Chapter 5

Isolation of the biologically active compound nimbolide from *Azadirachta indica* (Neem) leaves and bioevaluation for anticancer and antioxidant capacity

5.1 Introduction

Figure 5.1: Photograph of *A. indica* leaves with seeds

The promising properties of *Azadirachta indica* (commonly known in India as Neem; Family: Meliaceae) have been realized worldwide.\(^1\) Almost all parts of the tree offer tremendous potential for medicinal, agricultural and industrial exploitation. Various parts of the *A. indica* tree are used in traditional *Ayurvedic* medicines in India. Some of the traditional uses\(^1\) of different parts of the tree is given in Table 5.1. There are a number of formulations in *Ayurvedic* system of medicine which contain *A. indica*. In addition to its therapeutic efficacies, *A. indica* has already established its potential as a source of naturally occurring insecticides, pesticides and agrochemicals. The environmental compatibility of the *A. indica* products, the lack of resistance developed to them, their harmless nature against non target
organisms and lack of toxicity, all have significantly enhanced the use of *A. indica* in integrated pest control. For thousands of years the beneficial properties of the *A. indica* tree have been recognized in India and it is perhaps the country’s most utilized useful traditional medicinal plant.\(^2\)

**Table 5.1:** Medicinal properties of different parts of neem tree

<table>
<thead>
<tr>
<th>Part</th>
<th>Medicinal uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>Treatment of leprosy, eye problems, intestinal worms, anorexia, skin ulcers.</td>
</tr>
<tr>
<td>Flowers</td>
<td>Bile suppression, elimination of intestinal worms</td>
</tr>
<tr>
<td>Twig</td>
<td>Relieves cough, asthma, piles, spermatorrhoea, diabetes</td>
</tr>
<tr>
<td>Bark</td>
<td>Analgesic, antipyretic.</td>
</tr>
<tr>
<td>Fruit</td>
<td>Relieves piles, intestinal worms, urinary disorder, eye problem, diabetes etc</td>
</tr>
<tr>
<td>Gum</td>
<td>Effective against ring worms, scabies, wounds etc.</td>
</tr>
<tr>
<td>Seed pulp</td>
<td>Leprosy and intestinal worms.</td>
</tr>
<tr>
<td>Oil</td>
<td>Leprosy and intestinal worms.</td>
</tr>
<tr>
<td>Root, bark, leaf, flower and fruit</td>
<td>Biliary afflictions, itching, skin ulcer, burning sensation and leprosy.</td>
</tr>
</tbody>
</table>

*A. indica* tree is native to the Indian subcontinent and South East Asia. There are two closely related species, *A. indica* and *A. azedarch*. The former is popularly known as Indian neem or Indian lilac and the later as the Persian lilac.\(^3\) *A. indica* is a large, hardy, fast growing tree, 10-15m in height with spreading branches. Leaves are 20-40 cm long, are pinnately branched and have ovate-lanceolate, bright green leaflets, measuring up to 5cm in length and 1.5cm in width. Leaves have disagreeable odour and very bitter taste.\(^4\) Almost all parts of the *A. indica* tree (stem, bark, roots, leaves, gum, seeds, fruits, flowers etc) have been in use as traditional medicine for household remedies against various ailments.\(^5\) These spectacular properties of *A. indica* have attracted organic chemists, biologists, clinicians and agriculturalists
around the world to undertake systematic research on this unique plant in various directions.³

It is reported that the aqueous extract of *A. indica* leaves significantly reduces blood sugar levels and prevent adrenaline and glucose induced hyperglycemia⁶ as well as decreases hyperglycemia in streptozotocin induced diabetes.⁷ *A. indica* leaf extract has shown significant antiulcer and antisecretory effects in rats.⁸ It also possess antimalarial activity. Extracts of the leaves have been studied for their effect on *in vitro* and *in vivo* growth and development of the human malarial parasite *Plasmodium falciparum* and found to have highly appreciable results on parasites resistant to antimalarial drugs such as chloroquine and pyrimethamine.⁹ The aqueous extract of the leaf showed antioxidant activities.¹⁰ The chemoprotective property of the leaf extract was also confirmed.¹¹ The antioxidant activity of *A. indica* seed extract has also been demonstrated.¹² *A. indica* is very effective against common skin diseases like acute and chronic eczema, ring worm and scabies.¹³ The acetone extract of the leaf showed significant anxiolytic activity in rats.¹⁴ The ethanol extract of the stem bark and root bark showed hypotensive, spasmyolytic and diuretic activities. The chemical constituent sodium nimbidinate, was found to be a potent diuretic agent in dogs.¹⁵ Oral administration of an aqueous extract of *A. indica* leaf also showed antifertility effects in mice.¹⁶ The anti-inflammatory activity and antihypertensive effect of *A. indica* has also been reported.¹⁷

More than 140 compounds have been isolated so far from different parts of *A. indica*. Indeed, organic chemists, especially natural product chemists are still carrying out research on the active principles of *A. indica*. The compounds found in *A. indica* may be divided into two classes:

(i) Isoprenoids like diterpenoids and triterpenoids including protomelaiacin, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type compounds and nimbin, salanin and azadirachtin.
(ii) Non isoprenoids which include proteins, aminoacids, carbohydrates, sulfur compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcones, coumarins, tannins, aliphatic compounds etc.

Although a large number of compounds have been isolated from various parts of neem tree, especially from the seeds, only a few of them have been investigated so far for biological activity.\textsuperscript{18,19} Structures of some of the important compounds isolated from \textit{A. indica} is given in chart 5.1 and some important compounds isolated from the leaves are given in chart 5.2.

\textbf{Chart 5.1:} Structures of important compounds (known) isolated from \textit{A. indica}

![Chart 5.1: Structures of important compounds](image_url)
Chart 5.1 continued...

Isomargolonone

Mahmoodin

Melianol

Quercetin

(-)-Epicatechin

Isoazadirolide

Melianodiol

Azadirachtanin

Catechin

Sulanin
Chart 5.1 continued...

Salannic acid

Azadirachtol

Azadirachtanin

Chart 5.2: Structures of important compounds (known) isolated from the leaves of A. indica

Nimocinol

2',3'-Dehydosalannol

4-Epinimbin

Azadirachtanin
5.2 Aim and scope of the present investigation

From the review of literature, it was clear that there are detailed reports on the biological activity of the compounds isolated from the seeds. But less known is the activity of the components isolated from the leaves. Only few reports are available on the anti-cancer studies of nimbolide, which is one of the major constituent of \textit{A. indica} leaf.\textsuperscript{20} Therefore it was of interest to study the anti cancer activity of nimbolide and antioxidant activity of different leaf extracts. During an earlier study conducted in our group on the leaves of \textit{A. indica}\textsuperscript{21} a methodology for the rapid isolation of nimbolide was found. From this study, pure sample of nimbolide was also available that could be used for TLC comparison purposes.
5.3 Extraction of A. indica leaves and isolation and characterization of nimbolide

5.3.1 Plant material and extraction

A. indica leaves were collected from NIIST campus, dried and powdered. 450 g of the powdered leaves was extracted successively with hexane, acetone and methanol at room temperature. Solvents were removed under reduced pressure using a rotary evaporator to get 12 g, 21 g and 32 g of the hexane, acetone and methanol extracts respectively. TLC was performed on the extracts along with a standard sample of nimbolide which indicated that acetone extract contained maximum amount of nimbolide. Thus the acetone extract was subjected to column chromatographic separation for obtaining nimbolide.

5.3.2 Isolation of nimbolide from the acetone extract

The acetone extract (21 g) was then subjected to careful column chromatography using silica gel (400 g, 100-200 mesh) starting with hexane as eluant and thereafter gradually raising the polarities with ethyl acetate depending on the separation obtained after examining the fractions by TLC. 360 fractions of 80-90 ml were collected which were finally pooled to ten fraction pools according to the similarities in TLC’s. The first fractions contained β-carotene as a major constituent upon examination of the TLC with a standard sample of β-carotene.

The seventh fraction pool (150-189; 1 g) obtained on elution with 25% ethyl acetate in hexane contained a major compound upon examination by TLC. On crystallization in dichloromethane-ethyl acetate mixture to get white crystals were obtained (116 mg, m.p: 204-205 °C). The compound was analyzed using various spectroscopic techniques. $^1$H NMR spectrum (Figure 5.2) confirmed the presence of four methyl groups at δ 1.22, 1.37, 1.47 and 1.70. The –OMe protons were confirmed by the presence of a sharp singlet
integrating for three protons at $\delta$ 3.54. Multiplet between $\delta$ 7.32-7.22 confirmed the presence of three protons in the furan ring. The $\alpha$ protons of the $\alpha$, $\beta$-unsaturated ketone was observed as the doublet integrating for one proton at $\delta$ 5.92 ($J = 11.8$ Hz). Further by comparison of the Mass, $^1$H and $^{13}$C NMR data with the values reported earlier$^{22}$ it was confirmed that the compound was nimbolide whose structure is shown below.

![Nimbolide](image)

**NIMBOLIDE**

![NMR Spectrum](image)

**Figure 5.2: $^1$H NMR spectrum of nimbolide**
Figure 5.3: $^{13}$C NMR spectrum of nimbolide

Figure 5.4: DEPT – 135 NMR spectrum of nimbolide
5.4 Biological activity studies of *Azadirachta indica*

5.4.1 *In-vitro* cytotoxicity of nimboide

The *in vitro* cytotoxicity of nimboide against human cancer cell line was determined in collaboration with RRL, Jammu. The cell lines studied include colon (colo-205, HCT-15, HT-29, SW-620); liver (Hep-2); lung (A-549) and prostate (Du-145). It was observed that nimboide showed more than 50% growth inhibition at $1 \times 10^{-6}$ M against four cell lines (HCT-15, HT-29, Hep-2 and Du-145) and 37% growth inhibition for the cell line colo 205. Its effect was evaluated at higher concentrations in case of seven cell lines and the growth inhibition was found to be greater than 50%. The growth inhibition was also studied at $2.5 \times 10^{-7}$ M, $5 \times 10^{-7}$ M and at concentrations lower than $1 \times 10^{-6}$ M and in all cases it was less than 50%. The results of the colon cell lines were compared with 5-fluorouracil and in all cases nimboide was found to be better than the reference drug. In case of liver cell line (Hep-2), nimboide showed better result than 5-fluorouracil but it was less active than Mitomycin C. The lung (A-549) cell line showed better effect with the
compound as compared to Paclitaxel at $1 \times 10^{-5}$ M. The results of colon cell lines indicate that in all these cases, the compound was better than the reference drug. The details are shown in Table 5.2. A graphical representation of the cytotoxicity is depicted in figure 5.6.

**Table 5.2:** % Growth inhibition of cancer cells by nimbolide

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concen. (M)</th>
<th>Cell line / tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colo-205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colon</td>
</tr>
<tr>
<td>Nimbolide</td>
<td>2.5 $\times 10^{-7}$</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>5 $\times 10^{-7}$</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1 $\times 10^{-6}$</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td><strong>2 $\times 10^{-6}$</strong></td>
<td><strong>85</strong></td>
</tr>
<tr>
<td></td>
<td>3 $\times 10^{-6}$</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>4 $\times 10^{-6}$</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>1 $\times 10^{-5}$</td>
<td>87</td>
</tr>
<tr>
<td>5-Flourourasil</td>
<td>1 $\times 10^{-5}$</td>
<td>26</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1 $\times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1 $\times 10^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>
The results indicate that the compound nimbolide obtained from the medicinal plant *Azadirachta indica* has significant cytotoxicity against human colon cancer cell lines derived from different tissues. The cytotoxic effect is dose dependent and degree of growth inhibition was cell line specific. Therefore, nimbolide has the potential for further development as an anticancer drug.

### 5.4.2 Antioxidant activity of *Azadirachta indica* using the ferric reducing antioxidant power (FRAP) assay

FRAP assay\(^2\) provides information on the reducing ability of polyphenols which seems to be an important factor for dietary antioxidant activity and free radical scavenging ability of these compounds. In this method, carotenoids which are known antioxidants do not participate and

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**Figure 5.6: Cytotoxicity by nimbolide**

[Graph showing cytotoxicity results for different cell lines]
therefore do not contribute to the ferric reducing ability. As a result, the method is commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts rich in carotenoids.  

FRAP assay is based on electron transfer reaction. The FRAP assay is a simple and reproducible method which can be applied to study the antioxidant activity of plasma or antioxidants. Here a ferric salt (Fe [III] [TPTZ]_2 Cl_3) is used as an oxidant (TPTZ is 2,4,6 tripyridyl s-triazine). This assay depends on the reduction of ferric tripyridyl triazine (Fe (III)-TPTZ) complex to ferrous tripyridyl triazine (Fe (II) TPTZ) by a reductant at low pH which has intense blue color which is monitored at 593 nm. Results are expressed in trolox equivalents. Trolox, a powerful antioxidant is 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). It is a water soluble derivative of Vitamin E

![Chemical structures](image)

Hexane, acetone and methanolic extracts were analyzed for their antioxidant activities by FRAP assay. The ferric reducing ability is expressed in gram equivalents of Trolox/100g of extracts. As per this assay, the methanol extract contained 17.6 ± 2.36 mg Trolox equivalent/100 g of dry leaves, whereas the hexane and the acetone extracts respectively contained 8.96 ± 1.14 mg and 5.36 ± 1.06 mg Trolox equivalent/100 g dry leaves of A. indica. Thus the methanol extract has the highest percentage of reductones
that could contribute largely to the antioxidant activity of the extracts. Nimbolide did not show any antioxidant activity by the FRAP assay.

The extracts as well as nimbolide were also tested for the free radical scavenging activity by DPPH'.

5.4.3 DPPH' radical scavenging capacity

The hexane, acetone and methanol extracts of the leaves of *A. indica* as well as nimbolide were evaluated for free radical scavenging capacity. The samples were tested at a concentration range of 2500-4000 ppm. Among the extracts, the methanol extract exhibited the highest free radical scavenging capacity (92% at 4000 ppm) with an EC$_{50}$ value of 1830 ppm whereas the hexane and acetone extracts showed free radical scavenging capacity of 64% and 39% at 4000 ppm respectively (their respective EC$_{50}$ values are 3100 and 3480 ppm). Thus the free radical scavenging capacity decreases in the order methanol extract > hexane extract > acetone extract. Nimbolide, isolated from the acetone extract of the leaves of *A. indica* did not show any DPPH free radical scavenging capacity even at higher concentrations. The plot of the DPPH' radical scavenging capacity of the extracts at different concentration is presented in figure 5.6.

![Figure 5.6: DPPH radical scavenging capacities at different concentrations of (□) methanol extract; (○) hexane extract and (△) acetone extract of *A. indica* leaves.](image-url)
5.4 Experimental

General experimental procedures and chemicals used are as reported in chapter 2 of this thesis. RPMI-1640 medium, glutamine, streptomycin, fetal bovine serum, penicillin, gentamicin, TPTZ and Trolox were purchased from Sigma-Aldrich. All solvents and reagents used were of spectroscopic grade.

5.4.1 Extraction

Fresh *A. indica* leaves were collected from NIIST campus. It was then thoroughly cleaned, chopped into small pieces and dried for 48 hours at 50 °C in a convection air drier. This was coarsely powdered and 450 g of this material was subjected to extraction at room temperature (27 °C) using hexane, followed by acetone and methanol. Each extract was then concentrated under reduced pressure in a rotary evaporator.

5.4.2 Isolation of nimbolide from the acetone extract

The acetone extract (21 g) was dissolved in 1:1 mixture of hexane:ethylacetate and then loaded on to a column packed with silica gel (400 g, 100-200 mesh). The column was eluted starting with 5 % ethyl acetate in hexane and then gradually increasing the polarity. A total of 162 fractions of 40-50 ml each were collected and pooled together in 22 fraction pools according to the similarities in TLC. The seventh fraction pool contained a major compound on examination by TLC. The fraction was kept for crystallization in dichloromethane-ethyl acetate mixture. 116 mg of pure white crystals of nimbolide was obtained upon recrystallisation. The structure was assigned from the spectral data as shown below:

<table>
<thead>
<tr>
<th>FT-IR (KBr, ( v_{\text{max}}/\text{cm}^{-1} ))</th>
<th>2978, 1778, 1730, 1672, 1433, 1296, 1238, 1192, 1153, 1069, 951, 827, 750</th>
</tr>
</thead>
</table>

\[ ^1H \text{ NMR (300 MHz, CDCl}_3) \]
\[ \delta 7.32 - 7.22 (3H, m), 6.26 (1H, s), 5.92 (1H, d, J = 11.8 Hz), 4.66 (1H, dd, J = 3.67, 12.5 Hz), 4.27 (1H, d, J = 3.6 Hz), 3.67 (1H, d, J = 8.6 Hz), 3.54 (3H, s), 3.23-3.16 (2H, m), 2.74 (1H, t, J = 5.6 Hz), 2.37 (1H, dd, J = 5.8, 16.2 Hz), 2.21-2.13 (2H, m), 1.70 (3H, s), 1.47 (3H, s), 1.37 (3H, s), 1.22 (3H, s) \]

\[ ^{13}C \text{ NMR (75 MHz, CDCl}_3) \]
\[ \delta 200.7, 174.9, 172.8, 149.4, 144.7, 143.0, 138.7, 136.3, 130.8, 126.4, 110.2, 88.2, 82.8, 73.3, 51.6, 50.2, 49.3, 47.6, 45.1, 43.5, 41.1, 41.0, 32.0, 18.4, 17.0, 15.0, 12.7 \]

DEPT-135 (75 MHz, CDCl3)
\[ \delta (i) 149.5, 143.1, 138.8, (ii) 41.2, 32.1 \]

FAB-MS m/z: \( 257.12 [M+1] (100) \), \( C_{15}H_{12}O_4 \), requires 256.07

m.p. (°C): 204-205 °C, lit\textsuperscript{21} 204-205 °C

\[ 5.4.3 \text{ In-vitro cytotoxicity assessment of nimbolide} \]

A stock solution of \( 2X10^{-2} \) M of nimbolide was prepared in DMSO. The stock solutions were serially diluted to obtain working test solutions with
complete growth medium (RPMI-1640 medium with 2mM glutamine, 100µg/ml streptomycin, pH 7.4, sterilized by filtration and supplemented with 10% fetal bovine serum and 100 units/ml penicillin before use) containing 50 µg/ml of gentamicin to obtain working test solutions. The working test solutions were not filtered /sterilized but microbial contaminations was controlled by addition of gentamicin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

*In vitro* cytotoxicity against human cancer cell lines was determined\(^\text{23}\) using 96-well tissue culture plates. The cells were grown in tissue culture flasks in complete growth medium at 37°C in an atmosphere of 5% CO\(_2\) and 90% relative humidity in a carbon dioxide incubator. The cell suspension of required cell density (1-2 lakhs/ml) depending on the mass doubling time of cell lines was prepared in complete growth medium for determination of cytotoxicity. Aliquots of 100 µl of cell suspension were added to each well on a 96- well tissue culture plate. The cells were incubated for 24 hours. The blank wells contained complete medium in place of suspension. Simultaneously, control experiment with positive controls containing known anticancer agent 5-fluorouracil was also carried out.

The test materials (100µl in each well) were added after 24 hours to the wells containing cell suspension and blank wells. The cells were allowed to grow in presence of test material by further incubating the plates for 48 hours. At the end of incubation period the cell growth was stopped by gently layering trichloroacetic acid (50% TCA, 50µl /well) on top of the medium in all the wells. The plates were incubated at 4 °C for 1 hour. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc and was air-dried.

The cell growth was measured by staining with Sulphorhodamine B. Sulphorhodamine B (SRB, 0.4% in 1% acetic acid, 100µl/ well) was added to each well and plates were allowed to stand at room temperature for 30
minutes. The plates were washed with 1% acetic acid four times and then dried. Tris-HCl buffer (0.01 M, pH 10.5, 100µl/well) was added to each well to solubilize the dye. The plates were shaken gently for 10mts on a shaker and the optical density was recorded on ELISA reader at 540 nm. The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in absence of any test material as 100 % and in turn percent growth inhibition in presence of test material was calculated. All the experiments were carried out in quadruplicate.

5.4.4 Trolox equivalent antioxidant capacity by FRAP method

The automated method for measuring the Ferric Reducing Antioxidant power (FRAP)\textsuperscript{19} was used for the present study. The reagents include (i) Acetate buffer, 300 mmol/l, pH 3.6, (ii) 10 mmol/l 2,4,6-Tripyridyl-s-triazine (TPTZ) in 40 mmol/l HCl and (iii) 20 mmol/l FeCl\textsubscript{3} x 6H\textsubscript{2}O in distilled water. The FRAP working solution is the mixture of the above three solutions in the ratio 25:2.5:2.5. The working solution must be always freshly prepared. 1.5 ml of the working solution was mixed with 0.1 ml of the extract. It was incubated at 37 °C for 20 min. Trolox was the standard and a standard curve was drawn by reading the absorbances at 593 nm. The results are expressed in trolox equivalents.

5.5 References


