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Antibacterial and anti-adhesion activity of the pentacyclic triterpenoids isolated from the leaves and edible fruits of *Carissa macrocarpa*

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Four pentacyclic oleanane triterpenes (β-amyrin, methyl oleanolate, oleanolic acid and 3β-hydroxyolean-11-en-28,13β-olide) were isolated from the fruits of *Carissa macrocarpa* whilst the ursane triterpene, ursolic acid, was isolated from the leaves. 3β-hydroxyolean-11-en-28,13β-olide has only been found once previously (in the Lamiaceae) and its lactone ring, which contributes significantly to the expression of antibacterial activity in oleanane triterpenes, makes it an interesting molecule for synthetic and biological studies. The immune boosting properties of the triterpene rich edible fruits of *C. macrocarpa* are important, especially in South Africa, due to high incidences of Human immunodeficiency virus (HIV) and hepatitis in this country.

Key words: *Carissa macrocarpa*, amatungula, oleanane triterpenes, ursolic acid.

INTRODUCTION

The dependence on uncultivated resources in sub-Saharan Africa, where purchased food is substituted with indigenous or wild fruit and vegetable, is well documented (UNAIDS, 1999). The dependence on traditional medicine and medicinal plants by the rural poor for the treatment of HIV related infections such as venereal diseases, tuberculosis, diarrhea and appetite loss have also been reported (Bodeker et al., 2000; King, 2000). The reliance on wild foods and medicinal plants for nutrition, treatment and care is primarily a function of economic and physical accessibility. This can result in the decline of natural resources of commonly used medicinal plants as indicated by traditional healers at the 13th International Acquired immune deficiency syndrome (AIDS) Conference in Durban, South Africa, in 2000. This necessitates the identification of other natural resources, with the same nutritional and medicinal benefits. One such plant is *Carissa macrocarpa* 'Tomlinson' (Ecklon) A. DC, of the family Apocynaceae, which is indigenous to KwaZulu-Natal (KZN), South Africa (Botha and Botha, 1997).

*C. macrocarpa* is a small, evergreen, twiggy shrub, found in abundance in KZN, with star-shaped white scented flowers, Y-shaped thorns and tasty red fruit (known as the Amatungula by the Zulu people of South Africa) that are enjoyed by both birds and children (Botha and Botha, 1997). The fruit is reputed to be rich in vitamin C, Ca, Mg and P (Wehmeyer, 1966). Leaves of *C. macrocarpa* are used by the Zulu people to treat diarrhea in livestock. Different morphological parts are used in South African folk medicine to treat coughs and venereal diseases (National Research Council, 2008).

Other *Carissa* species are also used in traditional systems of medicine in different parts of the world. Of these, *Carissa edulis* is used in African traditional medicine (Ibrahim et al., 2005); *Carissa carandas* in Ayurvedic systems of medicine (Hegde et al., 2009); and *Codonopsis lanceolata* by the Aboriginal communities of Western Australia (Lindsay et al., 2000). These *Carissa* species have received widespread scientific attention.
(Nedi et al., 2004; Siddiqui et al., 2002) whilst no phytochemical studies have been done on *Carissa macrocarpa* despite its role in South African traditional medicine. Furthermore, since the fruits are eaten by children, a phytochemical study to determine the types of compounds present in the fruit is essential. Thus far, a preliminary phytochemical analysis and pharmacological screening has been done on the crude extracts of *C. macrocarpa* and some ubiquitous triterpenoids (lupeol, β-sitosterol and ursolic acid) were identified using thin layer chromatography, a technique that is neither quantitative nor absolute (Wehmeyer, 1986; Zaki et al., 1981). In this study, the isolation, characterization and identification of the phytocompounds from the edible fruits and leaves of *C. macrocarpa* was undertaken. Compounds isolated from other *Carissa* species include *inter alia* cardiac glycosides (Rastogi et al., 1969), lignans (Achenbach et al., 1983), sesquiterpenes (Achenbach et al., 1985) and triterpenes (Siddiqui et al., 2002). The antimicrobial activities of selected isolated compounds were also determined to evaluate the plant’s potential to control microbial manifestations on biotic or abiotic surfaces.

**MATERIALS AND METHODS**

**General experimental procedure**

Infrared (IR) spectra were obtained using a Perkin Elmer Spectrum 100 FT-IR spectrometer with Universal ATR sampling accessory. NMR spectra were recorded in deuterated chloroform (CDCl$_3$) or dimethyl sulfoxide (DMSO) at room temperature on a Bruker Avance$^\text{®}$ 400 spectrometer with tetramethylsilane (TMS) as internal standard. The $^{13}$C-NMR spectral assignments were made by comparison of chemical shifts with literature data (Mahato and Kundu, 1994; Pereda-Miranda and Delgado, 1990), by analysis of DEPT spectra for the determination of primary, secondary and tertiary carbons and by use of 2D techniques (COSY, HSQC and HMBC). GC-MS data were recorded on an Agilent GC-MS D apparatus and are uncorrected.

**Plant materials**

The fruits and leaves from *C. macrocarpa* were collected from the University of KwaZulu-Natal (UKZN), Westville campus, South Africa, in May 2009. These were identified by taxonomist, Prof. A. Nicholas, from the School of Biological and Conservation Sciences, UKZN, Westville and a voucher specimen (Moody, R1) was deposited in the ward herbarium at UKZN.

**Extraction and isolation**

Ground fruit (900 g) and ground leaves (450 g) were subjected to exhaustive extraction with hexane, dichloromethane (DCM) and methanol (MeOH) by maceration and continuous shaking on an orbital shaker at room temperature for 48 h. The solvent extracts were concentrated by use of a rotary evaporator and the crude extracts were stored at 4°C for further analysis. The crude extracts were subjected to column chromatography (Merck Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM) on suitably sized columns and the fractions monitored by TLC (Merck silica gel 60, 20 x 20 cm F254 aluminium sheets) which was analyzed under UV (254 nm) and visualized using anisaldehyde spray reagent (97: 2: 1; MeOH: conc. H$_2$SO$_4$: anisaldehyde). For the crude DCM extract from the fruits (3.0 g), a hexane: ethyl acetate gradient was used on a 4 cm diameter column starting with 10% ethyl acetate in hexane. This was increased to 20%, then 50% ethyl acetate and finally 100% ethyl acetate. Twenty five fractions of 40 mL each were collected for each solvent system. Fractions 26 to 44 were combined to yield fraction A and fractions 71 to 82 were combined to yield fraction B. Fraction A was rechromatographed over silica gel in a 2 cm column with 100% hexane to yield compound 1 (12.65 mg) which eluted after 20 mL.

Fraction B was rechromatographed over silica gel in a 2 cm column with 100% DCM (50 mL) followed by 10% EtOAc in DCM (50 mL) and 20% EtOAc in DCM (50 mL). Fifteen fractions of 10 mL each were collected, with fractions 7 to10 yielding compound 5 (12.61 mg). Fractions 12 to15 were combined and recrystallisation with CHCl$_3$: MeOH (1:1) afforded compound 2 (40.52 g). The crude MeOH extract (10.0 g) from the fruits was dissolved in water and subjected to partitioning with an equal volume of EtOAc. The EtOAc fraction was then dried with anhydrous Na$_2$SO$_4$ and subjected to column chromatography on a 4 cm diameter column with 100% hexane for 1 h, then with 20% ethyl acetate and finally 100% ethyl acetate. Ten fractions of 50 mL each were collected. Compound 3 (50.35 mg) eluted in fractions 6 to 10 yielding white crystals upon evaporation. The crude DCM extract from leaves (8.85 g) was subjected to column chromatography on a 4 cm diameter column. For elution, a mobile phase consisting of a hexane: DCM and DCM: ethyl acetate gradient was used, starting with 100% hexane to 50% DCM, which was further stepped to 20 and 40% ethyl acetate. Ten fractions of 50 mL each were collected in each step. Fractions 36 to 38 with similar TLC profiles were combined and purified on a 1.5 cm diameter column using 100% DCM (3 x 40 mL) to 50% ethyl acetate (3 x 40 mL) and finally 100% ethyl acetate (3 x 40 mL). Compound 4 (1.78 g) was obtained in fractions 6 to 9.

The physical and spectroscopic data for compounds 1 to 4 matched those of Mahato and Kundu (1994). The nuclear magnetic resonance (NMR) data for compound 5 matched those of Pereda-Miranda and Delgado for most, but not all resonances, therefore NMR data is provided (Pereda-Miranda and Delgado, 1990). 3β-hydroxyolean-11-en-28,13β-olide (5), colourless crystals, melting point 260-262°C; EI-MS *m/z*: 454 [M]$^+$, 345, 281, 207, 55, 43; $^{1}$H-NMR (CDCl$_3$, 400 MHz): 6.07 (3H, s, Me-29), 0.86 (3H, s, Me-28), 0.89 (3H, s, Me-26), 0.95 (3H, s, Me-27), 0.96 (3H, s, Me-30), 1.03 (3H, s, Me-23), 1.22 (3H, s, Me-29), 3.20 (1H, dd, J=11.55, 4.77 Hz, H-3), 3.41 (1H, dd, J=10.35, 3.03 Hz, H-11), 6.02 (1H, d, J=10.35 Hz, H-12); $^{13}$C-NMR (CDCl$_3$, 400 MHz): 51800.00 (C-28), 135.83 (C-12), 126.92 (C-11), 89.80 (C-13), 78.83 (C-3), 54.78 (C-5), 53.21 (C-9), 50.54 (C-18), 44.03 (C-17), 41.60 (C-14), 41.41 (C-8), 38.92 (C-4), 38.26 (C-1), 37.34 (C-19), 36.35 (C-10), 34.37 (C-7), 33.26 (C-29), 31.42 (C-20), 31.16 (C-21), 29.68 (C-22), 27.75 (C-23), 27.16 (C-2), 27.01 (C-16), 25.39 (C-15), 23.54 (C-30), 18.96 (C-27), 18.27 (C-26), 17.93 (C-24), 17.65 (C-6) and 14.90 (C-25).
microtitre plate protocol (Basson et al, 2008). Bacterial strains were cultured over night in TSB, then washed and resuspended in sterile distilled water to obtain final inoculums of 5×10^8 cfu/mL, equivalent to a 0.5 McFarland standard (Andrews, 2001). Stock solutions of the isolated phytocompounds were at 10 mg/mL in 100% DMSO. The 96 well microtitre plates were prepared by dispensing 90 µL of Mueller-Hinton (M-H) broth into each well after dispensing 90 µL of the test compound stock solutions were dispensed into the wells to obtain two-fold dilutions of test concentrations ranging from 0.04 to 32 µg/mL, was used as the standard antimicrobial agent for comparison. All plates contained a control for both assays was un-inoculated LB to test for sterility and non-specific binding of media, while the positive control was LB with respective cell suspensions without oleanolic acid, ursolic acid or methyl oleanolate. OD\textsubscript{595nm} values of treated cells were compared to untreated cells to investigate the increase or decrease in adhesion as a result of antimicrobial agent exposure. Treated and untreated samples were compared statistically using paired t-tests and Wilcoxon signed rank tests if normality failed (SigmaStat V3.5, Systat Software, Inc).

**RESULTS AND DISCUSSION**

**Structure elucidation**

The DCM extract from the fruits of *C. macrocarpa* yielded three compounds, β-amyrin (1), methyl oleanolate (2) and 3β-hydroxyolean-11-en-28,13β-olide (5) whilst the methanol extract yielded one compound, oleanolic acid (3). The DCM extract from the leaves of *C. macrocarpa* yielded ursolic acid (4) only. EI-MS, IR spectroscopy, 1D-NMR (1\textsuperscript{H}, 13\textsuperscript{C}-NMR and DEPT) and 2D-NMR spectroscopy (COSY, HSQC and HMBC) together with data published in the literature were used to identify the compounds. Compounds 1 to 4 (Figure 1) are common triterpenes found widely in many plant species; however compound 5 (Figure 2) has only been isolated previously from *Hypitis albidus* in the Lamiaceae family (Pereda-Miranda and Delgado, 1990). For compounds 1 to 3, the 1\textsuperscript{H}-NMR spectrum showed characteristic resonances at 5.26 (H-12) and a doublet at 3.21 (H-3). The only structural difference in these compounds occurred at C-30 and therefore the resonances of H-3, H-12 and H-18 are similar. The H-3 and H-12 resonances of 4 are also similar; however the H-18 resonance occurred at 2.25, consistent with that of ursolic acid and the methyl group at C-19 instead of the tertiary carbon atom with two methyl groups at C-20. The 13\textsuperscript{C}-NMR and DEPT spectra for compounds 1 to 4 had the required methyl, methylene, methine and quaternary carbon resonances for β-amyrin, methyl oleanolate, oleanolic acid, and ursolic acid and the
molecular ion peaks were observed at m/z 426 for 1, 470 for 2 and 456 for 3 and 4, respectively. The NMR data compared well with the data in the literature for these compounds (Mahato and Kundu, 1994). The 1H-NMR spectrum for compound 5 showed a resonance at δH 3.21, for H-3, similar to the other four compounds. Two olefinic resonances at δH 5.39 (1H, dd, J=10.35, 3.03 Hz, H-11) and δH 6.02 (1H, d, J=10.35 Hz, H-12) were also observed. The 13C-NMR spectrum for compound 5 resolved 30 carbon resonances comprising 7 methyl, 9 methylene, 6 methine, 7 quaternary and 1 carbonyl resonance, identified using the DEPT spectrum. The carbon resonances in the A and B rings were similar to compounds 1, 2 and 3. This is typical of oleanane triterpenes where the geometry of the D/E ring junction does not cause significant alterations in the shielding of carbons in the A/B rings (Mahato and Kundu, 1994). The C-11 and C-12 resonances occurred at δC 126.90 and δC 135.80, respectively and the carbonyl resonance at δC 180.00 was assigned to C-28 which correlated to H-18 in the HMBC spectrum. The C-13 resonance occurred at δC 89.80. The data above compared well with the data in literature for 3β-hydroxyolean-11-en-28,13β-olide (Pereda-Miranda and Delgado, 1990) as confirmed by the molecular ion peak, M+ at m/z 454, corresponding to the formula, C30H46O3.

Antimicrobial activity

The pentacyclic triterpenoids, oleanolic acid and ursolic acid, and their derivatives show appreciable antibacterial activity (Krystyna et al., 2010) with the position of the hydroxy group influencing the activity of the compound (Djoukeng et al., 2005). The present study shows that the pentacyclic triterpenes, β-amyrin (1), methyl oleanolate (2), oleanolic acid (3) and ursolic acid (4), have moderate antibacterial activity (MICs in the range 0.12 to 1.0 mg/mL) whilst the oleanolic acid derivative, 3β-hydroxyolean-11-en-28,13β-olide (5), has good antibacterial activity (MICs in the range 0.06 to 0.12 mg/mL) for all bacterial strains studied (Table 1). The addition of a lactone ring contributes significantly to the expression of antibacterial activity in oleanane triterpenes. No difference in the extent of activity between Gram-positive and Gram-negative bacteria was observed indicating that the mode of antibacterial activity of these pentacyclic triterpenes is unaffected by the cell wall structural differences between these organisms. Most of the isolated compounds have a bacteriostatic (bacteria-inhibiting) effect on the studied microorganisms which is favoured in some cases especially when treating immuno-competent patients with urinary tract infections (Gleckman, 1975). Increased adhesion to abiotic surfaces was observed for 100% of bacterial strains following all three oleanolic acid (3) exposures (Figure 3a), of which the increases observed following MIC and Supra-MIC exposures were statistically significant. Oleanolic acid (3) which increases the adhesion of Gram-positive and Gram-negative bacteria may be used in applications that need to limit the migration of pathogenic bacteria like in groundwater aquifers (Li and Logan, 1999).

For methyl oleanolate (2), variable effects were observed (Figure 3b), with 57% of bacterial strains displaying decreased adhesion. The decreased adherence for E. coli, S. aureus and S. saprophyticus indicated that methyl oleanolate (2) interfered with the ability of these microorganisms to adhere to polystyrene surfaces. Increased adhesion was observed for the remaining three microorganisms demonstrating the ability of this compound to promote their biofilm formation. Decreased adherence was also observed for E. coli and S. aureus bacterial strains with all three concentrations of ursolic acid (4) (Figure 3c). For P. aeruginosa, decreased adhesion was observed only with sub-MIC and MIC exposures, whilst increased adhesion was observed at supra-MIC exposure. In contrast, S. saprophyticus adhesion was increased at sub-MIC exposure but decreased following MIC and supra-MIC exposures. Of the three compounds tested, ursolic acid (4) demonstrated the greatest ability to prevent bacterial colonization, with 71% of bacterial strains showing decreased adhesion at most ursolic acid (4) exposures. It was interesting to note that the adhesion of K. pneumonia, the gram-negative opportunistic pathogen most frequently implicated in nosocomial infections, was increased following exposure to all three compounds at all concentrations. The reason for this is unclear.

Importance of the phytochemical constituents in the edible fruits

Oleanolic acid is known for its hepatoprotective effects
Table 1. Minimum inhibitory concentrations (MICs), in mg/mL, of compounds 1-5 isolated from *Carissa macrocarpa*, against Gram-positive and Gram-negative bacteria.

<table>
<thead>
<tr>
<th>MIC (mg/mL)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> (ATCC 25923)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td><em>S. aureus</em> (ATCC 43300)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
<td>2</td>
</tr>
<tr>
<td><em>E. faecium</em> (ATCC 19434)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.12</td>
<td>8</td>
</tr>
<tr>
<td><em>S. saprophyticus</em> (ATCC 35552)</td>
<td>0.25</td>
<td>0.25</td>
<td>1.0</td>
<td>0.25</td>
<td>0.12</td>
<td>4</td>
</tr>
<tr>
<td><em>E. coli</em> (ATCC 25922)</td>
<td>0.12</td>
<td>0.25</td>
<td>1.0</td>
<td>0.25</td>
<td>0.06</td>
<td>2</td>
</tr>
<tr>
<td><em>K. pneumonia</em> (ATCC 700603)</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.12</td>
<td>8</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (ATCC 35032)</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.06</td>
<td>32</td>
</tr>
</tbody>
</table>


Figure 3. Alterations in adhesion profiles of Gram-positive and Gram-negative bacteria following sub-MIC, MIC and supra-MIC exposures of (a) oleanolic acid (b) methyl oleanolate and (c) ursolic acid.
Isolates

Figure 3. Contd.
(Oliveira et al., 2005). Consequently, it is used to treat acute and chronic hepatitis in China (Liu, 1995). In South Africa, due to the number of people living in informal settlements, the spread and prevalence of the hepatitis virus is high (Tucker et al., 1996). Consumption of the easily accessible Amatungula fruit that has hepatoprotective effects should therefore be encouraged. Oleanolic acid is known for its anti-inflammatory effects without causing ulcerations, unlike Aspirin (Singh et al., 1992); β-amyrin is more potent than Aspirin in inhibiting platelet aggregation (Ching et al., 2010) therefore consumption of the Amatungula fruit that is rich in oleanolic acid and β-amyrin can be a natural alternative to Aspirin, which is not readily available in the rural parts of South Africa, and may promote the health and well-being of the local people. Children eating the Amatungula fruit also benefit from the anti-cariogenic properties of the compounds that it holds (Hada et al., 1990). Oleanolic acid also exhibits hypoglycemic (Hao et al., 1989), anti- lipidemic (Liu et al., 1987) and anti-cancer activities (Li et al., 2002) so the benefits of ingestion of the Amatungula fruit are numerous.

Conclusion

Five pentacyclic triterpenes were isolated from the leaves and fruits of *C. macrocarpa*. All four of the triterpenes isolated from the fruits of *C. macrocarpa* had the oleanane skeleton and appear to be derived biosynthetically from oleanolic acid whilst only the ursane triterpene, ursolic acid, was isolated from the leaves. MICs show that the addition of a lactone ring to the oleanane backbone contributes significantly to the expression of antibacterial activity in pentacyclic oleanane triterpenes. For most microorganisms studied, oleanolic acid tended to promote adhesion to abiotic surfaces whilst methyl oleanolate and ursolic acid tended to inhibit it.

The anti-adhesion effect of the three studied compounds were in the increasing order of oleanolic acid < methyl oleanolate < ursolic acid. The immune boosting properties of the compounds contained in the fruits of *C. macrocarpa* are important, especially for children in KZN, due to high incidences of HIV and hepatitis in this area. The cost of immune boosting supplements is out of reach for the indigenous people of South Africa therefore freely available fruit such as the Amatungula is a welcome source of these compounds. This study lends scientific credence and validity to the ethnomedicinal use of *C. macrocarpa* and highlights the medicinal benefits of consuming the indigenous edible fruit.

REFERENCES


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