CHAPTER III

Isolation and Identification of Active Compound in the ethanolic root extract of Zaleya decandra

5.1 Introduction

Natural products have been used to treat human disease for thousands of years and play an increasingly important role in drug discovery and development. In fact, scientists have been taking advantage of natural products for such purpose, approximately 40% of the drugs approved for use in the past few years were somehow related to natural products (Newman and Cragg, 2007). The discovery of penicillin from the filamentous fungus Penicillium notatum by Fleming in 1929 had a great impact on the investigation of nature as a source of new bioactive agents (Bennett and Chung, 2001). The isolation of the analgesic morphine from the opium poppy, Papaver somniferum, in 1816 led to the development of many highly effective pain relievers (Benyhe, 1994).

Medicinal plants have an important role in the management of diabetes mellitus especially in developing countries. A large number of crude plant extracts and purified substances from plants have been tested in clinical trials for treatment of diabetes. Useful throughout history for their medical as well as other benefits, plant-derived compounds have gained particular importance recently, due to environmental factors. The use of traditional medicine is wide spread and plants are still a large source of natural antioxidants that might serve as leads for the development of novel drugs. The therapeutic effects of several plants and vegetables, which are used in traditional medicine, are usually attributed to their antioxidant compounds (Anokwuru et al., 2011). These factors have inspired the widespread screening of plants for possible medicinal and antioxidant properties, the isolation and characterization of diverse phytochemicals and the development and utilization of antioxidants of natural origin (Jayaprakasha et al., 2001).

Different physicochemical methods have, since the beginning of the 20th century, permitted the characterization of a plethora of complex structures and at the
present time the technical advances both in chromatographic techniques and in identification tools particularly the diverse forms of mass-spectrometry allow us to meet the challenges of separation and characterization of compounds of increasing complexity, of high molecular weight, low stability and low abundance (Whiting, 2001).

The aim of the present study was to isolate and identify the active constituents in the ethanolic root extract of *Zaleya decandra*.

### 5.2 Materials and Methods

#### 5.2.1 Column chromatography

The chromatographic column was packed with 150g silica gel using petroleum ether as solvent. 10 g ethanolic root extract of *Zaleya decandra* was laid on the top of the silica gel. The column was then sequentially eluted with petroleum ether, chloroform, ethyl acetate and methanol.

#### 5.2.2 Thin layer chromatography

Thin layer chromatography is an easy and highly useful technique in research laboratories to separate and identify unknown compounds. Thin layer chromatography is for the separation of a mixture into individual components using a stationary and mobile phase (Sadasivam and Manikam, 1992).

**Principle**

The principle of separation is adsorption. One or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate. The mobile phase solvent flows through because of capillary action towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster. Thus the components are separated on a thin layer chromatographic plate based on the affinity of the component towards the stationary phase.

**Procedure**

The optimized conditions were used for the identification of active constituents present in the plant extract. The fractions collected from chromatographic columns were
monitored by thin layer chromatography (TLC) in different solvent systems as in Table 5.1. These plates were placed in the solvent chamber containing mobile phase. The solvent was allowed to rise to the maximum height of the TLC plate, then they were removed from solvent chamber, dried and the spots were detected by placing the TLC plates in a chamber containing iodine vapour.

Table 5.1 Thin layer chromatographic solvent systems for separation of active constituents

<table>
<thead>
<tr>
<th>S. No.</th>
<th>TLC mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>2.</td>
<td>Petroleum ether / Ethyl acetate (8:2)</td>
</tr>
<tr>
<td>3.</td>
<td>Chloroform / Methanol (8:2)</td>
</tr>
</tbody>
</table>

5.2.3 Analysis of the functional groups by Fourier transform infrared (FTIR) spectroscopy

The infrared spectrum originates from the vibrational motion of the molecule. The vibrational frequencies are a kind of fingerprint of the compounds. This property is used for characterization of organic, inorganic and biological compounds. The band intensities are proportional to the concentration of the compound and hence quantitative estimations are possible.

Description

The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise to the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer.

The Shimadzu FTIR Spectrum instrument consists of globar and mercury vapour lamp as sources, an interferometer chamber comprising of KBr and Mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm\(^{-1}\) is covered by this instrument. The spectrometer works under purged conditions. Solid samples are dispersed in KBr or polyethylene pellets depending on the region of
interest. This instrument has a typical resolution of 1.0 cm\(^{-1}\). Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible.

**Instrument details**

- Instrument made by Shimadzu
- Model- IR Affinity I
- Detector- DLATGS

**Sample required**

1:200 (1 mg extract in 200 mg KBr)

**Applications**

Infrared spectrum is useful in identifying the functional group like -OH, CN, -CO, -CH, -NH\(_2\) etc.

**5.2.4 Identification of the structure of the active constituents by Nuclear Magnetic Resonance (NMR) spectroscopy:**

NMR spectroscopy is used to determine the molecular structure based on the chemical environment of the magnetic nuclei like \(^1\)H, \(^{13}\)C, 2D NMR, etc., even at low concentrations. This is one of the most powerful non destructive techniques in elucidating the molecular structure of biological and chemical compounds.

**Description**

In NMR spectroscopy, a strong RF pulse excites the entire range of precessional frequencies of a given nuclear species whose time response is known as free induction decay (FID) containing all the information. A Fourier transform of FID gives the NMR spectrum. This technique is used in JEOL GSX 400 NB FT-NMR spectrometer. The spectra of samples containing low abundant nuclei like \(^1\)H, \(^{13}\)C, 2D NMR, etc., are thus easily obtained. Also dynamic studies are possible by relaxation measurements. Homo and hetero \(^1\)H decoupling are also possible.

JEOL GSX 400 NMR operates at 300 MHz (for proton) with a magnetic field of 9.3 Tesla. Hence a supercon magnet is used. A PDP-11/73 computer is an integral part of the instrument for the purpose of Fourier transformation and spectral manipulation. The spectrum is plotted on a HP plotter and data can be obtained on a printer.
The probes available are $^1$H/$^{13}$C combined and multinuclei probe to study the nuclei like $^{23}$Na, $^{27}$Al, $^{43}$Ca, $^{37}$Cl, $^{79}$Br etc.

Instrument details

JEOL GSX 400 NB, 300 MHz FT NMR Spectrometer

Sample required

5 mg for $^1$H and 15 mg for $^{13}$C, Solubility: 10 mg/ml for $^1$H and 50 mg/ml for $^{13}$C

Solvents available: CDCl$_3$, D$_2$O, C$_6$D$_6$, CD$_3$COCD$_3$ and DMSO-d$_6$.

Applications

Widely used in organic chemistry, biology, medicine, pharmaceuticals, etc. for characterization of compounds.

5.2.4.1 Two-dimensional Nuclear Magnetic Resonance (2D NMR) spectroscopy:

Two-dimensional nuclear magnetic resonance (2D NMR) provides valuable information for resolving and identifying the nature of chemical sites in a variety of chemical and biochemical systems.

Two-dimensional (2D) spectroscopy fulfills a central role in the application of nuclear magnetic resonance (NMR) to chemistry, Biochemistry, and Medicine (Jeener, 1971; Aue et al., 1976; Ernst et al., 1987). 2D NMR experiments separate and correlate spin interactions along independent frequency domains, providing an enhancement in resolution and information that is unavailable in unidimensional spectral counterparts.

5.2.4.2 2D NMR Techniques

HSQC (Heteronuclear Single Quantum Correlation)

This two dimensional NMR technique correlates $^{13}$C nuclei with directly attached protons. The resolution is very high since the experiment is proton detected $^1$H-$^{13}$C correlation. Only one bond couplings ($^1$J$_{C,H}$) are detected and two bond three bond carbon-hydrogen couplings are eliminated.

$^1$H-$^1$H COSY (Homonuclear Correlation Spectroscopy)

HOMOCOSY NMR technique reveals correlations between coupled protons. Cross peaks are obtained for all protons that have measurable coupling. Diagonal peaks are due to chemical shift equivalent protons and they do not provide useful information.
**HMBC (Heteronuclear Multiple bond Correlation)**

This is also two dimensional proton detected $^1$H-$^{13}$C correlation experiment. In this technique one bond couplings ($^1$J$_{C,H}$) are sacrificed and two and three bond carbon-hydrogen couplings are detected.

**5.2.5 Determination of the molecular weight of the compound by Mass spectroscopy (MS)**

Mass spectrometry has become a vital tool in the hands of organic chemists and biochemists because of its potential to supply definitive, qualitative and quantitative information on molecules based on their structural compositions.

**Description**

The Mass spectrometer consists of an ion source, an analyzer and a detector maintained at a vacuum of $10^{-8}$ torr. The vaporized molecules are first bombarded by a stream of high energy electrons converting some of the molecules into molecular ions and fragment ions. The ions are accelerated and separated according to their mass to charge ratios in the magnetic field (analyzer). These are velocity focused in an electric field. The ions are detected in terms of their mass to charge ratios by the detector namely a secondary electron multiplier. The output is amplified and fed to the recorder for processing. The mass spectrum, a graph of intensity of the ions detected vs. m/z value is presented on the screen and printed. An IBM compatible PC is used to control the Mass spectrometer and also to acquire process and print out the spectral data.

**Instrument details**

Finnigan MAT 8230 JEOL GC Mate GC-MS Spectrometer

**Sample required**

About 1 mg. The sample required can be in the solid or liquid state. Sample should be pure and free from solvents and metal ions.

**5.2.6 Elucidation of the structure of the isolated compound**

For the structural elucidation of the active constituent UV, IR, $^1$H NMR $^{13}$C NMR and 2D NMR were carried out and the spectra were analyzed. Mass spectroscopy was carried out to find out the molecular weight.
5.2.7 α-glucosidase inhibition assays (Tadera et al., 2006).

The yeast α-glucosidase was dissolved in 100mM phosphate buffer, pH 6.8 was used as enzyme source; 10mM paranitrophenyl-α-D-glucopyranoside was used as substrate. *Evolvulus alsinoides* extract powder was weighed and mixed with dimethylsulfoxide to get a concentration of 10-100µg/ml. The different concentration of plant extract was mixed with 320µl of 100mM phosphate buffer (pH 6.8) and 50 µl of 10mM PNPG in the buffer and then it was incubated at 30 °C for 5 minutes. After the incubation, 20µl of the buffer containing 0.5 mg/ml of the enzyme was added and further incubated at 30°C for five minutes. Finally, 3.0 ml of 50mM sodium hydroxide was added to the mixture and the absorbance (A) was measured at 410nm on a spectrophotometer. The enzyme without plant extract was used as a control.

\[
% \text{ Inhibition} = \frac{A_{410} \text{ control} - A_{410} \text{ test}}{A_{410} \text{ control}} \times 100
\]

The IC\textsubscript{50} values (inhibitor concentration at which 50% inhibition of the enzyme activity occurs) of the plant extracts were determined by performing the assay as above with varying concentrations of the plant extracts ranging 20 to 100µg. The IC\textsubscript{50} values were determined from plots of percent inhibition vs log inhibitor concentration and calculated by non-linear regression analysis from the mean inhibitory values.

5.3 Results and Discussion

5.3.1 Column chromatography

A total of 138 fractions of 50 ml each were collected from the column as shown in Table 5.2.

**Table 5.2 Separation of active constituents by column chromatography**

<table>
<thead>
<tr>
<th>% of solvents</th>
<th>Fraction number</th>
<th>No of Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>1 - 38</td>
<td>38</td>
</tr>
<tr>
<td>Chloroform</td>
<td>39 - 107</td>
<td>69</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>108 -124</td>
<td>17</td>
</tr>
<tr>
<td>Methanol</td>
<td>125 -138</td>
<td>14</td>
</tr>
</tbody>
</table>
5.3.2 Thin layer chromatography

The fractions 8 to 13 showed single, clear spot (Figure 5.1) with the same R_f value (0.82 cm) in TLC indicating the presence of a single compound, were mixed together. 24 mg of the pure compound was obtained from petroleum ether fraction.

Figure 5.1 Thin Layer Chromatography (TLC) of the isolated compound from *Zaleya decandra* root extract with petroleum ether and ethyl acetate (8:2) solvent system

5.3.3 Analysis of the functional groups by Fourier transform infrared (FTIR) spectroscopy

Analysis of the functional groups by Fourier transforms infrared (FTIR) spectroscopy was next carried out 1:200 (1 mg. extract in 200 mg KBr) in order to identify the functional group like -OH, CN, -CO, -CH, -NH_2 among others.

FTIR is very useful for the analysis of many types of materials (organic compounds, polymers etc). Infrared spectrum is useful in identifying the functional groups present in a compound like -OH, CN, -CO, -CH, -NH_2 etc. The data of the isolated compound from the ethanolic extract of *Zaleya decandra* are shown in figure 5.2 and table 5.3. The compound showed absorption bands in the IR spectrum at 1732 cm^{-1} for a carbonyl group, 2927 cm^{-1} for C-H group, 1072 cm^{-1} for C-O group and a series of small absorption bands in the range of 1600 cm^{-1} due to the presence of aromatic ring system.
Figure 5.2 FTIR spectrum of the ethanolic extract of *Zaleya decandra*
Table 5.3  IR absorption frequencies of functional groups in *Zaleya decandra*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Characteristic Absorption (cm(^{-1}))</th>
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<tr>
<td>1</td>
<td>2927</td>
<td>C-H</td>
</tr>
<tr>
<td>2</td>
<td>1732</td>
<td>Carbonyl group (C=O)</td>
</tr>
<tr>
<td>3</td>
<td>1600</td>
<td>Aromatic ring</td>
</tr>
<tr>
<td>4</td>
<td>1072</td>
<td>C-O</td>
</tr>
</tbody>
</table>

5.3.4 Determination of the molecular weight of the compound by Mass spectroscopy (MS)

The fragment ions present in the mass spectrum suggest the presence of respective structural moieties in the compound.

5.3.5 Structure of the compound by using FTIR, \(^1\)H-NMR, \(^{13}\)C-NMR, HSQC, \(^1\)H-\(^1\)H COSY and HMBC Spectrum:

FTIR is very useful for the analysis of many types of materials (organic compounds, polymers etc). Infrared spectrum is useful in identifying the functional groups present in a compound like -OH, CN, -CO, -CH, -NH\(_2\) etc. The data of the isolated compound from the ethanolic extract of *Zaleya decandra* are shown in table 5.4. The compound showed absorption bands in the IR spectrum at 1732 cm\(^{-1}\) for a carbonyl group, 2927 cm\(^{-1}\) for C-H group, 1072 cm\(^{-1}\) for C-O group and a series of small absorption bands in the range of 1600 cm\(^{-1}\) due to the presence of aromatic ring system.

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<td>C-O</td>
</tr>
</tbody>
</table>
In its \textsuperscript{1}H-NMR spectra the pair of triplets at δ 0.83 overlapped with one another each integrating for three protons indicates the presence of two methyl groups (H-11’ H-11’’). The broad singlet at δ 1.18 is attributed to the long chain methylene groups (H-4’, 4’’ to H-10’ H10’’). The peak at δ 1.60 are due to H-2’ and H-2’’ and the signal at δ 4.17 (H-1’ & H- 1’’) is due to oxy methylene group (OCH\textsubscript{2}). The pair of doublet of doublets at δ 7.63 and 7.45 each integrating for two protons indicates the presence of four aromatic protons (Table 5.5).

\textbf{Table 5.5: \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR Spectral data of the compound}\textsuperscript{*}

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Signal(δ)</th>
<th>Proton</th>
<th>Signal(δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>131.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>129.84</td>
<td>H-2</td>
<td>7.63 (dd)</td>
</tr>
<tr>
<td>3</td>
<td>127.80</td>
<td>H-3</td>
<td>7.44 (dd)</td>
</tr>
<tr>
<td>4</td>
<td>131.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>127.80</td>
<td>H-5</td>
<td>7.44 (dd)</td>
</tr>
<tr>
<td>6</td>
<td>129.84</td>
<td>H-6</td>
<td>7.63 (dd)</td>
</tr>
<tr>
<td>7 &amp; 8</td>
<td>166.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1’ 1’’</td>
<td>65.00</td>
<td>H-1’ 1’’</td>
<td>4.176 (m)</td>
</tr>
<tr>
<td>2’ 2’’</td>
<td>37.77</td>
<td>H-2’ 2’’</td>
<td>1.61</td>
</tr>
<tr>
<td>3’ 3’’</td>
<td>29.39</td>
<td>H-3’ 3’’</td>
<td>1.56</td>
</tr>
<tr>
<td>4’ 4’’ - 10’ 10’’</td>
<td>28.68,27.93,22.78,21.97</td>
<td>H-4’, 4’’ to H-10’ H10’’</td>
<td>1.18 (br.S)</td>
</tr>
<tr>
<td>11’ 11’’</td>
<td>13.01, 9.94</td>
<td>H-11’ H-11’’</td>
<td>0.825, 0.851</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Assignments were made based on the HSQC data

The above data was complemented by its \textsuperscript{13}C-NMR spectral data. The weak signal at δ 166.72 is attributed to the carbonyl carbon. The three signals at
δ 131.30 (weak signal due to the quaternary carbon atoms C-1 and C-4) and at δ 129.84 and 127.80 (each for two carbon atoms C-2 & C-6; C-3 & C-5) confirms the presence of a symmetrically substituted aromatic ring system. The signal at δ 66.00 confirms the presence of -OCH₂ group. The signals at δ 9.94 and 13.01 (C-11’ & C-11’’) and the signals between δ 21.97 and 37.77 are due to the long chain hydrocarbon.

**Figure 5.3.** ¹H NMR spectrum of the isolated compound from _Zaleya decandra_

Further to this ¹H-¹H COSY three characteristic cross peaks were observed between the signals at δ 7.63 (H-2, H-6) & 7.45 (H-3 & H-5), between the signals at δ 4.2 (H-1’ & H1’’) and 1.8 (H-2’ & H-2’’), and between the signals at 0.89 (H-11’ & H-11’’) & 1.18 (H-2’ & H-2’’) indicating that they are coupled with each other.

In the HMBC spectra long range coupling was observed between the protons at δ 4.2 (H-1’ & H1’’) with C-1’ and C-2’. The proton at δ 1.8 (H-2’ & H-2’’) showed cross peak with C-1’, C-3’ & C-11’. The signal at δ 1.56 (H-3’) showed cross peaks with C-1’, C-2’, C-4’.
References

Antidiabetic activity of *Zaleya decandra* root extract in diabetes induced rats

Figure 5.4. $^{13}\text{C}$ spectrum of the isolated compound from *Zaleya decandra*

Figure 5.5. $^1\text{H}$ spectrum of the isolated compound from *Zaleya decandra*
Antidiabetic activity of *Zaleya decandra* root extract in diabetes induced rats
In the Mass spectra the ion at m/z 171.8 [CH\_3- CH\_2- (CH\_2)\_8-CH\_2-OH]^+,
indicates the presence long chain hydrocarbon with an oxygen function. The ion at m/z
141.8 which is 30 units less suggests the fragmentation of the –OCH\_2 group leaving
[CH\_3- CH\_2- (CH\_2)\_8-H]^+ ion. Peaks at m/z 113.9 (28 units less for 2 methylene groups)
and the above data indicates the presence of the C-11 hydrocarbon chain.

5.3.6 Elucidation of the structure of the isolated compound

From all the above spectral studies (IR and NMR) the isolated compound was
characterized and the assumed structure of the compound is as follows

**Figure 5.8 Structure of the isolated compound from Zaleya decandra**

![Structure of the isolated compound](image)

**Molecular formula** = C\(_{30}\)H\(_{50}\)O\(_{4}\)

**Molecular weight** = 474.71 grams/mole.

**Compound Name** = Diester of undecyl alcohol of 1,4-benzene dicarboxylic acid.

The FTIR analysis revealed the presence of functional groups in Zaleya
decandra root extract compound. The \(^1\)H NMR, \(^1\)C NMR, HSQC, \(^1\)H- \(^1\)H COSY and
HMBC spectrum revealed the presence of an aromatic ester compound from Zaleya
decandra root extract. The present study of selected medicinal plant and phytochemistry
study revealed a good aid in the treatment of diabetes mellitus.
5.3.7 α-glucosidase inhibitory activity of crude ethanolic extract and isolated compound of Zaleya decandra at varying concentrations

Diabetes mellitus (DM) is a common endocrine system disease that causes metabolic disorders and which leads to multiple organ damage syndrome. Clinical admiral diabetes is divided into two types, with more than 90% of patients having Type II diabetes (Harborne, 1987). The number of diabetes cases was 171 million in 2000 and is expected to rise to 366 million in 2030 (Sadasivam and Manickam, 1996). Inhibition of α-glucosidase (EC 3.2.1.20) and α-amylase (EC 3.2.1.1), enzymes involved in the digestion of carbohydrates, can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet and therefore can be an important strategy in the management of postprandial blood glucose level in type 2 diabetic patients and borderline patients (Singleton and Rossi, 1965, Ordon et al., 2006).

Figure 5.9. α-glucosidase inhibitory activity of crude ethanolic extract and isolated compound of Zaleya decandra at varying concentrations

Our studies demonstrated that Zaleya decandra extract and the compound ZD-1 has α-glucosidase inhibitory activity. The percentage inhibition at 100, 80, 60, 40 and 20 µg/ml concentrations of plant extract as well as the compound showed a concentration-dependent reduction in percentage inhibition (Figure 5.9). Thus the

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highest concentration of 100 µg/ml tested compound ZD-1 showed a maximum inhibition 57% and the plant extract showed the inhibitory activity of 47%. The plant extract produced a weak α-glucosidase enzyme inhibition when compared with compound ZD-1 and standard acarbose (65%).

Intestinal α-glucosidase is a glucosidase acting as a key enzyme for carbohydrate digestion, located at the epithelium of the small intestine. α-glucosidase has been recognized as a therapeutic target for the modulation of postprandial hyperglycemia, which is the earliest metabolic abnormality that occurs in Type II DM (Yao et al., 2010). Several natural α-glucosidase inhibitors including acarbose, voglibose and miglitol are clinically used as a treatment, but their prices are high and clinical side effects occur (Scott et al., 2000, Kim et al., 2000). Natural products are still the most available source of α-glucosidase inhibitors (Lee et al., 2008). Therefore, screening of alpha-amylase and glucosidase inhibitors in medicinal plants has received much attention. These medications are most useful for people who have just been diagnosed with type 2 diabetes and who have blood glucose levels only slightly above the level considered serious for diabetes. They also are useful for people taking sulfonylurea medication or metformin, who need an additional medication to keep their blood glucose levels within a safe range. Therefore, the retardation and delay of carbohydrate absorption with a plant-based α-glucosidase inhibitor offers a prospective therapeutic approach for the management of type 2 diabetes mellitus and borderline patients (McCue et al., 2004, Subramanian et al., 2008).

The FTIR analysis revealed the presence of function groups in Zaleya decandra root extract. The MS and NMR studies revealed the presence of an aromatic ester in the root of Zaleya decandra extract.