210
CII	0.76	0.24	0.58	219
CIII	0.76	0.20	0.44	41
CIV	0.54	ND	0.00	21

Key: ND, Not done

5.3.2. Characterization of compound CI

Compound **CI** was isolated as an amorphous colourless powder (210 mg). The ^{1}H NMR showed the presence of seven tertiary methyl groups at δ 0.98, 0.78, 0.84, 1.06, 0.96, 0.78 and 1.67. The ^{13}C NMR assignments of **CI** revealed the presence of seven methyl, 11 methylene and six methane carbons. The chemical shifts observed through ^{13}C NMR experiment were identical to those of lup-20(29)-en-3β-ol (lupeol) (Burns *et al.,* 2000) (Table 5.2, Fig. 5.2). The mass spectrum of **CI** revealed a

molecular ion peak at *m/z* 426 corresponding to the molecular formula $C_{30}H_{50}O$. The other fragment ion peak was at *m/z* 411 $[M\text{-}CH_3]^+$, which is characteristic for pentacyclic triterpene with an isopropenyl group (Saied and Begum, 2004). Compound **CI** was identified as lup-20(29)-en-3β-ol (lupeol) following comparison of our spectral data with published data (Fig. 5.2). The spectral data of **HI** was comparable to that of **CI**, suggesting that **HI** is also lupeol.

This triterpene, a common secondary metabolite of plants, has been isolated from many plant species. It is present in *Salvia horminum* (Ulubelen *et al.,* 1977), *Berbveris vulgaris* (Saied and Begum, 2004) and other species (Das & Mahato, 1983; Jassbi, 2006). This appears to be the first report of its presence and isolation from *Curtisia spp.*

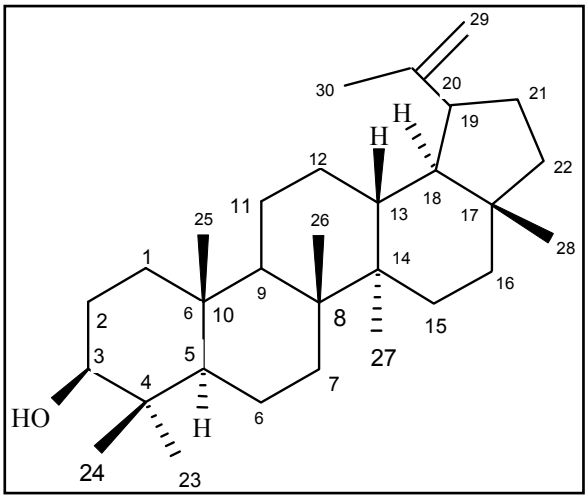


Figure 5.2. The structure of **CI** (lupeol) isolated from the leaves of *Curtisia dentata.*

5.3.3. Characterization of compound CII

Compound **CII** was isolated as a colourless amorphous powder (219 mg). The ^{13}C NMR spectra revealed 30 carbon signals (Table 5.3.) which were shown by DEPT experiments to be five methyls, five quaternary carbons, one carboxylic acid, and two olefinic carbons suggesting that compound **CII** is a triterpenic acid having five rings (Siddiqui *et al.,* 1988). The ^1H NMR spectrum of compound **CII** showed the signals for five tertiary methyl groups at δ 0.74, 0.85, 0.85, 0.63 and 0.91, one isopropenyl moiety at δ 1.77, 4.55 and 4.67, indicating a lupane-type skeleton (Fig. 5.3). The mass spectrum of **CII** displayed a molecular ion peak (M^+) at *m/z* 456 corresponding to the formula $C_{30}H_{48}O_3$. Compound **CII** was identified as 3β-hydroxy-lup-20(29)-en-

28-oic acid, betulinic acid by direct comparison of its spectral data with reported values (Table 5.3.) (Siddiqui *et al.,* 1988).

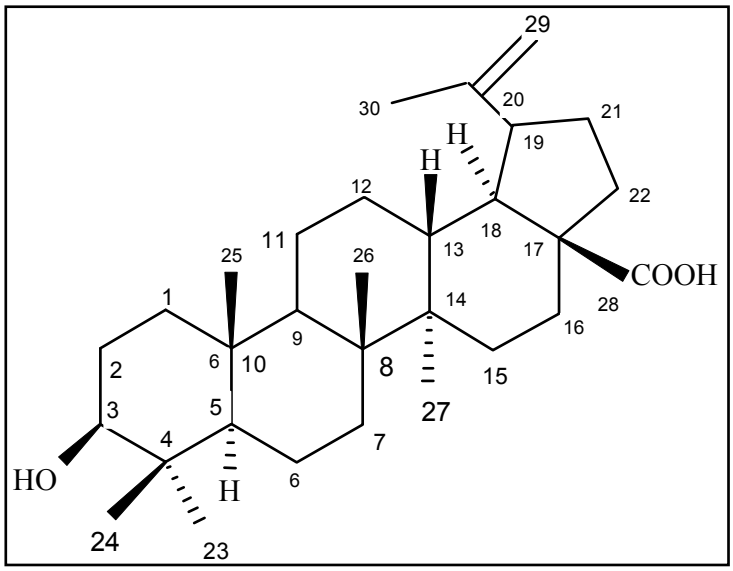


Figure 5.3. The structure of **CII** (betulinic acid) isolated from the leaves of *Curtisia dentata.*

This pentacyclic triterpene (betulinic acid) is a common secondary metabolite of plants. It is very common and abundant (up to 22% yield) in the *Betula* species (Betulaceae) used by Native Americans as folk remedy to treat intestinal problems such as diarrhoea and dysentery (Yogeeswari and Sriram, 2005). It was isolated from species such as *Ziziphus mauritiana* Lam (Rhamnaceae) collected in Zimbabwe (Pisha *et al.,* 1995), *Arbutus menziesii* (Robinson & Martel, 1969) and many other tree species that are valuable for timber purposes (Pavanasasivan & Sultanbawa, 1974). In the Cornaceae family, betulinic acid was isolated from *Cornus florida* Hook (Robertson *et al.,* 1939). The bark of *Cornus florida* is reported to contain about 2% betulinic acid (Robertson *et al.,* 1939). Bhakuni *et al.* (1987) reported the presence of triterpenes from *Cornus capitata,* some of which were of the lupane family. This appears to be the first report of the presence of betulinic acid in *Curtisia dentata.*

5.3.4. **Compound CIII**

The mass spectral data of compound **CIII** showed the molecular ion peak at *m/z* 456 and a base peak at *m/z* 248 along with a strong peak at *m/z* 203 due to retro-Diels-Alder fragmentation, typical of Δ^{12}-oleanene or ursine triterpene with the molecular formula $C_{30}H_{48}O_3$ (Mahato and Kundu, 1994). The structure of **CIII** is shown in Fig. 5.4 while the retro-Diels-Alders fragmentation is displayed in Fig. 5.5. The ^{1}H NMR

spectrum of **CIII** exhibited signals of an olefinic proton at δ5.11 and seven methyls characteristic of the oleanene skeleton. This structural type was further supported by the ^{13}C NMR spectrum, which contained resonance for olefinic carbons (δ_c 125.2 [C-12], 138.8 [C-13]) and a carboxylic acid at δ_c 178.9. The complete ^{13}C NMR data is given in Table 5.4. and Appendix 1. Full characterization of **CIII** was done by comparison with reported data for 3β-hydroxy-urs-12-en-28-oic acid (ursolic acid) (Hamzah & Lajis, 1998).

Ursolic acid has been isolated as the bioactive chemical entity from several plant species of importance in traditional and folk medicine (Mallavadhani *et al.,* 1998). It was isolated from *Hedyotis berbacea* (Hamzah & Lajis, 1998), *Rosmarinus officinalis* (Collins & Charles, 1987) and others (Mahato *et al.,* 1988). However, this appears to be the first report of its isolation from *Curtisia dentata.*

5.3.5. Compound CIV

The spectral data of **CIV** (21 mg) were very similar to that of compound **CIII.** The ^{13}C NMR spectrum displayed signals for 30 carbon atoms, which were one carbonyl (δ_c 178.4), two ethylenic carbon atoms, two oxymethines, seven methyls, eight methylene, five methines and six quaternary carbons (Table 5.5, Fig. 5.5 and appendix 1). The ^{1}H NMR spectrum showed an olefinic proton signal at δ5.01. The presence of a double bond at C-12 was confirmed by the chemical shifts of C-12 (δ_c 124.56) and C-13 (δ_c 138.32) characteristic of triterpene of Δ^{12}-ursene type (Mahato & Kundu, 1994). Compound CIV was identified as 2α-hydroxy ursolic acid by comparison of ^{1}H and ^{13}C NMR spectra with those of CIII and spectral data in literature (Bandaranayake *et al.,* 1975; Kojima & Ogura, 1989).

The compound 2α-hydroxyursolic acid has been isolated from many plant species (Olafsdottir *et al.,* 2001; Begum *et al.,* 2002; Yamagishi *et al.,* 1988). Its isolation from *C. dentata* has not been reported in literature.

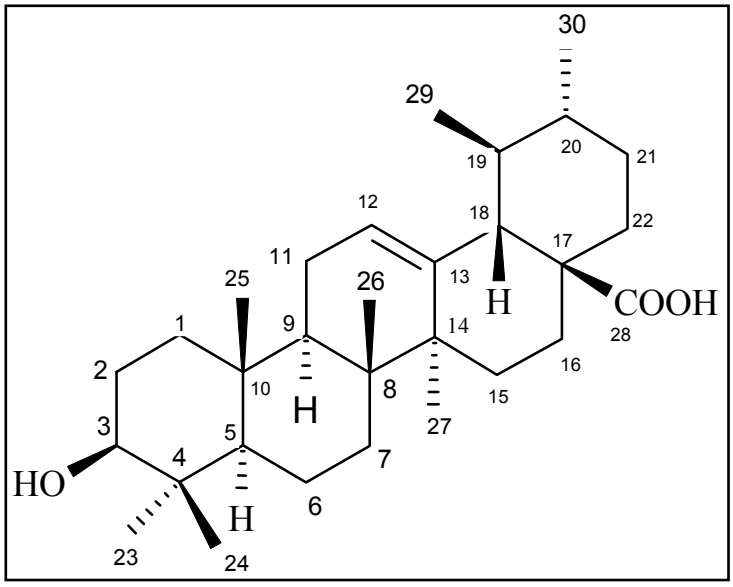


Figure 5.4. The structure of **CIII** (ursolic acid) isolated from the leaves of *Curtisia dentata.*

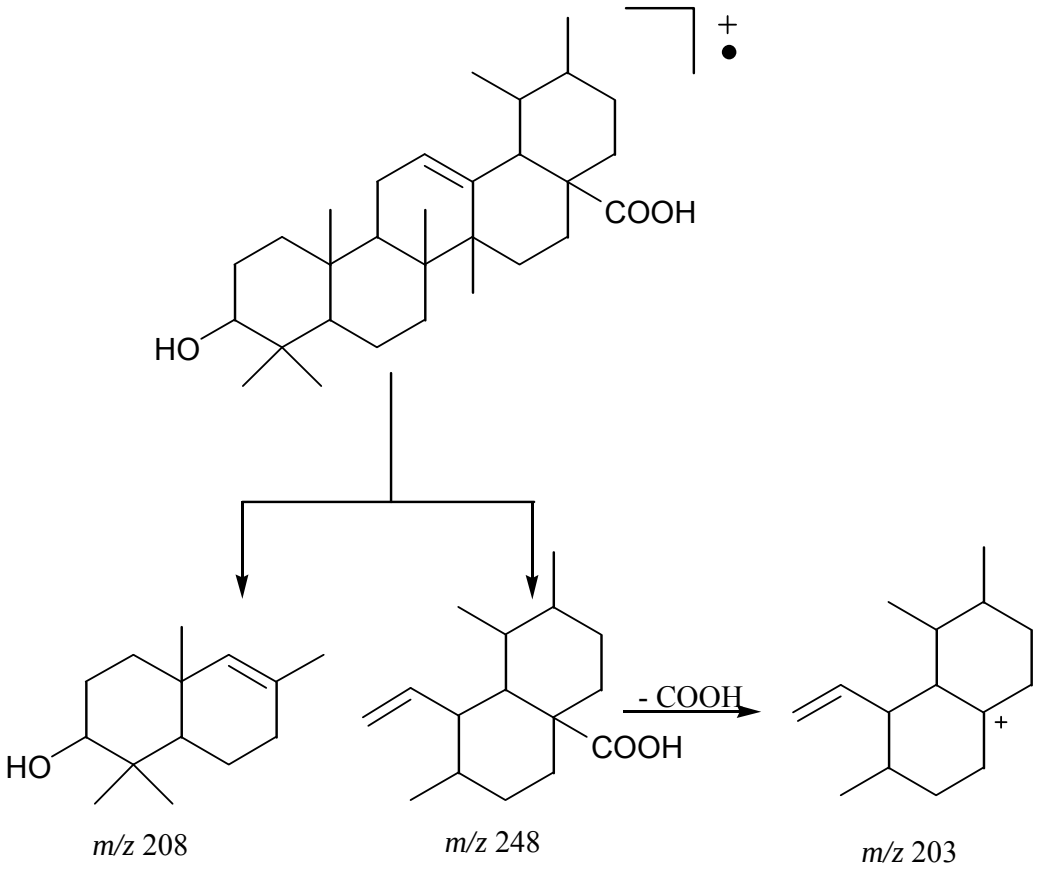


Figure 5.5. Retro-Diels-Alders fragmentation pattern of ursolic acid (**CIII**) isolated from the leaves of *Curtisia dentata.*

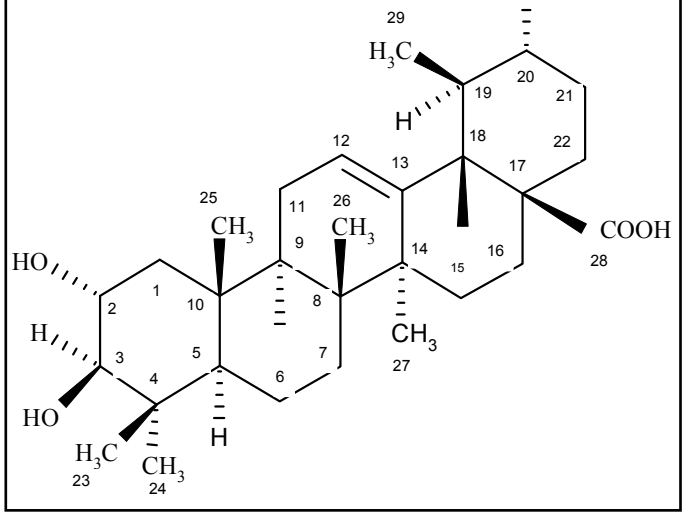
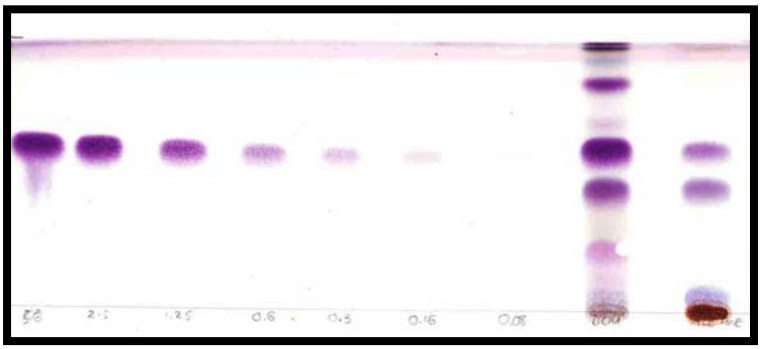


Figure 5.6. The structure of **CIV** isolated from leaves of *Curtisia dentata.*

5.3.6. Estimation of betulinic content of *C. dentata* leaves

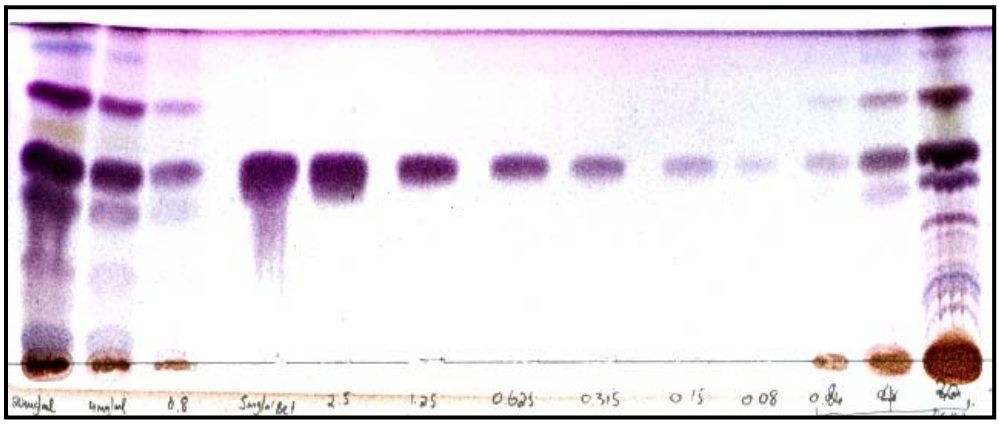
Betulinic acid was the most abundant of the isolated compounds, present in most of the bulk fractions of *C. dentata.* The quantity of betulinic acid was estimated by analyzing known concentrations alongside crude acetone and dichloromethane fractions on TLC plates. The amounts of betulinic acid ranged from 0.8-50 µg. For both DCM and acetone crude fractions 100 µg was loaded on TLC plates. The plates were sprayed with vanillin-sulphuric acid spraying reagent and heated for 5 min at 100^{o}C to visualize the compounds. The intensity of betulinic acid in the bulk dichloromethane fraction was comparable to the intensity in the lane loaded with 25 µg of betulinic acid. In 100 µg of the bulk dichloromethane fraction about 25% (25 µg) is the betulinic acid content. The same method was used to estimate the concentration of betulinic acid in the bulk acetone fraction. The quantity of betulinic acid was estimated at 6% (about 6 µg) in the acetone fraction of *C. dentata* leaves (Fig. 5.7). The estimated quantity of betulinic acid in the dichloromethane is lower than to the quantity found in *Betula* species (Betulaceae), at approximately 22% (Yogeeswari and Sriram, 2005).



µg loaded	50	25	12.5	6.0	3.0	1.6	0.8	DCM	ACN

Figure 5.7. Estimation of betulinic acid content in the dichloromethane and acetone fractions of *Curtisia dentata* leaves. DCM, dichloromethane fraction; ACN, acetone fraction.

The quantity of betulinic acid was also estimated in both the acetone and dichloromethane individual extracts as described above. It appeared that the betulinic acid band in 200 µg of acetone extract was equivalent to the band corresponding to 50 µg of betulinic acid standard. This means that betulinic acid concentration in the acetone extracts is around 25%. The betulinic acid band in the 40 µg of methanol extract appeared equivalent to the band loaded with 6.3 µg of betulinic acid standard, suggesting the betulinic acid level in the methanol extract is about 15%.



µg loaded	200	40	8	50	25	12.5	6.3	3.2	1.6	0.8	8	40	200
	Acetone										**MeOH**		

Figure 5.8. Estimation of betulinic acid content in the acetone and methanol extracts of *Curtisia dentata* leaves. DCM, dichloromethane fraction; ACN, acetone fraction. MeOH, methanol extract.

Table 5.2. ^{13}C NMR data of compound **CI** compared with literature data (Burns *et al.,* 2000). Both experiments were conducted in $CDCl_3$ as the solvent.

Carbon	Lupeol (Burns *et al.*, 2000)	Compound CI
1	38.72	37.72
2	27.43	27.42
3	79.02	79.05
4	38.87	38.86
5	55.31	55.28
6	18.33	18.32
7	34.29	34.28
8	40.84	40.84
9	50.35	50.43
10	37.18	37.17
11	20.94	20.93
12	25.16	25.15
13	38.07	38.05
14	42.85	42.83
15	27.46	27.94
16	35.60	35.59
17	43.01	43.00
18	48.32	48.29
19	48.00	47.98
20	150.98	150.97
21	29.86	29.69
22	40.02	40.00
23	28.00	28.03
24	15.38	15.40
25	16.13	16.08
26	15.99	16.08
27	14.56	15.32
28	18.02	17.96
29	109.32	109.31
30	19.32	20.92

Table 5.3. ^{13}C NMR chemical shift assignments for betulinic acid (Siddiqui *et al.,* 1988) and compound **CII**

Carbon	Betulinic Acid (Solichin *et al.*, 1980;Siddiqui *et al.*, 1988)	Compound CII
1	39.1	37.6
2	28.1	27.2
3	78.1	76.8
4	39.4	38.5
5	55.7	55.4
6	18.6	18.0
7	34.7	33.9
8	40.9	40.3
9	50.8	49.9
10	37.3	38.3
11	21.1	20.5
12	25.9	25.1
13	38.4	36.7
14	42.4	42.0
15	31.1	31.7
16	32.7	33.9
17	56.3	54.9
18	47.6	46.6
19	49.5	48.5
20	150.7	150.3
21	30.1	30.1
22	37.5	36.4
23	28.5	28.1
24	16.3	15.9
25	16.3	15.8
26	16.2	15.7
27	14.8	14.4
28	178.7	177.3
29	110.3	109.7
30	19.4	18.9

Table 5.4. Ursolic acid (CIII) ^{13}C NMR assignments compared with literature data (Seebacher *et al.,* 2003).

Carbon	Ursolic acid (Seebacher *et al.,* 2003)	Compound CIII
1	39.2	39.1
2	28.2	28.2
3	78.2	77.5
4	39.6	39.8
5	55.9	55.5
6	18.8	18.7
7	33.7	33.4
8	40.1	41.1
9	48.1	47.5
10	37.5	38.9
11	23.7	23.5
12	125.7	125.2
13	139.3	138.9
14	42.2	42.3
15	28.8	27.7
16	25.0	24.5
17	48.1	47.5
18	53.6	53.0
19	39.5	37.0
20	39.4	37.2
21	31.1	30.9
22	37.4	37.2
23	28.8	28.9
24	16.5	16.8
25	15.7	15.9
26	17.5	17.6
27	24.0	23.9
28	179.7	179.0
29	17.5	17.7
30	21.4	21.8

Table 5.5. 2α-hydroxyursolic acid ^{13}C NMR spectral data (Olafdottir *et al.,* 2001) compared with NMR data obtained for compound CIV.

Carbon	hydroxyursolic acid (Olafsdottir *et al.*, 2001)	Compound CIV
1	46.9	47.0
2	67.0	67.2
3	82.1	82.3
5	54.6	54.8
6	17.9	18.1
7	32.5	32.7
9	46.7	46.9
10	38.3	38.5
11	22.8	23.0[b]
12	124.4	124.6
13	138.1	138.3
14	41.6	41.8
15	27.4	27.5
16	23.7	23.9
17	47.0	47.1
18	52.3	52.4
19	37.5	37.6
20	38.4	38.7
21	30.1	30.2
22	36.2	36.4
23	28.7	28.9
24	17.0	17.3
25	16.3	16.5
26	16.8	17.0[a]
27	22.8	23.0[b]
28	178.2	178.4
29	21.0	21.2
30	16.8	17.0[a]
	C4 & C8 about 40.0 hidden under solvent peak.	

CHAPTER 6

BIOLOGICAL ACTIVITY OF ISOLATED COMPOUNDS

6.1. INTRODUCTION

Four pentacyclic triterpenoids, isolated from *Curtisia dentata* using column chromatography, were identified using NMR spectroscopy and mass spectrometry as lupeol (**CI**), betulinic acid (**CII**), ursolic acid (**CIII**) and 2α-hydroxy ursolic acid (**CIV**). Various biological activities of these compounds have been reported.

The activities of pentacyclic triterpenoids of ursane, lupane and oleanone skeleton, which accumulate in a number of terrestrial plants, free or linked to carbohydrate moieties (Liu, 1995), have been extensively reported (Ringbom *et al.,* 1998; Saraswat *et al.,* 2000). Ursolic acid demonstrates activities such as hepatoprotective, anti-inflammatory, antiviral and antitumor activities (Shishodia *et al.,* 2003), as well as growth inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* and with MIC values of 50 µg/ml (Mallavadhani *et al.,* 2004). Zeletova *et al.* (1986) also reported activity of ursolic acid against *S. aureus.* Ursolic acid is also identified as one of the active components of *Rosmarinus officinalis* inhibiting growth of food-associated bacteria and yeast (Collins & Charles, 1987). Ursolic acid isolated from *Crataegus pinatifida* is active against HIV-1 protease at a concentration of 100 µg/ml (Pengsuparp *et al.,* 1994). The C-28 carboxylic acid moiety was suggested and proven as the chemical group responsible for enhanced pharmacological activity of ursolic acid (Kashiwada *et al.,* 2000; Ma *et al.,* 2000). Corosolic acid (2α-hydroxyursolic acid), also called colosolic acid or botanical insulin, enhances uptake of glucose by Ehrlich ascites tumor cells and induced hypoglycemic effects (Murakami *et al.,* 1993).

A variety of biological activities resulting from betulinic acid and other lupane triterpernoids have been published extensively. Betulinic acid is active against *Mycobacterium tuberculosis* with an MIC value of 50 µg/ml (Chaiyadej *et al.,* 2004) and *E. coli* with an MIC value of 64 µg/ml (Rahaman *et al.,* 2002). Betulinic acid is also active against *Bacillus subtilis* at a concentration of 1 000 µg/disc, resulting in a zone of inhibition of 18.8 mm^2 (Chandramu *et al.,* 2003). Betulinic acid isolated from *Syzgium claviflorum* is active against HIV with an inhibitory concentration (IC_{50} value) of 13 µM (Fujoka and Kshiwada, 1994). Betulinic acid derivatives inhibit HIV-1 entry

(Mayaux *et al.,* 1994), HIV-protease (Xu *et al.,* 1996) and reverse transcriptase (Pengsuparp *et al.,* 1994). Esterification of betulinic acid at C-3 results in increased inhibitory potency against HIV protease activity (Ma *et al.,* 1999). Preclinical studies of this modified betulinic acid (PA-457) indicates significant activity against drug-resistant virus, activity in an animal model of HIV and that the potential drug substance is suitable for combination therapy with other anti-HIV drugs (Sigh *et al.,* 2005). Isolation, characterization and determination of biological activity of betulinic acid from *C. dentata* have not been reported.

Diverse ranges of biological activities triggered by lupeol have been reported. The stimulation of programmed cell death in human leukemia cell line (HL-60) by lupeol was observed and reported (Aratanechemuge *et al.,* 2003). Lupeol triggered a 33% reduction in granuloma weight in a chronic granuloma rat model (Agarwal & Rangari, 2003). Geetha and Varalakshmi (2001) also showed that lupeol reduced paw swelling by 39% in an adjuvant arthritis rat model. Lupeol is further reported to possess antimicrobial activity (Ragasa *et al.,* 2005). However, Chaiyadej *et al.* (2004) published that lupeol, in a study to evaluate biological activities of compounds isolated from *Sonneratia alba,* failed to show any activity against *Mycobacterium tuberculosis* and *Plasmodium falciparum.*

6.2. MATERIALS AND METHODS

6.2.1. Bioautography

Bioautography was performed as described in chapter 2. The isolated compounds (**CI – CIII**) were dissolved in acetone at a concentration of 1 mg/ml and 10 μg was loaded on TLC plates. The amount of **CIV** (21 mg) was not enough for bioautographic investigation. Duplicate TLC plates were developed in CEF and dichloromethane: ethyl acetate mixture (DE) (4:1) before spraying with overnight culture suspensions of the test organisms. *Candida albicans* was used as a test fungal organism while *E. coli, Enterococcus faecalis* and *S. aureus* were the selected bacterial organisms. Clear zones on TLC plates indicated inhibition of growth of the test microorganism by a specific compound.

6.2.2. Minimal inhibitory concentration (MIC) determination

The determination of MIC values was performed as outlined in Section 2.3.4. *Candida albicans* was tested together with *Sporothrix schenckii, Aspergillus fumigatus, Microsporum canis*, *Cryptococcus neoformans, Candida guilliermondii and Candida spicata.* Amphotericin B was used as a positive control against fungal test organisms. *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus* were used as bacterial test organisms. Gentamicin was used as a positive control against bacterial test organisms.

6.2.3. Cytotoxicity

Cytotoxity assay was performed as described in Section 2.3.5. With the exception of lupeol and betulinic acid, the compounds were not isolated in sufficient quantities to allow cytotoxicity testing. As a result, only lupeol and betulinic acid were tested against Vero cells.

6.3. RESULTS

6.3.1. Antifungal activity

Bioautography was used in antibacterial and antifungal activity assays to 1) indicate the purity of the isolated compounds and 2) to ascertain that the isolated compounds are similar to those observed in crude extracts. The three compounds investigated using bioautographic experiments were active against *C. albicans.* Ursolic acid showed the most pronounced zones of inhibition against *C. albicans.* Lupeol and betulinic acid were active against *C. albicans* on bioautograms though not to the degree shown by ursolic acid (Fig. 6.1).

The MIC values resulting from each compound against fungal test organisms were recorded (Table 6.1). All the compounds were active against several of the fungal species investigated. Lupeol failed to display appreciable activity against *C. albicans* and *C. spicata* with the highest concentration used (250 µg/ml) failing to inhibit fungal growth. However, lupeol displayed high activity against *S. schenckii* and *M. canis* with MIC values calculated as 12 and 16 µg/ml, respectively. The average MIC values of each compound calculated against all the fungal species tested are displayed in Fig. 6.2. The amphotericin B MIC average was 0.27 µg/ml. The lowest

MIC average resulted from betulinic acid. The highest MIC average was calculated for lupeol to be 128 µg/ml. The average MIC values against each fungal test organism indicated that *S. schenckii* and *M. canis* were the most sensitive to the isolated compounds (Fig. 6.3).

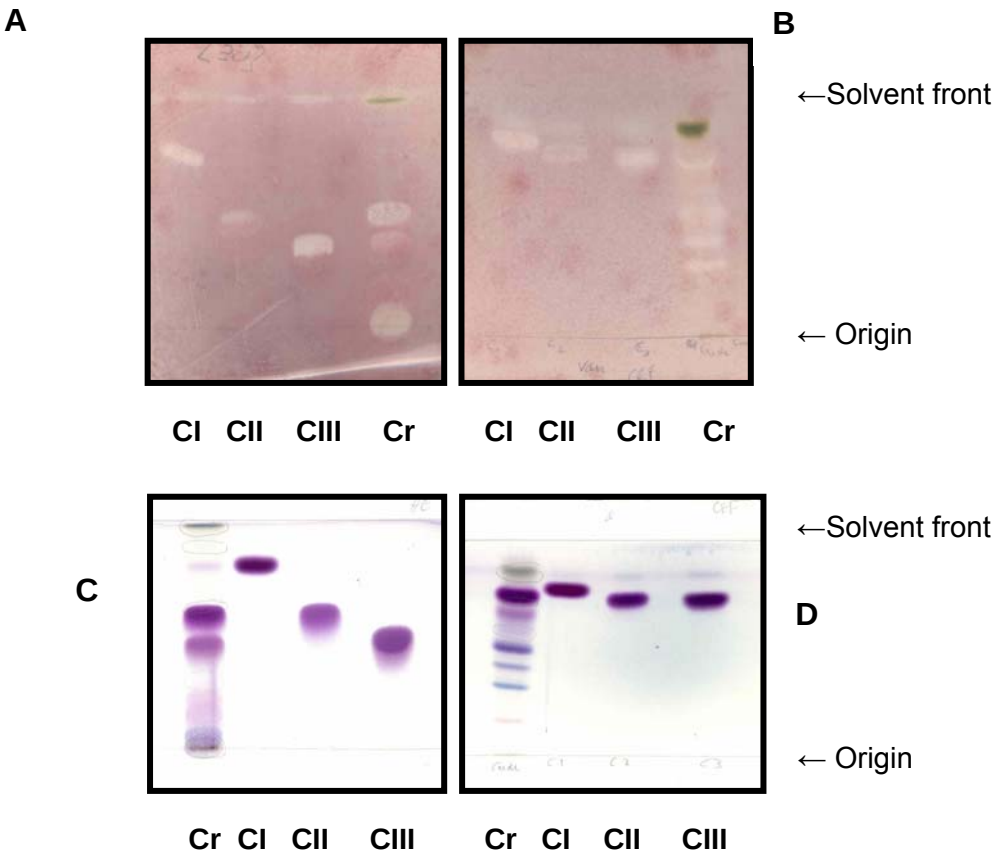


Figure 6.1. TLC bioautograms showing activity of three isolated compounds against *Candida albicans.* TLC plate was developed in DE (4:1) (A & C) & CEF (B & D). CI, lupeol; CII, betulinic acid; CIII, ursolic acid; CIV, hydroxyursolic acid; Cr, crude DCM extract.

Betulinic acid, ursolic acid and 2α-hydroxyursolic acid resulted in appreciable inhibition of fungal growth with MIC values ranging from 8 to 63 µg/ml (Table 6.1). The lowest MIC recorded was 8 µg/ml resulting from hydroxyursolic acid against *M. canis.* The crude extract was active against all species tested with MIC values ranging from 30 µg/ml against *S. schenckii* to 200 µg/ml against *A. fumigatus.* The overall average MIC values for each of the compounds tested against all fungal species investigated was calculated as 56.9 µg/ml (Fig. 6.2). With an average MIC value of 128 µg/ml lupeol was the least active of the compounds whereas the

average MIC values (overall) for betulinic acid, ursolic acid and hydroxyursolic acid ranged between 23.2 and 38.9 µg/ml. Comparisons of the MIC averages calculated for each fungal species revealed that *S. schenckii* (MIC average of 19.3 µg/ml) was the most sensitive (Fig. 6.3).

Table 6.1:. The minimal inhibitory concentration (µg/ml) of isolated compounds against fungal test organisms.

Compound	MIC (µg/ml) and test organism						
	SS	***MC***	***AF***	***CA***	***CN***	***CG***	***CSP***
Lupeol	12	16	93.5	250	180	94	>250
Betulinic acid	16	12	24	16	32	15.6	47
Ursolic acid	32	12	24	63	63	15.6	63
Hydroxyursolic acid	24	8	32	32	32	11.7	63
DCM crude extract	30	120	200	60	160	78	160
Amphotericin B	0.2	0.8	0.2	0.16	0.16	0.2	0.2

Key: SS, *Sporothrix schenckii;* MC, *Microsporum canis;* AF, *Aspergillus fumigatus;* CA,, *Candida albicans;* CN, *Cryptococcus neoformans;* CG, *Candida guilliermondi*, CSP; *Candida spicata.*

6.3.2. Antibacterial activity

The compounds lupeol, betulinic acid and ursolic acid were further investigated to determine their antibacterial activity using the bioautography method. Test bacterial species selected for the investigation were *E. coli, Enterococcus faecalis* and *S. aureus.* All the compounds tested were active against bacterial test organisms. The most intense zones of inhibition against bacterial growth resulted from betulinic acid and ursolic acid. Though lupeol was active against all the bacterial species tested, the intensity of the zones of inhibition was not comparable to those resulting from ursolic and betulinic acid. Against *E. faecalis,* ursolic acid led to the most intense zones of inhibition compared to lupeol and betulinic acid (Fig. 6.4).

MIC values calculated indicated that ursolic acid and hydroxyursolic acid were active against all bacterial test organisms with the exception of *E. coli* (Table 6.2). The ursane triterpenes ursolic acid and colosolic acid were more active than the lupane triterpenes, betulinic acid and lupeol. MIC averages of each compound against all the

bacterial species indicated that the compounds ursolic acid and betulinic acid were more active than lupeol and betulinic acid (Fig. 6.5)**.** The average MIC values calculated for each the test organisms indicated that *E. coli* was most resistant to the test compounds while *E. faecalis* was most sensitive (Fig. 6.6). In other studies ursolic acid markedly inhibited the growth of *S. aureus* but failed to inhibit Gram-negative bacteria and yeasts (Braghilori *et al.,* 1996).

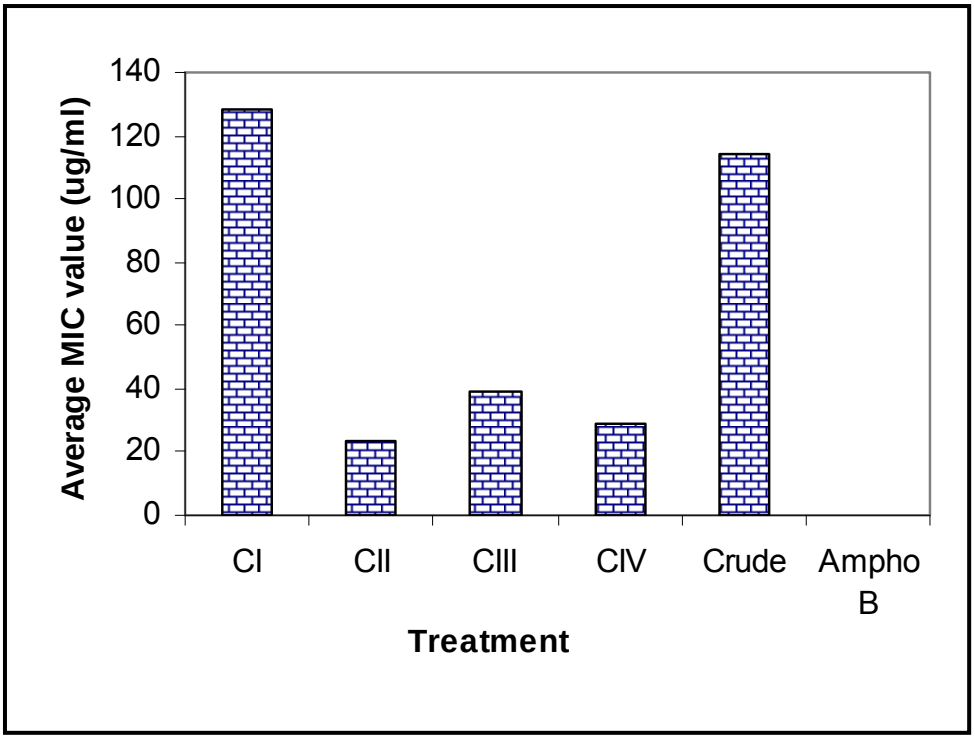


Figure 6.2. Average MIC values of each compound against all the fungal test organisms.

6.3.3. Cytotoxicity

The cytotoxicity of lupeol and betulinic acid was determined against Vero cells using the MTT assay. The results are presented in Figs. 6.7 and 6.8. Berberine was used as a positive control and was found to be toxic with an LC_{50} of about 10 µg/ml. Lupeol, with an LC_{50} of about 90 µg/ml, was less toxic than berberine and betulinic acid. The latter was comparable to berberine with an LC_{50} of about 11 µg/ml. The toxicity of betulinic acid may account for its broad-spectrum activity against both bacteria (both Gram-negative and Gram-positive) and fungi.

The selectivity index for betulinic acid and lupeol was calculated using the cytocotoxic concentrations of these compounds. A high number indicates selective

activity against pathogens whilst a low number is indicative of high toxicity. The selectivity index (SI) was calculated as:
Selectivity index (SI) = LC_{50} against Vero cells/MIC values.

Lupeol had high selectivity index values against both *Sporothrix schenkii* (7.4) and *Microsporum canis* (5.5), while against other fungal species the values remained below 1. The average selectivity index calculated for betulinic acid was 0.58.

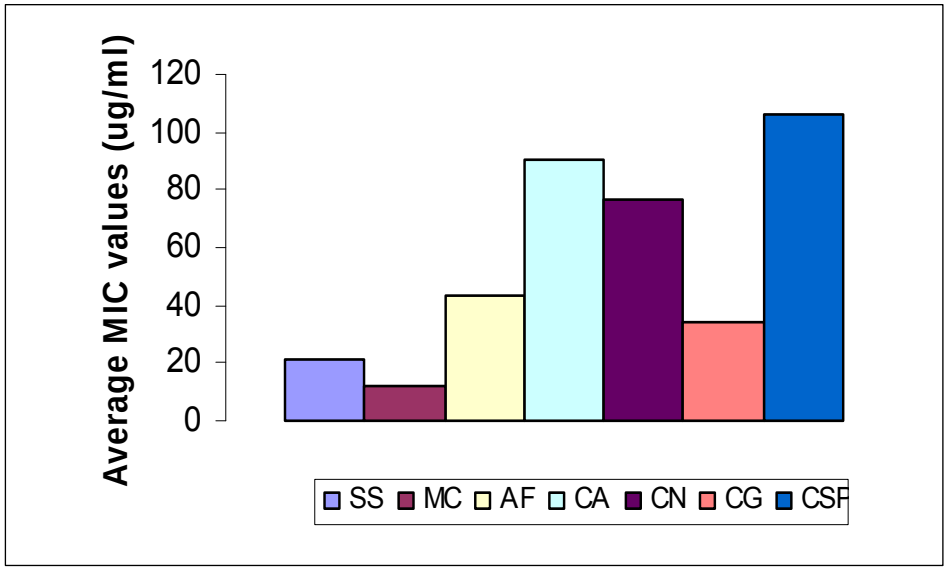


Figure 6.3. Sensitivity of each tested fungal species to all the isolated compounds. MIC averages were calculated from MIC values of all the isolated compounds against each of the fungal species.

Betulinic acid inhibited the growth of bacteria and fungi as evidenced by zones of inhibition and low MIC values. Literature reports outlining the activities of betulinic acid against growth of *C. albicans* and other fungal species are scarce. The antibacterial activity of betulinic acid has been reported. For instance, Chandramu *et al.* (2003) reported that betulinic acid isolated from the leaves of *Vitex negundo* had growth inhibitory activity against *Bacillus subtilis* at a concentration of 1000 µg/ml, resulting in a zone of inhibition of 18.8 mm^2. Other activities of betulinic acid have been reported extensively in literature.

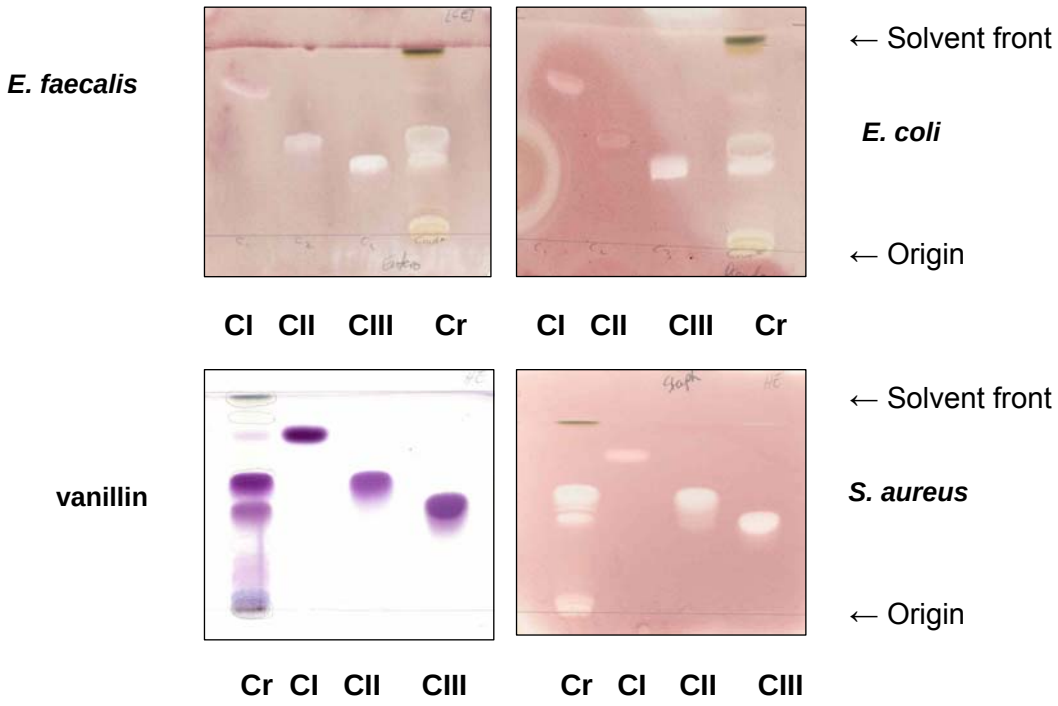


Figure 6.4. Antibacterial activity of isolated compounds against three bacterial test organisms. TLC plates were developed in DE (4:1) before spraying with concentrated suspensions of culture of test organism cultures. CI, lupeol; CII, betulinic acid; CIII, ursolic acid; CIV, hydroxyursolic acid; Cr, crude DCM extract.

Table 6.2: MIC (µg/ml) of compounds isolated from *C. dentata* against bacterial test organisms

Compound	*S. aureus*	*E. coli*	*P. aeruginosa*	*E. faecalis*
Lupeol	250	250	250	63.0
Betulinic acid	250	250	250	16.0
Ursolic acid	62.5	250	4.0	47.0
Hydroxyursolic acid	32.0	250	7.8	38.0
Gentamicin	0.2	8.0	0.2	1.6

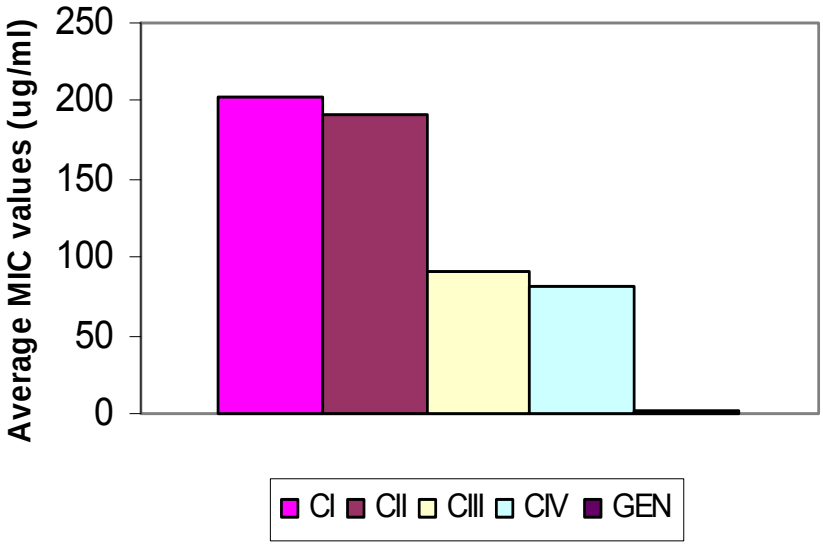


Figure 6.5. Averages of MIC values of each compound against all bacterial test organisms.

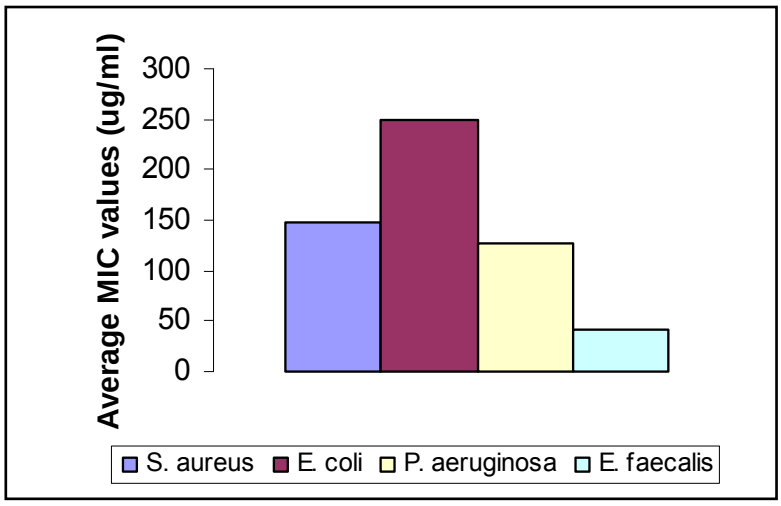


Figure 6.6. Average MIC values against each bacterial test organisms by all the isolated compounds.

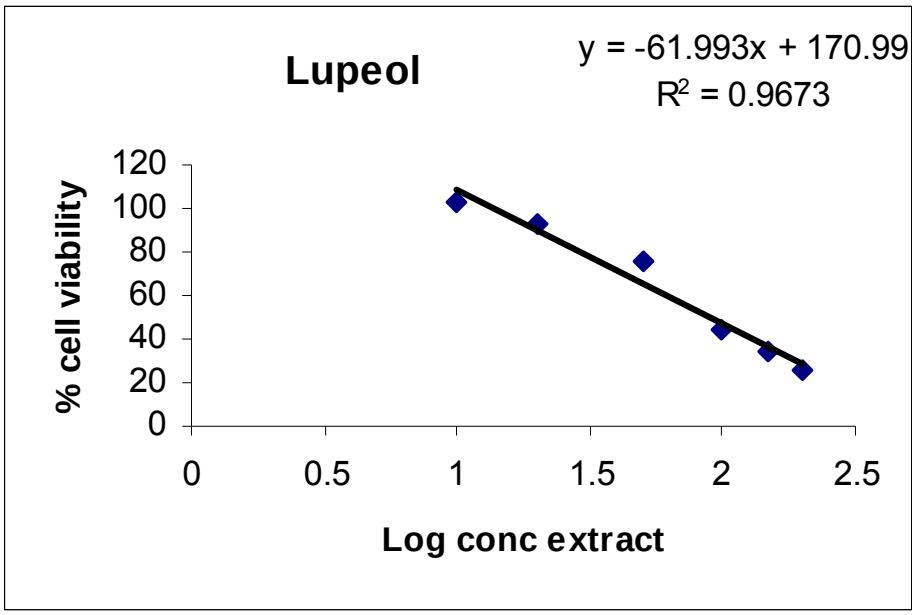


Figure 6.7: Cytotoxicity of lupeol (**LC_{50}** = 89.4688722 ug/ml) against Vero cells

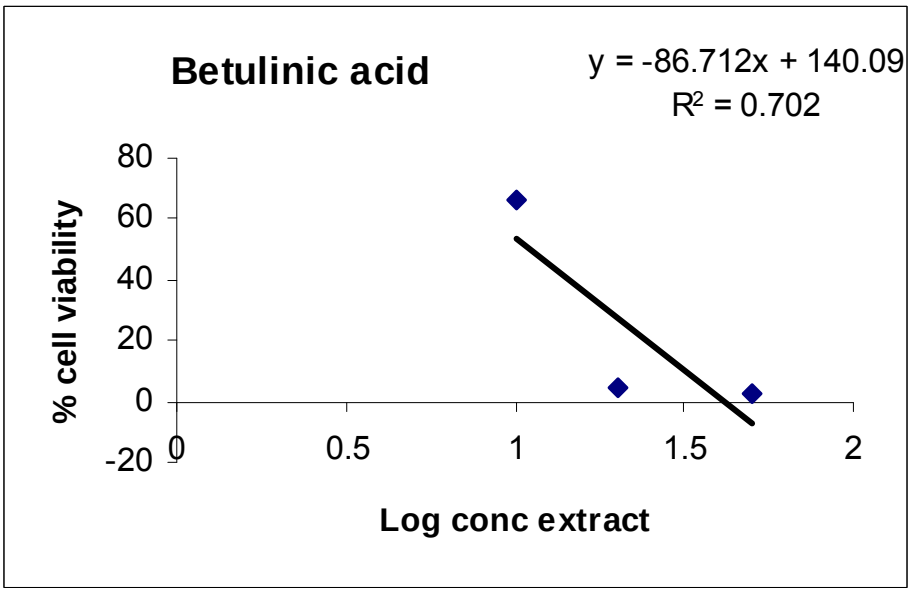


Figure 6.8. Cytotoxicity of betulinic acid (**LC_{50}** = 10.93846914 ug/ml) against Vero cells

Table 6.3. Selectivity index of lupeol and betulinic acid isolated from extracts of *Curtisia dentata* leaves. Key; Ca, *Candida albicans*; Cn, *C. neoformans*; Mc, *M. canis*; Ss, *S. schenckii*; Af, *Aspergillus fumigatus*.

Compounds	Ss	Mc	Af	Ca	Cn	Cg	Csp	average
Lupeol	7.4	5.6	0.95	0.35	0.49	0.95	0.35	2.30
Betulinic acid	0.69	0.92	0.46	0.69	0.34	0.71	0.23	0.58
Amphotericin B	140	35	140	175	175	140	140	135
Average	49.36	13.84	47.14	58.68	58.61	47.22	46.86	45.96

6.4. DISCUSSION

The triterpenic family of compounds to which the four isolated compounds belong, has been reported to possess antifungal and antibacterial activity. For instance, Collins and Charles (1987) identified ursolic acid as one of the active components in rosemary claimed to inhibit growth of some food-associated bacteria and yeasts (Becker *et al.,* 2005). Ursolic acid and its derivatives inhibited the growth of *S. aureus* and *Microsporium lenosum* (Zeletova, 1986). Ursolic acid has been reported as the active constituent from a number of plant species used in traditional and folklore medicine (Ringbon *et al.,* 1988; Saraswat *et al.,* 2000).

6.4.1. Lupeol and betulinic acid

Literature reports that 28-COOH and ester functionality at C-3 are important in determining the pharmacological activities of pentacyclic triterpenes (Mallavadhani *et al.,* 2004). Betulinic acid and lupeol, both of which are lupane pentacyclic triterpenes, slightly differ structurally. Betulinic acid posses a carboxylic (-COOH) function at C-28 whereas lupeol contains a methyl ($-CH_3$) group at position 28, making lupeol more hydrophobic than betulinic acid. Since betulinic acid was more active against microbial growth (in this study) than lupeol it would seem that the carboxylic acid moiety at C-28 plays an important role in activity of these triterpenes. In another study Chaiyadej *et al.* (2004) reported that lupeol was not active against *Mycobacterium tuberculosis* H37Ra. Betulinic acid was active (with an IC_{50} value of 50 µg/ml) against *M. tuberculosis* H37Ra.

Triterpenes possessing a carboxyl group at C-28 show more cytotoxic activity against several cancer cell lines (Chiang *et al.,* 2005; Baglin *et al.,* 2003; Sakai *et al.,* 2004) and induction of apoptosis (Hata *et al.,* 2002). Hiroya *et al.* (2002) reported that the protective activity of betulinic acid derivatives against cadmium toxicity in hepatocytes is closely related to polarity at C-3 and C-28. In another study it was shown that substitution of COOH at C-28 by $CONH_2$ led to improved antiviral activity of lupane triterpenes (Flekhter *et al.,* 2003). In addition, the hydroxyl function on C-3 is alleged to play a role in the activity of these triterpenes. For instance, changing lupeol to lupeol acetate reduces antimutagenic activity (Guevara *et al.,* 1996).

6.4.2. Ursolic acid and hydroxyursolic acid

Ursolic acid and colosolic acid differ structurally due to the presence of an additional hydroxyl group (-OH), in the α-configuration, at C-2 in the latter compound. Some literature publications indicate that the presence of a hydroxyl group at C-3 is important for activity of ursolic acid. For instance, esterification of 3-OH and 28-COOH results in loss of cytotoxic activity of ursolic acid (Ma *et al.,* 2005). This evidence suggests that a hydrogen donor group at either C-3 or C-28 is essential for activity of triterpenoids. In this study, the antifungal activities of ursolic acid and colosolic acid were comparable as indicated by the MIC values (averaging at 38.9 and 29.0 µg/ml, respectively). It would seem that the additional hydroxyl group on C-2 of colosolic acid did not increase its activity. Ma *et al.* (2005) declared that the configuration at C-3 was important for activity of ursolic acid. They arrived at this conclusion after observing that β-orientated hydrogen bond-forming group at C-3 displayed more pronounced (20-fold increase) cytotoxic activity than α-orientated counterpart.

One of the problems associated with triterpenic compounds is poor solubility in aqueous media. For instance, betulinic acid and ursolic acid suffer from low water-solubility, resulting in low biological efficacy (Baglin *et al.,* 2003). This poor solubility behavior was also displayed in this study in acetone by lupeol, betulinic acid and to a slight extent by ursolic acid.

6.4.3. Cytotoxicity

Betulinic acid and berberine were cytotoxic to Vero cells with LC_{50} values of 10 and 11 µg/ml. In other studies, the anticancer activity of betulinic acid was associated

with little toxicity (Pisha *et al*., 1995). Vero cells may be relatively sensitive to betulinic acid in comparison with other cell types. It would be worthwhile to investigate the toxicity of betulinic acid using other human and animal cell lines, including primary liver cells.

The amount of ursolic and hydroxyursolic acid isolated were not adequate for cytotoxicity testing. Some literature reports suggest that these ursane triterpenes are cytotoxic to tumour cell lines. Ma *et al.* (2005) reported that ursolic acid was cytotoxic to HL-60, BGC, Bel-7402 and Hela cells with ED_{50} values of 72.0, 53.7, 45.0 and 49.4 µg/ml, respectively. The cytotoxicity of 2α-hydroxyursolic acid on these cells was comparable with that resulting from ursolic acid (Ma *et al.,* 2005).

Lupeol, though not as toxic as berberine, was a non-selective mild inhibitor of both fungal and bacterial growth. The calculated toxicity indexes indicate that amphotericin B has very pronounced antifungal activity associated with little cytotoxicity to the Vero cells. The low SI value (0.58) calculated for betulinic acid suggests that betulinic acid has antifungal activity accompanied by high toxicity. The selectivity index values of betulinic acid against all the fungal species tested suggested that toxicity was accountable for the low MIC values of this compound. The SI values of betulinic acid were lower than 1 against all fungal species tested.

Betulinic acid was selectively cytotoxic against several human melanoma cancer cell lines. It was also found to be active *in vivo* against athymic mice carrying human melanoma with little toxicity (Pisha *et al.,* 1995). The mechanism of action of betulinic acid on mammalian cells is thought to involve the induction of apoptosis (Pisha *et al.,* 1995). Preclinical developments towards topical formulations including betulinic acid and its synthetic derivatives as the active pharmaceutical ingredients are underway at the University of Illinois at Chicago (Balunas & Kinghorn, 2005).

6.5. CONCLUSIONS

In this study all the triterpenic compounds tested inhibited the growth of several fungal species with low MIC values. Using bioautography the inhibition of growth of fungal and bacterial species was clearly demonstrated, as indicated by clear zones on TLC chromatograms. The MIC values calculated suggested that lupeol was the least active against test fungi. The COOH group at C-28 and the C-3 hydroxyl group of triterpenoids are important for antibacterial and antifungal activity. Lupeol has –

CH_3 at C-28, while betulinic acid has –COOH at the same position. The activity observed with the isolated compounds further validates the traditional uses of *Curtisia dentata* for treating fungal and bacterial infections. Investigation of efficacy of the isolated compounds on *Candida albicans* infected wounds in animal models could indicate the potential uses of these triterpenes for topical treatment and prevention of infections in wounds. Based on the calculated activity indexes betulinic acid and lupeol, though active against some fungi, are not comparable with amphotericin B. The value of the antifungal activity of betulinic acid is outweighed by its associated cytotoxicity. Lupeol, though less toxic, has relatively low antifungal activity, leading to a low selectivity index.