CHAPTER - IV

EXTRACTION, ISOLATION & IDENTIFICATION OF
ACTIVE COMPOUNDS FROM

GLYPTOPETALUM CALOCARPUM
ABSTRACT

Background: *Glyptopetalum calocarpum* is a plant indigenous to Andaman and Nicobar Islands, and has not been reported elsewhere. Preliminary phytochemical investigation and antibacterial studies revealed a significant antibacterial activity, and the presence of alkaloids, flavonoids, triterpenes and tannins. The aim of the present study was to isolate and purify the bioactive compounds from the methanolic extract of *Glyptopetalum calocarpum* (Kurz.) Prain leaves.

Materials and Methods: Plant material was collected from Car Nicobar of Andaman and Nicobar Islands. The air dried leaves (1kg) were grinded, made into powder, and soaked in methanol for one week. The resulting extract was concentrated under vacuum, to obtain a residue, which was subjected to repeated column chromatography. This was further purified by preparative thin layer chromatography and flash chromatography. The structures were determined by one dimensional (1D) (\(^1\)H and \(^{13}\)C) and two dimensional (2D) NMR spectra (H-H COSY, HMQC and HMBC).

Results: The methanol extract of *G. Calocarpum* leaves were subjected to column chromatography on silica gel, and six triterpene compounds were obtained. These compounds were identified as \(\alpha\)-Lupene, Lupeol, Lupenone, Stigmasterol, \(\beta\)-Amyrin and \(\beta\)-Amyrin acetate, from \(^1\)H, \(^{13}\)C NMR data which were reported in literature.

Conclusions: In this study, six triterpene compounds were purified from the indigenous plant, *G. calocarpum*. However, further in vitro and in vivo studies are needed to investigate the pharmacological and toxicological properties of *G. calocarpum* extract, before it can be considered as a new antimicrobial agent.

INTRODUCTION

One of the cardinal objectives in medicinal plant research and development is identification, isolation and characterization of the bioactive components present in an extract. Medicinal plant extracts are inherent complex mixture of diverse chemical components. Separation of active components of plant phytochemicals from the inactive components can be categorized into three parts: extraction, purification and chromatography. Various chromatographic methods are available for qualitative and quantitative estimation. These include TLC fingerprint, high performance liquid chromatography (HPLC) fingerprints. For isolation of compounds, open column
chromatography (OCC), vacuum liquid chromatography (VLC), HPLC, high-speed counter-current chromatography (HSCCC), gas-liquid chromatography (GLC) and gel permeation chromatography (GPC) are used. The principle of separation is based on molecular size, adsorption to the stationary phase, polarity and solubility in the mobile phase.

**Column chromatography**

Isolation of compounds is usually carried out by open column chromatography under gravitation force using silica gel, sephadex, polyamides or reverse phase (RP) mode on C8 or C18-bonded silica gel stationary phase. The separation of individual compounds from the complex extract mixture is based on the characteristic ability of the compound for the stationary phase in the column, relative to the polarity of the mobile phase. Changing the polarity (gradient elution) of the mobile phase will allow all the target compounds to elute in a sequential manner. The chromatographic process should be rapid, and should not lead to decomposition of compounds, material loss or formation of artefacts.

Open column chromatography is simple, cheap and universally practiced, despite few obvious disadvantages of being slow, and often produces irreversible adsorption of sample onto the stationary phase. The method is also encumbered with large sample and solvent requirement. The bioactive compounds of interest in this study are non-volatile (Shahid, 2012).

**Mass spectrometry**

Mass spectrometry (MS) is an important physico-chemical tool applied for structural elucidation of compounds from natural products, including medicinal plants (Diana et al., 2007). The fundamental principle of MS is to use different physical means for sample ionization, and to separate the ions based on their mass (m) to charge (z) ratio (m/z) (Negi et al., 2013). The ionization techniques available include, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB) and matrix-assisted laser desorption ionization (MALDI). Mass spectrometry have high sensitivity, with detection limit of femtogram, compared to NMR with a sensitivity limit of nanogram range and above. The high sensitivity and the flexibility for hyphenation with other chromatographic technique make MS a versatile analytical instrument.
**NMR spectrometry**

Structural information on isolated compounds are usually obtained from different spectroscopic techniques namely: nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), and to a lesser extent infrared spectroscopy (IR) and ultraviolet-visible spectroscopy (UV-visible). The characteristic features of each NMR experiment is summarized in Table 4.1.

**Table 4.1: NMR experiments commonly applied for natural product structural elucidation (Shahid, 2012).**

<table>
<thead>
<tr>
<th>NMR experiment</th>
<th>Information/ interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proton NMR (1D $^1$H NMR)</td>
<td>Quantitative overview of the distribution of protons in a sample.</td>
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<tr>
<td>Carbon-$^{13}$NMR (1D $^{13}$C NMR)</td>
<td>Can provide a quantitative overview of the carbon distribution.</td>
</tr>
<tr>
<td>Distortionless enhancement through polarization transfer (DEPT) (1D $^{13}$C NMR)</td>
<td>Separate the carbon of a compound into primary (CH$_3$), secondary (CH$_2$), Tertiary (CH) and quaternary (C) spectra.</td>
</tr>
<tr>
<td>$^1$H-$^1$H correlation spectroscopy (COSY) (2D $^1$H NMR)</td>
<td>Connectivity information of protons on adjacent carbons. Cross-peaks connect the chemical shifts of protons that are coupled. Symmetrical cross peaks appear around a central diagonal.</td>
</tr>
<tr>
<td>$^1$H-$^{13}$C heteronuclear single quantum correlation (HSQC) and heteronuclear multiple quantum correlation (HMQC) (2D $^1$H-$^{13}$C NMR)</td>
<td>$^1$H-$^{13}$C 1 bond correlation. Cross peaks represent carbon chemical shifts in one dimension and proton chemical shifts in the other dimension.</td>
</tr>
<tr>
<td>$^1$H-$^{13}$C heteronuclear bond multiple correlation (HMBC) (2D $^1$H-$^{13}$C NMR)</td>
<td>$^1$H-$^{13}$C 2-4 bond correlations. Quaternary carbons are observed. Connectivity information is read as vertical lines.</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

*Description of plant Glyptopetalum calocarpum (Kurz.) Prain*

![Image](image.png)

Kingdom: Plantae  
Phylum: Tracheophyta  
Class: Magnoliopsida  
Order: Celastrales  
Family: Celastraceae  
Genus: Glyptopetalum  
Species: *Glyptopetalum calocarpum*

**Figure 4.1. Glyptopetalum calocarpum**

*Traditional use*

*Glyptopetalum calocarpum* (Kurz.) Prain known as Miroonlō (Nicobarese language), is endemic to Andaman and Nicobar Islands (Pandey and Diwakar, 2008). It is also known as Andaman Spindle bush, and belongs to *Celastraceae* family. It is endemic to Andaman and Nicobar Islands, and found along the sea shores of Car Nicobar and Nancowry Islands. It is a small tree, bearing greenish-white flowers, globose capsules and red seeds (Dagar and Singh, 1999). Capsule is the size of a cherry, round, obscurely 4-lobed. There is a single seed in each compartment (Sclater and Walsh, 1892). Nicobarese use its leaves as a remedy for fever, body ache, joint pain and body swelling.

A preliminary study indicated that, methanol extract of *Glyptopetalum calocarpum* was found to be active against the bacterial pathogens tested. Considering its medicinal properties and its indigenous nature in this Island, it was selected for identifying its bioactive constituents.

The present study was carried out to isolate the active metabolites from the methanolic extract of *Glyptopetalum calocarpum* (Kurz.) Prain leaves, along with their antimicrobial activity. The compounds were identified and characterized on the basis of various spectral techniques including 1D, Mass, and IR spectral data. To the best of our knowledge, this is the first report on the isolation of chemical constituents of the plant, *Glyptopetalum calocarpum*.  

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**Plant material**

The leaves of *G. calocarpum* were collected from the forest area of Topoiming village (N 09°12.419 E 092°47.499) at Car Nicobar Island, during March 2013. The plant material was brought to the research laboratory of Regional Medical Research Centre (ICMR), Port Blair. Plant specimen was identified at Botanical Survey of India, Port Blair, and the voucher specimen was maintained in the Institutes’ library (Voucher No.: ANH/CN/290).

**Extraction and isolation**

The air dried leaves (1kg) were grinded, made into powder, and soaked with methanol for one week. The resulting extract was concentrated under vacuum, to obtain a residue. This residue (90 g) was subjected to column chromatography over silica gel (60-120 mesh) using an eluent system of increasing polarity of n-hexane: ethyl acetate (100: 0 to 0: 100), to obtain nine major fractions (F1-F9). Further chromatographic analysis of fraction F1 (solvent system using n-hexane: ethyl acetate 97:03) yielded compound 1 (5 mg), while chromatographic analysis (solvent system using n-hexane: ethyl acetate 90:10) of fraction F2 yielded compound 2 (5 mg). Compound 5 (4 mg) and 3 (6 mg) were isolated from fraction F3, when subjected to preparative TLC using n-hexane: ethyl acetate 95:05. Fraction F4 on repeated column chromatography yielded the compound 4, with minor impurity. This was further purified (using n-hexane: ethyl acetate 94:06, isocratic solvent system) to obtain compound 4 (7mg) in pure form. Fraction F5, on subjecting to repeated chromatography (solvent system n-hexane: ethyl acetate 85:15) eluted compound 6 (8 mg) (Figure 4.2).

**Characterization of the isolated compounds**

**NMR spectroscopy**

One dimensional (1D) (¹H and ¹³C) and two dimensional (2D) NMR spectra (H-H COSY, HMQC and HMBC) were recorded on a Varian-NMR-vnmrs 600 spectrometer with tetramethylsilane (TSM) as internal standard. Standard pulse sequences were used for homo and hetero nuclear correlation experiments. ¹H NMR resonances were determined by DEPT experiments. All NMR experiments were performed at constant deuteriomethanol or deuteriodimethylsulphoxide as solvent on the basis of the solubility of the sample and literature data.
Figure 4.2: Schematic representation of the isolation procedure

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Leaves of Glyptopetalum calocarpum (1Kg)
<table>
<thead>
<tr>
<th>Methanol maceration</th>
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<tbody>
<tr>
<td>Methanol extract (90g)</td>
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<tr>
<td>Column chromatography</td>
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</table>

Extraction, fractionation and isolation of bioactive compounds from the leaf extract of G. calocarpum

**Mass spectroscopy**

Electrospray ionization mass spectrometric analyses (negative and positive mode) were carried out to obtain the molecular weight and fragmentation patterns of the isolated compound(s) using TOF mass spectrometer (WATERS HPLC).

**UV spectroscopy**

The UV-spectrum of the isolated bioactive compound(s) was recorded using Agilent 1200 UV-Visible spectrophotometer.

**RESULTS AND DISCUSSION**

The methanolic leaf extract of *G. calocarpum* were repeatedly subjected to gravity column chromatography to yield six pure antimicrobial triterpenoids. The structure of the compounds were determined by extensive NMR techniques and chemical methods mainly by 1D NMR (¹H and ¹³C) and 2D NMR (COSY, HMQC and HMBC), ESIMS, UV-visible spectra and by compared with the literature data.
Spectroscopic characterization of the isolated compounds

Compound 1 was obtained as an amorphous white powder with melting point 181-182° C. $^1$H NMR (CDCl$_3$, 300 MHz): 4.69 (brs, 1H), 4.58 (brs, 1H), 2.52-2.31 (m, 3H), 2.06-1.84 (m, 3H), 1.68 (s, 3H), 1.52-1.34 (m, 19H, overlapped each other), 1.63-1.52 (m, 2H), 1.07 (s, 2 x 3H), 1.03 (s, 3H), 0.96 (s, 3H), 0.88 (s, 3H), 0.80 (s, 3H). $^{13}$C (CDCl$_3$, 75 MHz): 150.8, 109.5, 54.8, 49.7, 48.2, 47.9, 47.3, 42.9, 42.8, 40.7, 39.9, 39.6, 38.1, 36.8, 35.4, 34.1, 33.5, 31.9, 29.3, 27.4, 26.3, 25.1, 22.6, 21.4, 21.0, 19.6, 17.9, 15.9, 15.7, 14.1. EI-MS $m/z$ 410 [M $^+$]$.^7$ The comparison of these NMR data with the literature, confirmed the compound to be as $\alpha$-Lupene (Dharmassree et al., 1981).

Figure 4.3: $^1$H NMR & $^{13}$C NMR spectrum of $\alpha$-Lupene
Compound 2 was obtained as a white powder with melting point 172-174 °C; IR (KBr; \( \nu_{\text{max}}, \text{cm}^{-1} \)) 3311, 2946, 2870, 1638, 1464, 1189, 1035, 996, 680; \(^1\)H NMR (CDCl\(_3\), 300 MHz): 4.68 (1H, brs), 4.56 (1H, brs), 3.18 (dd, \( J =5.0, 10.7 \) Hz, 1H), 2.37 (1H, m), 1.20-0.90 (25H, all peaks merged with each other), 1.68 (3H, s), 1.03 (3H, s), 0.96 (3H, s), 0.94 (3H, s), 0.83 (3H, s), 0.79 (3H, s), 0.76 (3H, s). \(^{13}\)C (CDCl\(_3\), 75 MHz): 203.5, 151.0, 109.3, 78.9, 55.2, 50.5, 48.3, 47.9, 43.0, 42.7, 40.8, 39.9, 38.8, 38.6, 38.0, 37.1, 35.5, 34.2, 29.8, 28.0, 27.3, 25.1, 20.9, 19.6, 18.3, 17.9, 16.0, 15.9, 15.3, 14.5; EI-MS \( m/z \) 427 [M + H]\(^+\). The comparison of these NMR data with the literature confirmed the compound as Lupeol (Jain and Bari, 2010).

**Figure 4.4: \(^1\)H NMR & \(^{13}\)C NMR spectrum of Lupeol**
Compound 3 was obtained as a white powder with melting point mp 168-170 °C; IR (KBr; \( \nu_{\text{max}} \), cm\(^{-1} \)) 2,940, 1,700, 1,580, 1,454, 1,290, 1,140, 975, 767; \(^1\)H NMR (CDCl\(_3\), 300 MHz): 4.66 (d, \( J = 2.4 \) Hz, 1H), 4.57 (d, \( J = 2.4 \) Hz, 1H); 2.52-2.45 (m, 1H), 2.43-2.35 (m, 2H), 1.97-1.87 (m, 2H), 1.73-1.70 (m, 1H), 1.68 (s, 3H), 1.65-1.61 (m, 2H), 1.52-1.26 (16H, merged with each other), 1.23-1.18 (m, 1H), 1.07, 1.06, 1.03, 0.96, 0.93, 0.80 (each 3H, s, Me \( \times 6 \)). \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 218.2, 150.8, 109.3, 54.9, 49.7, 48.2, 47.9, 47.3, 42.9, 42.8, 40.7, 39.9, 39.6, 38.1, 36.8, 35.4, 34.1, 33.5, 29.8, 27.4, 26.6, 25.1, 21.4, 21.1, 19.6, 19.3, 18.0, 15.9, 15.7, 14.4. EIMS for C\(_{30}\)H\(_{48}\)O \( m/z \): 425 [M+ H]\(^+\). The comparison of these NMR data with the literature confirmed the compound as Lupenone (Prakash and Prakash, 2012).

**Figure 4.5:** \(^1\)H NMR & \(^{13}\)C NMR spectrum of Lupenone
Compound 4 was obtained as a white powder with melting point: 174-176 °C; IR (KBr; $\nu_{\text{max}}$, cm$^{-1}$): 3435, 3019, 2940, 1636, 1422, 1380, 1215, 1020, 756, 669. $^1$H NMR (CDCl$_3$, 300 MHz): 5.29 (t, $J = 6.1$Hz, 1H), 5.10(m, 1H), 4.93 (m,1H), 3.49 (m,1H), 2.51- 2.07 (m, 5H), 1.97-1.91(m, 3H), 1.69 (3H, s), 1.59 (m,1H), 1.55 (m, 2H), 1.31- 1.40 (m, 3H),1.16 (m, 2H), 1.15 (m, 2H), 1.06 (s, 3H), 1.04 (s, 3H), 1.00 (m, 1H), 0.95 (m, 1H), 0.90 (d, $J = 6.2$ Hz, 3H), 0.82 (t, $J = 7.2$ Hz, 3H), 0.81(d, $J = 6.6$ Hz, 3H), 0.79 (d, $J = 6.6$ Hz, 3H), 0.71 (s, 3H). $^{13}$C NMR (CDCl$_3$,75MHz): 141.0, 138.5, 129.4, 121.6, 72.0, 56.6, 56.1, 50.4, 46.3, 42.6, 42.4, 40.4, 39.8, 37.5, 36.4, 32.3, 31.8 (2C), 29.4, 29.2, 25.2, 24.1, 21.6, 21.4, 20.1, 19.7, 18.7, 12.1, 12.0. The comparison of these NMR data with the literature confirmed the compound as Stigmasterol (Jain and Bari, 2010).

**Figure 4.6: $^1$H NMR & $^{13}$C NMR spectrum of Stigmasterol**

![Figure 4.6: $^1$H NMR & $^{13}$C NMR spectrum of Stigmasterol]
Compound 5 was obtained as a white powder with melting point: 189-191 °C; IR (KBr; \( \nu_{\text{max}} \), cm\(^{-1} \)) : 3360 and 1650. \(^1\)H NMR (CDCl\(_3\), 300 MHz): 5.25 (d, \( J = 6.0 \) Hz, 1H), 3.2 (dd, \( J = 4.9 \), 10.8 Hz, 1H), 2.10-1.85 (m, 2H), 1.82-1.68 (m, 4H), 1.64-1.49 (m, 11H, overlapped with each other), 1.45-1.27 (m, 7H, overlapped), 1.04 (s, 3H), 1.0 (s, 3H), 0.97 (s, 2x3H), 0.95 (s, 3H), 0.85 (s, 3H), 0.77 (s, 3H), 0.74 (s, 3H). \(^{13}\)C NMR (CDCl\(_3\), 75MHz): 139.8, 118.8, 79.0, 55.2, 47.6, 47.2, 42.3, 42.1, 41.0, 39.2, 38.8, 38.7, 37.1, 36.7, 36.2, 34.3, 34.1, 29.6, 27.9, 27.6, 27.3, 27.0, 22.5, 21.5, 18.3, 17.6, 16.2, 15.9, 15.3, 14.6. Comparison of these NMR data with the literature confirmed the compound as \( \beta \)-Amyrin (Shan et al., 2014).

**Figure 4.7:** \(^1\)H NMR & \(^{13}\)C NMR spectrum of \( \beta \) - Amyrin
Compound 6 was obtained as a colourless needles, melting point 241-242.5 °C; IR (KBr; $\nu_{\max}$, cm$^{-1}$): 1722, 1635, 1240, and 812. $^1$H NMR (CDCl$_3$, 300 MHz): 5.21 (t, $J = 3.5$ Hz, 1H), 4.54 (dd, $J = 11.6$ Hz, 1H), 2.07 (s, 3H), 2.03-1.84 (m, 5H), 1.80-1.72 (m, 1H), 1.69-1.60 (m, 5H), 1.59-1.45 (m, 4H), 1.43-1.38 (m, 4H), 1.36-1.30 (m, 3H), 1.09-1.03 (m, 1H), 1.14 (s, 3H), 0.98 (s, 3H), 0.96 (s, 3H), 0.88 (s, 2 X 3H), 0.87 (s, 3H), 0.86 (s, 3H), 0.83 (s, 3H). $^{13}$C NMR (CDCl$_3$, 75MHz): 171.0, 145.1, 121.6, 80.9, 55.2, 47.5, 47.2, 46.7, 41.6, 39.7, 38.2, 37.7, 37.1, 36.8, 34.7, 33.3, 32.5, 32.4, 31.0, 28.3, 28.0, 26.9, 26.1, 25.9, 23.6, 23.5, 21.3, 18.2, 16.7, 16.6, 15.5 EIMS $m/z$: 468 [M + $^+$]. Comparison of these NMR data with the literature confirmed the compound as $\beta$-Amyrin acetate (Matsunaga et al., 1988).

**Figure 4.8: $^1$H NMR & $^{13}$C NMR spectrum of $\beta$-Amyrin acetate**
CONCLUSIONS

Six compounds were purified from the plant extract of *Glyptopetalum calocarpum* (Kurz.) Prain. However, further in vitro and in vivo studies are needed to investigate the pharmacological and toxicological properties of this compounds, before it can be considered as a new antimicrobial agents.