**Introduction** (Bruguiera cylindrica)

Mangroves are productive wetland restricted to tropical and subtropical estuarine zones, serve as feeding and spawning grounds for commercial finfish and shell fishes, approximately 55 species of mangroves from 22 genera are distributed in and around Indian ocean. Considering the utility of mangrove plants, a study has been undertaken on *Bruguiera cylindrica* Blume (Rhizophoraceae) to explore the chemical entities present in the stem bark of the plant which are responsible for its medicinal properties.

**Taxonomical Classification**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Eudicots</td>
</tr>
<tr>
<td>Order</td>
<td>Malpighiales</td>
</tr>
<tr>
<td>Family</td>
<td>Rhizophoraceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Bruguiera</td>
</tr>
<tr>
<td>Species</td>
<td>cylindrica (L) Blume</td>
</tr>
</tbody>
</table>

**General description**

*Bruguiera cylindrica* is a small tree growing up to 20 metres (66 ft) tall but often grows as a bush. The bark is smooth and grey, with corky raised patches containing lenticels which are used in gas exchange and the trunk is buttressed by roots. The aerial roots or pneumatophores project from the soil in knee-shaped loops and have many lenticels which allow air into the interconnecting roots while excluding water. The roots spread out widely to provide stability in the waterlogged soil. The glossy green leaves are opposite, simple and elliptical with pointed ends. The flowers are in small bunches of 2-5 in the axils of the leaves. They have 8 long green sepals and 8 smaller, greenish-white petals with several little bristles on the tip. The flowers are pollinated by insects and release a cloud of pollen when probed at the base by the insect's mouthparts. The seed does not
detach itself from the flower stalk but germinates where it is and is known as a propagule. It grows into a slightly curved cylinder up to 15 cm (6 in) long, with the upturned calyx still attached, and looks rather like a slender, dangling cucumber.

**Common names:**

White Burma mangrove (English), Kakandan, Madama (Hindi), Kandel, Kari–kandel (Malayalam), Kakandan, Kaakkandal, Pannukkucci (Tamil), Vurada, Varavada (Telgu)

**Distribution range**

*Bruguiera cylindrica* is found in south east Asia with the range extending from India and Sri Lanka through Malaysia, the Philippines, Indonesia and Queensland, Australia. It is one of the commonest mangroves, *Bruguiera cylindrica* is found on new deposits of silt, often behind other mangroves such as *Avicennia* which are more salt tolerant. Unlike some other mangroves, its regeneration is not easy from broken off branches, Indian mangroves forests are mostly concentrated in the southwest coast of India.

**Traditional uses**

The timber of *Bruguiera cylindrica* is dense, reddish and strong and is used in construction. It burns well as firewood and can be converted into charcoal. The crushed bark has an unusual odour which is repulsive to fish and this wood is not therefore used for fish traps. Nevertheless, extracts are made from the pneumatophores which are used in the manufacture of perfume. Parts of the tree are eaten; the root tips are relished in Thailand; the bark supplies a spice and the young shoots are boiled and served as a vegetable. In Maldives the propagules are boiled and eaten as a vegetable in the islands where it grows. In traditional medicine, the skin of the fruit is used to stop bleeding and the leaves are used to lower blood pressure.

**REVIEW OF LITERATURE**

**A. Phytochemistry**

The genus of *Bruguiera* has six species and one variety which derives from *B. sexangula*, including *B. cylindrica*, *B. exaristata*, *B. gymnorrhiza*, *B. hainessi*, *B. parviflora*, *B. sexangula* and *B. sexangula* var. *rhynchopetala*, various class of compounds have been isolated from genus
Bruguiera cylindrica

Bruguiera. [2-18].hich are as follows (1) Alkaloids (2) Flavonoids, (3) Steroids (4) Lipids (5) Carbohydrates (6) Coumarins (7) Sulphur containing compounds (8) Diterpenes and triterpenes

Structures of compounds from genus Bruguiera

![]()

Tropine

![]("

Brugine

![]("

Tropine acetate

1,2-Dithiolan-4-ol,phenylcarbamate

Tropine iso-butyrate

Tropine iso-valerate

Tropine propionate

Tropine butyrate

Tropine benzoate

Bruguierol A

Bruguierol B

Bruguierol C

2,5-Hexanediol,1,3-hydroxyphenyl

1H-2-Benzopyran-6,8-diol, 3,4-dihydro-3-(3-hydroxybutyl)-1,1-dimethyl
Bruguiera cylindrica

[4,8*-Biflavan]-3,3',4,4',5,5',7,7'-octoacetoxy-
4'-[2-(3,4-dimethoxyphenyl)-3-hydroxy-5,7-dimethoxy-
4-chromanyl]-oxy]

4,8*-Biflavan]-3-diol,3',3'',4,4'',5,5'',7,7''-octamethoxy-
4''-[2-(3,4-dimethoxyphenyl)-3-hydroxy-5,7-dimethoxy-
4-chromanyl]-oxy]

4,8*-Biflavan]-3,3',4,4',5,5',7,7'-octamethoxy-
4'-[2-(3,4-dimethoxyphenyl)-3-hydroxy-5,7-dimethoxy-
4-chromanyl]-oxy]

4,8*-Biflavan]-3,3',4,4',5,5',7,7'-octoacetoxy-
4'-[2-(3,4-dimethoxyphenyl)-3-hydroxy-5,7-dimethoxy-
4-chromanyl]-oxy] pentadecacacetate

Page 109
Bruguiera cylindrica

1-D-1-O-Methyl-muco-inositol
Ellagic acid
3,3'-Dimethoxy ellagic acid

ent-Kaur-16-ene-13,19-diol
ten-Kaur-16-ene-13,19-endo-hydroxy-19-oic acid
ent-8(14)-Pimarene-1a,15R,16-triol

Isopimar-7-ene-15S,16-diol
ten-Kaur-16-ene-13,19-endo-hydroxy-19-al
Methyl-ent-Kaur-9(11)-en-13,17-epoxy-16-hydroxy-19-oate

2'-Phenanthrene-carboxaldehyde, 1,2,3,4,4a,5,5,6,7,8,8a,9-dodecahydro-2,4b,8,8-tetramethyl
1,2-Ethanediol, 1-[(2S,4aS,4bS,8S,8aS)-1,2,3,4,4a,5,5,6,7,8,8a,9-dodecahydro-2,4b,8,8-tetramethyl-2-phenanthrenyl]-diacetate
Bruguiera cylindrica

(16R)-13,17-Epoxo-16-hydroxy-ent-kaur-9(11)-en-19-al
Methyl-ent-13,17-dihydroxy-ent-kaur-9(11)-en-19-oate
16β H-17-Hydroxy-ent-kauran-19-oic acid
Methyl-ent-13,17-epoxy-16-hydroxy-kauran-19-oate
16,17-Dihydroxy-19-nor-ent-kauran-9(11)-en-3-one

Apiculol
Gramrinone
Tetracosanoic

2,6 Dimethoxy-1,4-benzoquinone
(-)-3,4-Dihydro-3-hydroxy-7-methoxy-2H-1,5-benzodithiopine-6,9-dione
Bruguiera cylindrica
Bruguiera cylindrica

$3\alpha$-E-Coumaroyltaraxerol

$3\alpha$-E-Caffeoyltaraxerol

$3\alpha$-Z-Coumaroyltaraxerol

Diosiupesin A

$3\alpha$-Z-Coumaroyllupeol

$3\beta$-E-Coumaroyllupeol

$3\beta$-Z-Coumaroyllupeol

3 β-E-Feruoyllupeol
Bruguiera cylindrica

3 β-E-Caffeoyllupenol

3 β-Z-Caffeoyllupenol

3 β-E-Caffeoyltaraxerol

3 β-Z-Caffeoyllupenol

Bruguierin A

Bruguierin B

Bruguierin C

Rhynchosides A

Rhynchosides B
**B. Pharmacology**

![Diagram showing biological activities of Bruguiera genus]

**Biological activities**

The genus *Bruguiera* is characterized by the presence of large number of compounds, many of which show broad range of biological activities. These include insect antifeedant activity, antifungal, cytotoxicity, antimalarial and antibacterial activity. Until now, more than 200 bioactive metabolites have been isolated from true mangroves of tropical and subtropical populations and large number them belong to *Bruguiera*.

**Anti cancer activity**

*Bruguiera cylindrica* has shown anticancer activity, out of the six pentacyclic triterpenoid esters isolated from the fruits, two have exhibited weak cytotoxic activity against NCI-H187 cell line, namely 3R-Z-feruloyltaraxerol and 3R-Z-coumaroyltaraxerol[2]. Bruguerin A isolated from *Bruguiera gymnorrhiza* also inhibited phorbol ester-induced NFκB (nuclear factor-kB) luciferase activation with an IC$_{50}$ value of 1.4 μM. [3]
Anti malarial activity

A new lupane caffeoyl ester, 3-(Z)-caffeoyllupeol, isolated from the fruits of *Bruguiera* parviflora, has exhibited antimalarial activity with an EC\(_{50}\) value of 8.6 mg/ml [4].

Anti diabetic activity

The enzyme PTP1B plays important role both in physiologically and pathologically regulating the signaling of the insulin receptor, studies have shown that PTP1B-deficiency displays enhanced insulin sensitivity and resistance to diet-induced obesity [19]. PTP1B thus becomes a promising target in the treatment of type-II diabetes and obesity, compounds gymnorrhizol and bruguiesulfurol from *B. gymnorrhiza* have shown promising inhibitory activity against PTP1B, with an IC\(_{50}\) value of 17.6 µM for both.

Antibacterial Activity:

The invitro antibacterial activity revealed that the methanolic extract of *B.cylindrica* has vibriocidal activity, *B. cylindrica* has low activity but has inhibited the growth of two bacteria, *V. alcaligenes* (7 mM) and *V. alginolyticus* (10 mM). [20]

Ichthyotoxic Activity

The mangroves have shown *in vitro* ichthyotoxic activity at various concentrations, *A. marina* showed 100% mortality in tilapia after 4 h at a concentration of 200µg/ml. The extract of *B. cylindrica* and *A. ilicifolius* exhibited 100% mortality at 250 and 300 µg/ml respectively. The mechanism of action of these mangroves may be due to the inhibition of nervous system of fish. It has been revealed in the studies that the extracts of mangrove bring out more or less same sort of behavioral changes in the fishes. Bandaranayake, reviewed that saponins are the main factor responsible for the piscicidal activity. From the above findings, it could be inferred that mangrove extracts can be used for the management of weed and predatory fishes. [20, 21]

Antifouling Activity:

Natural products have been used as anti- fouling agents against such organisms, many studies have been done worldwide, the methanol extract from *A. marina* followed by extracts of *B. cylindrica* and *A. ilicifolius* have shown appreciable antifouling activity against *P. vulgate* [20, 22]
**Lipid lowering activity**

*Bruguiera cylindrica* extract has exhibited lipid lowering as well as antioxidant activity in triton model, serum lipids were found to be lowered by different fractions at 200 mg/kg, b.w in triton induced hyperlipidemia, the lipid lowering action of the extract was found to be mediated through inhibition of hepatic cholesterol biosynthesis, increased faecal bile acid excretion and enhanced plasma lecithin:cholesterol acyltransferase activity. [23]

**Aim and design of work**

Large number of compounds have been isolated from the *Bruguiera cylindrica* as already evident from the literature, our aim is isolate the chemical constituents from the stem bark of *Bruguiera cylindrica* and test the isolates for their anticancer and antiparkinsonian effect.

**Present study**

The stem bark of *Bruguiera cylindrica* was identified and provided by the Division of Botany, CSIR- Central Drug Research Institute (CDRI), Lucknow (CDRI plant code No. 301). The chapter deals with the isolation of compounds from stem bark and modification of isolates acquired in quantity and biological evaluation of isolates as well as the analogues.

**Extraction, Fractionation, Isolation Procedure and characterization of compounds**

The dried and powdered plant material (stem bark) was extracted with 95% ethanol by percolation method. Solvent was evaporated under vacuum at 40°C. The ethanolic extract was fractionated into *n*-hexane (F001), chloroform (F002), Methanol (F003) and aqueous (F004) fractions. The chloroform fraction (F002) fraction was taken for detailed chemical investigation. Repeated column chromatography of F002 fraction afforded four known compounds designated as BC-1 to BC-4. The compound BC-1 was isolated in quantity and was modified to get new analogues (Scheme 1). Compounds were characterized using IR, NMR, mass and comparing the data with those reported in literature for known compounds. These were also compared with authentic samples on thin layer plates.

A summary of isolation procedure is given in flowchart- 1 and the isolated compounds are given in table-1.
Flowchart-1 Summary of extraction, fractionation and isolation procedure for *Bruguiera cylindrica* (stem bark)

Table-1: Compounds isolated from *Bruguiera cylindrica* stem bark

<table>
<thead>
<tr>
<th>Code no.</th>
<th>Molecular formula</th>
<th>Characterized as</th>
<th>Molecular wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1</td>
<td>C$<em>{30}$H$</em>{48}$O</td>
<td>Lupenone</td>
<td>424</td>
</tr>
<tr>
<td>BC-2</td>
<td>C$<em>{30}$H$</em>{50}$O</td>
<td>Lupeol</td>
<td>426</td>
</tr>
<tr>
<td>BC-3</td>
<td>C$<em>{27}$H$</em>{46}$O</td>
<td>Cholesterol</td>
<td>386</td>
</tr>
<tr>
<td>BC-4</td>
<td>C$<em>{3}$H$</em>{6}$OS$_{2}$</td>
<td>4-Hydroxy1,2 dithiolane</td>
<td>122</td>
</tr>
</tbody>
</table>
LUPENONE (BC-1)

Semi-synthetic scheme undertaken for synthesis of Lupenone derived Schiff’s base.

**SCHEME-1:** Synthesis of Lupenone derived Schiff bases

Representative procedure for the Synthesis of target compounds (K1-K10)

(a) **Synthesis of carboxamide (Intermediate)**

To a magnetically stirred solution of lupenone (1.0 mmol) in ethanol (20 ml), hydrazine hydrate (2.0 mmol) was added. The resulting reaction mixture was refluxed at 80 °C for 8 hrs, completion of reaction was monitored through TLC, the solvent was concentrated in vacuo and the residual solid was purified by column chromatography on silica gel (Eluent EtOAc/ hexane) affording the corresponding product carboxamide (yield 60% )
(b) Preparation of substituted Schiff base (General method)

To the magnetically stirred solution of carboxamide (1.0 mmol) in aprotic solvent like dimethyl sulfoxide (DMSO), different substituted aromatic aldehyde were added. The resulting reaction mixture was stirred at room temperature (25-28 °C) for 12-16 hrs, after completion of the reaction (Monitored by TLC), 50ml water was added to the mixture, shake well with glass rod for 5-10 minutes and filter the precipitated solid. The residual solid will be purified by column chromatography or crystallization method.

Characterization data for synthesized Lupenone derivatives Schiff bases

Melting points were recorded on Buchi-530 capillary melting point apparatus and are uncorrected. IR spectra were recorded on Perkin-Elmer AC-1 spectrometer. ¹H NMR spectra were run on Bruker Advance DPX 300 MHz and 200 MHz in CDCl₃. ¹³C NMR spectra were recorded at 75 MHz and 50 MHz in CDCl₃. Chemical shifts are reported as values in ppm relative to CHCl₃ (7.26) in CDCl₃ and TMS was used as internal standard. ESI mass spectra were recorded on JEOL SX 102/DA-6000. Chromatography was executed with silica gel (60-120 mesh) using mixtures of chloroform and hexane as eluents in varying ratio.

K1

![Chemical structure of K1](image)

Yield: 56%; mp: 180-182 °C; IR (KBr cm⁻¹): 3397, 2947, 2368, 1668, 1456, 1254, 1143, 1026. ¹H NMR: (300 MHz, CDCl₃) δ 7.5 (s, 1H), 7.08 (d, J = 8.49 Hz, 2H), 6.95 (s, 1H) 4.72 (s, 1H), 4.62 (s, 1H), 3.93 (s, 3H), 3.87 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-
1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H); HRMS (EI) calcd for [M+H]^+: C_{38}H_{57}N_{2}O_{2}: 573.4420 Found 573.4412.

\[ \text{NMR: } (300 \text{ MHz, CDCl}_3) \delta 7.4 (s, 1H), 6.64 (s, 2H), 4.69 (s, 1H), 4.58 (s, 1H), 3.88 (s, 3H), 3.85 (s, 6H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H). \]

HRMS (EI) calcd for [M+H]^+: C_{37}H_{54}N_{3}O_{2}: 572.4216, found 572.4198.

**K3**

\[ \text{Yield: 58%; mp: 192-194^0C; IR (KBr cm}^{-1}) : 3452, 2938, 2367, 1659, 1440, 1128, 1027. \]

\[ \text{H NMR: } (300 \text{ MHz, CDCl}_3) \delta 7.50 (s, 1H), 7.41 (d, J = 8.49 \text{ Hz}, 2H), 6.74 (d, J = 8.46 \text{ Hz}, 2H) 4.71 (s, 1H), 4.61 (s, 1H), 3.03 (s, 6H), 2.48-2.37 (m, 1H), 1.90-2.02(m, 1H), 1.75(s, 3H), 1.49-1.25(m, 24H), \]
Bruguiera cylindrica

1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H) HRMS (EI) calcd for [M + H]^+: C_{39}H_{58}N_3O_4 : 632.4427, Found 632.4419

**K4**

![Chemical structure of K4](image)

Yield: 57%; mp: 196-198 °C; IR (KBr cm⁻¹): 3477, 2957, 2365, 1598, 1251, 1029. \(^1\)H NMR: (300 MHz, CDCl₃) δ 7.48 (s, 1H), 7.42 (d, J = 8.52 Hz, 2H), 6.96 (d, J = 8.52 Hz, 2H), 4.74 (s, 1H), 4.64 (s, 1H), 3.86 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H) HRMS (EI) calcd for [M+H]^+: C_{38}H_{54}N_3 : 552.4318, Found 552.4302

**K5**

![Chemical structure of K5](image)

Yield: 49%; mp: 190-192 °C; IR (KBr cm⁻¹): 3329, 2944, 2365, 1565, 1586, 1456, 1142, 1025 \(^1\)H NMR (300 MHz, CDCl₃): δ 7.40-7.45 (m, 6H), 6.98 (m, 3H), 4.73 (s, 1H), 4.63 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H) .HRMS (EI) calcd for [M+H]^+: C_{38}H_{57}N_2O_2 : 573.4420, Found 573.4402
**Bruguiera cylindrica**

K6

\[
\text{C}_6\text{H}_4\text{H}_2\text{CO} \quad \text{H}_2\text{CO} \\
\]

(1\(E,2\)E)-1-(4-benzyloxy)-3-methoxybenzylidene)-2-(3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-2-yl)hexadecahydro-1\(H\)-cyclopenta[\(a\)]chrysene-9(5\(b\)H,10\(H\),13\(b\)H)-ylidene)hydrazine

Yield: 48%; mp: 188-190 °C; IR (KBr cm\(^{-1}\)) : 3227, 2942, 2370, 1592, 1459, 1350 \(^1\)H NMR (300 MHz, CDCl\(_3\)):\(\delta\) 8.18-8.25 (m, 2H), 7.70 (d, \(J = 9\) Hz, 1H), 7.62 (d, \(J = 9\) Hz, 1H), 7.58-7.49 (m, 1H), 4.71 (s, 1H), 4.61 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H)

HRMS (EI) calcd for [M +H]\(^+\): C\(_{45}\)H\(_{63}\)N\(_2\)O\(_2\): 663.4890, Found 663.4882

K7

\[
\]

(1\(E,2\)E)-1-(3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-2-yl)hexadecahydro-1\(H\)-cyclopenta[\(a\)]chrysene-9(5\(b\)H,10\(H\),13\(b\)H)-ylidene)-2-(3,4,5-trimethoxybenzylidene)hydrazine

Yield: 44%; mp: 170-172 °C; IR (KBr cm\(^{-1}\)) 3279, 2936, 2365, 1656, 1473, 1380; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.53 (d, \(J = 9\) Hz, 2H), 7.40 (s, 1H), 7.26 (d, \(J = 9\) Hz, 2H), 4.71 (s, 1H), 4.61 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H); HRMS (EI) calcd for [M +H]\(^+\): C\(_{40}\)H\(_{61}\)N\(_2\)O\(_3\): 617.4682, Found 617.4670
**K8**

\[
\text{Bruguiera cylindrica}
\]

![Chemical Structure](image)

*4-((E,)(E)-3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-2-yl)hexadecahydro-1H-cyclopenta[a]chrysene-9(5bH,10H,13bH)-ylidene)hydrazono)methyl)*N,N-dimethylaniline

Yield: 55%; mp: 184-186 °C; IR (KBr cm\(^{-1}\)) 3288, 2942, 2363, 1669, 1457. \(^1\)H NMR (300 MHz, CDCl\(_3\)): δ 7.43 (d, J = 6 Hz, 2H), 7.38 (d, J = 6 Hz, 2H), 7.32 (s, 1H), 4.73 (s, 1H), 4.63 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H) HRMS (EI) calcd for [M+H] \(^+\): C\(_{39}\)H\(_{60}\)N\(_3\) : 570.4787, Found 570.4779

**K9**

\[
\text{(1E,2E)-1-(2,4-dimethoxybenzylidene)-2-(3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-2-yl)hexadecahydro-1H-cyclopenta[a]chrysene-9(5bH,10H,13bH)-ylidene)hydrazine}
\]

Yield: 50%; mp: 190-192 °C; IR (KBr cm\(^{-1}\)): 3260, 2957, 2369, 1660, 1462, 1115. \(^1\)H NMR (300 MHz, CDCl\(_3\)): δ 8.28 (d, J = 9 Hz, 2H), 7.5 (d, J = 9 Hz, 2H), 7.49 (s, 1H), 4.73 (s, 1H), 4.63 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H) HRMS (EI) calcd for [M+H] \(^+\): C\(_{39}\)H\(_{59}\)N\(_2\)O\(_2\) : 587.4577, Found 587.4568
Bruguiera cylindrica

K10

\[
(1E,2E)-1-(2,6-dichlorobenzylidene)-2-(3a,5a,5b,8a,11a-hexamethyl-1-(prop-1-en-2-yl)hexadecahydro-1H-cyclopenta[cd]chrysene-9(5bH,10H,13bH)-ylidene)hydrazine
\]

Yield: 40%; mp: 174-176\(^{0}\)C; IR (KBr cm\(^{-1}\)): 3273, 2946, 2363, 1673, 1456. \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta\) 7.40 (m, 2H), 7.10 (m,3H), 4.71 (s, 1H), 4.61 (s, 1H), 2.48-2.37(m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17(s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H) HRMS (EI) calcd for [M + H]\(^{+}\): C\(_{37}\)H\(_{53}\)Cl\(_2\)N\(_2\) : 595.3586 , Found 595.3572.

**BIOLOGICAL ACTIVITY: ANTICANCER**

Homeostasis [24] is the basis which keeps a human healthy and in working condition, when this process is disturbed in the body for any metabolic route, it is bound to effect the concern organ, same is true in case of cancer and is signified by either increased cell proliferation rate or a decrease in cell death i.e: imbalance occurs between cell proliferation and cell death, conditions favouring its initiation and progression are a) activation of proto-oncogenes b) inactivation of tumor suppressor genes c) alteration of repair genes, which normally keep genetic alterations in check.[25] Cancer is a curse for humanity as it seems to be unmanagable if detected at later/advance stage and in most of the cases it is detected at progressive stage, drugs administered at this juncture are able to hamper its progression and prolong the life span of the patient from few months to few years, currently large number of drugs are available in the market which are able to curb the disease if detected at an early stage, treatment available today are all very costly and in a country like India an average man is unlikely to afford the treatment as an average cost of one cycle of chemotherapy is 40 to 50 thousand and generally 12 cycles of chemotherapy are considered in treatment, depending on the type of cancer so need to develop anticancer drugs that
are cost effective, potent enough to be effective at progressive stage of the disease and non toxic, so in order to search and develop an effective, non–toxic entity, we pursued phytochemical analysis of *Bruguiera cylindrica* which already has proven anticancer activity.

Out of the four compounds isolated from the CHCl$_3$ fraction, three were obtained in traces while one (BC-1) was isolated in reasonably good quantity, it was derivatised to give ten molecules which belong to class Schiff bases, were subjected to analysis for Breast and Prostrate cancer cell lines, the rationale for choosing these two types of cancer lies in the fact that these are most prominent types of cancer likely to effect women [26,27] and men[ 28,29] respectively in their 40’s and 50’s.

The analyzed results were found to be encouraging for Breast cancer cell lines as four compounds from the series exhibited antiproliferative activity at a concentration below 50µM which can be considered pretty heartening for semi synthetic entities, compound K5 showed the best activity so it was pursued and was further evaluated for various parameters like ROS, mitochondrial membrane potential ,caspase activity and western blot analysis in order to determine the apoptotic pathway followed.

**Anti-proliferative activity**

The antiproliferative activity of Schiff bases were determined through MTT assay.[30] Antiproliferative activity of all the compounds were evaluated for Breast cancer cell lines (MCF-7 and MDA-MB-231) and Prostrate cancer cell lines (PC-3 and DU-145). $1 \times 10^4$ cells/well were seeded in 96-well microculture plates in 100 µL DMEM, media supplemented with 10% FBS in each well and incubated in a CO$_2$ incubator for 24 h at 37 °C, all the compounds were diluted to the desired concentrations and added in the culture medium in the wells with respect to the vehicle control. After 48 h of incubation, media were removed and 100 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and the plates were further incubated for 4 h. Supernatant was removed carefully from each well, formazan crystals were dissolved in 100 µL of DMSO and the absorbance were recorded at 540 nm wavelength.

**Analysis of mitochondrial membrane potential (MMP).**

The MMP was measured by the uptake of unique fluorescent cationic dye, JC-1 (excitation at
488 nm and emission at 525 nm), to signal the loss of MMP. [31] This fluorescent probe exists as a green fluorescent monomer (emission 527 nm) at low MMP. Mitochondrial depolarization is indicated by an increase in green fluorescence (FL-1). The MCF-7 cells (0.2 × 10^6 cells) were plated in a 6-well plate, exposed to compounds at varying concentrations for 36 h, washed and finally harvested in chilled PBS containing JC-1 (1 µM). The samples were incubated at 37°C for 30 min in the dark, washed twice with chilled PBS and finally resuspended in 200 µL PBS. Mitochondrial permeability transition was subsequently quantified on FACS.

**Measurement of intracellular ROS**

For analysis of intracellular ROS, the oxidation-sensitive probe DCF-DA was used, to analyze the net intracellular generation of ROS by flow cytometry,[32] cells were detached by trypsinization after incubation for 36 h of incubation in the absence or presence of the different compounds at varying concentrations. The cellular fluorescence intensity was measured after 30 min incubation with (DCFDA 10 µM) by using the same flow cytometer described above.

**Caspase induction assay**

The homogeneous caspase assay kit (Roche, Germany) that includes caspases 2, 3, 6, 7, 8, 9 and 10 was used to measure caspase activities after apoptosis induction according to manufacturer’s instructions. Camptothecin treated U937 cells were used as positive control. MCF-7 cells were cultured under serum-deprived conditions for 4 h. Compound treatment was given for 24 h at various concentrations. Cells were then incubated with DEVD-rhodamine-110 (tetra-peptide sequence ‘aspartic acid–glutamic acid–valine–aspartic acid’ recognized by caspases). Upon cleavage of the rhodamine substrate by activated caspases, fluorescence from the released rhodamine-110 was measured.

**FACS ANALYSIS**

The percentage of cells undergoing apoptosis were determined using Annexin V FITC Assay kit (BD Biosciences). Annexin V, a Ca^2+ dependent phospholipid-binding protein, has high affinity for phosphatidylserine (PS), and fluorochrome-labeled Annexin V is used for the detection of exposed PS and Propidium Iodide (PI) for the differentiation from necrotic cells using flow cytometry. The MCF-7 cells were cultured and treated with test items for 24hr. At the end of this
time period cells were washed twice with PBS, and centrifuged at 1200 rpm for 5 min. Afterwards cell pellet was treated with 5 µl Annexin V and 5 µl of PI, incubated for 15 min. in dark. Finally 200 µl of binding buffer was added to the cell pellet and analyzed by flow cytometry (BD Biosciences)

**Western Blot Analysis**
Equivalent amounts (20 µg) of total protein was loaded onto 12% SDS-PAGE. The gels was transferred to nitrocellulose membrane using an electro-blotting apparatus (Bio-Rad, Richmond, CA) and reacted with each primary antibody. Blots were washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody. The membranes were developed with ECL reaction and analyzed using LAS-3000 Luminescent Image Analyzer (FujiFilm, Tokyo, Japan). We used Image Gauge Ver. 3.0 software to calculate changes in protein expression. β-Actin was used as internal control.

**Results**

**Antiproliferative activity of K5 against MCF-7 and MDA-MB-231.**

Compounds K5 exhibited appreciable antiproliferative activity in both the breast cancer cell lines (MCF-7 and MDA-MB-231) and the compound was active in comparison to control. IC$_{50}$ (Table-2) for MCF-7 was 14.9 µM (Fig.1) and for MDA-MB-231 was 18.4 µM (Fig. 2) while the Lupenone is effective at IC$_{50}$ > 50µM in both cases (data not shown), thus bases have been found to be more effective and this prompted us to further assess other parameters.

**Table-2: IC$_{50}$ (in µM) of compounds (Schiff bases) for various cancer cell lines.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound</th>
<th>MCF-7</th>
<th>MDAMB-231</th>
<th>PC-3</th>
<th>DU-145</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K1</td>
<td>23.2</td>
<td>18.1</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2</td>
<td>K2</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>3</td>
<td>K3</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>4</td>
<td>K4</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td></td>
<td>K5</td>
<td>14.9</td>
<td>18.4</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>5</td>
<td>K6</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>6</td>
<td>K7</td>
<td>16.8</td>
<td>22.4</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>7</td>
<td>K8</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>8</td>
<td>K9</td>
<td>19.4</td>
<td>43.1</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>9</td>
<td>K10</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

**Figure.1** IC$_{50}$ of compound (K5=14.9 µM) for MCF-7 cell line.
**Figure.2** IC\textsubscript{50} of compound (K5=18.4 μM) for MDA-MB-231 cell line.

**Effect of compound (K5) on Mitochondrial membrane potential (MMP).**

The compound K5 exhibited decreased mitochondrial membrane potential with increase in the concentration of the compound which is reflected by decrease in mean fluorescence (FL-1) with respect to control (Fig. 3). (MCF-7 and MDA-MB-231 displayed basal fluorescence indicating functional mitochondria), loss of MMP is directly proportional to membrane permeability which may be due to ROS generation.

**Figure.3** For the assessment of loss in MMP, trypsinized cells were incubated with the fluorescent cationic dye, JC-1 (excitation at 488 nm and emission at 525 nm) (1 μM) for 30 min at 37°C in the dark, washed twice with chilled PBS and finally resuspended in 200µL PBS. The mitochondrial permeability transition was subsequently quantified on FACS. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01, ***P < 0.001(as compared to control cells).

**Determination of ROS level**

As mentioned above, compound treatment resulted in MMP loss, it may be due to ROS generation so it becomes imperative to assess intracellular ROS, results reveal an increase in DCFDA fluorescence on treatment of MCF-7 (Fig.4) and MDA-MB-231(Fig.5) with compound K5 in comparison to control at higher concentration, so now it becomes evident that compound K5 generated ROS which caused loss of mitochondrial membrane potential in both cell lines.
Figure 4 For measuring ROS, the MCF-7 cells were exposed for 36h at different concentration (10, 15, 20 µM), washed twice with chilled PBS, followed by incubating the cells with 10µM DCFDA fluorophore for 30 min at 37°C in the dark, washed twice with chilled PBS and trypsinized. Finally, the stained cells were analyzed through flow cytometry. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05 (as compared to control cells).

Figure 5 The MDA-MB-231 cells were exposed for 36h at different concentration (14, 18, 22 µM), washed twice with chilled PBS, followed by incubating the cells with 10µM DCFDA fluorophore for 30 min at 37°C in the dark, washed twice with chilled PBS and trypsinized.
Finally, the stained cells were analyzed through flow cytometry. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01 (as compared to control cells).

**Caspase induction**

Caspases are cysteine proteases which are activated if the apoptosis is caspase dependent therefore in order to assess the path by which compound (K5) mediates apoptosis, measure of induction of caspase is quintessential, MCF -7 and MDA-MB-231 cells on treatment with K5 for 24 h with increasing concentration and later incubation with DEVD-rhodamine-110, activated caspases, cleave rhodamine substrate, fluorescence was measured at 560 nm from the released rhodamine-110, compound exhibited significantly induced basal caspase level in both the cell lines signaling for caspase dependent apoptosis.(Fig.6)

![Graph showing Induction of Caspase](image)

**Figure.6** Cells (MCF-7 and MDA-MB-231) were treated with compounds at various concentrations (10µM, 15 µM, 20µM) for 24 h and then incubated with DEVD-rhodamine-110. Fluorescence from the activated caspases-mediated release of rhodamine-110 was measured at 560 nm. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05 (as compared to control cells)
Assessment of apoptosis by Flow cytometry

After ascertaining the fact that apoptosis brought about by compound K5 is caspase mediated, the percentage of cells going into apoptosis is determined by FACS. Histogram represents percentage of apoptotic cells for MCF-7(Fig.7) at three different concentrations (10µM, 15µM and 20µM) and MDA-MB-231(Fig.8) cells after treatment with compound (K5) for 24 h at concentrations (14µM, 18µM, 22µM). Control indicate of untreated cells.

**Figure.7** Percentage of apoptotic cells at various concentration. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01, ***P < 0.001 (as compared to control cells).
**Figure.8** Percentage of apoptotic cells at various concentration. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01***P < 0.001 (as compared to control cells)

**WESTERN BLOTS**

The western blot analysis demonstrated that in both the breast cancer cell lines, pro-apoptotic proteins like Bax and p53 were upregulated and anti-apoptotic proteins like Bcl-2 were down regulated.

![Western Blot Images](image)

**Figure. 9** Role of concentration dependent expression. MCF-7 and MDA-MB-231 cells were treated with compounds at various concentration, (10, 15, 20 µM) equal amount (20 µg) was loaded on SDS–polyacrylamide gel, β-Actin was used as control.

The results are identical to the results of previous chapter (anticancer analysis of Chalcones), reason being the starting material in that chapter was Lupeol and in current chapter, the starting material is its oxidized form, Lupenone. Schiff bases have shown better potency in comparison Chalcones and four out of ten molecules are active against both the breast cancer cell lines which can be considered a decent frequency.
BIOLOGICAL ACTIVITY: ANTI-PARKINSONIAN EFFECT

Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that currently affects nearly 2% of the world population. In most populous countries, an estimated 4 million individuals on an average above 50[33, 34] had PD in 2005, and this number is likely to reach 9 million by 2030. PD [35-40], characterized by the loss of dopaminergic cells in the substantia nigra, displays the presence of protein aggregates known as Levy bodies (LBs) composed mainly of α-synuclein, a soluble neuronal protein that in pathological conditions evolves into insoluble oligomer. Clinically, people affected by PD display four signature symptoms: rigidity, tremor, dystonia, and bradykinesia, and occasionally akinesia. Physiologically, these symptoms result from a progressive loss of motor function due to the degeneration of the dopaminergic (DAergic) neurons within the substantia nigra, pars compacta. Epidemiologically, PD is classifies into familial (FPD) or IPD, depending on whether the disease is hereditary (FPD) or from unknown origin, possibly due to exposure to environmental neurotoxicants (IPD). Eleven genomic regions (PARK1 to 11) have been associated with FPD, eight of them have been identified as PARK1 (α-SYNUCLEIN), PARK2 (PARKIN), PARK4 (α-SYNUCLEIN), PARK5 (UCHL1), PARK6 (PINK1), PARK7 (DJ1), PARK8 (DARDARIN/LRRK2), and PARK9 (ATP13A2) On the other hand, various environmental contaminants were suspected in IPD cases, especially pesticides and metals.

In view of the above facts and no specific drug to inhibit the progression of PD at the later stage, without incurring side effects, our lab is currently involved in evaluating various natural products for the same, here we are presenting the findings of *Bruguiera cylindrica* Blume.[20] mangrove plant of the family Rhizophoraceae, native to many countries of southern and eastern Africa, Asia, and northern Australia. Previous studies have reported the presence of a wide range of phytochemicals, including phenol, flavonoid, steroid, sulfur-containing, and terpenoids[10,41-44] in fruit and bark [45] of this plant, its roots and leaves have been used for treating burns, it also exhibited anti-tumor activity against HepG2 hepatoma cells, antibacterial activity against Lactobacillus acidophilus and Bacillus subtilis, and termicidal activity against Coptotermes formosanus, respectively is found active in PD model.
Brief Considerations on Age-Related Effects

Oxidative stress and mitochondrial impairment leading to cell death are not specific to PD-associated neurodegeneration, as they naturally occur with aging. Aging is associated with mitochondrial iron overload, respiratory chain oxidative damage, increased free radical production, decrease in antioxidant response, DNA-repairing capabilities and increase in oxidation of lipids, proteins and nucleic acids. This sensitive state of the aging brain makes it more vulnerable to oxidative stress or other injuries, such as seizures. Similarly, it probably explains the prevalence of PD in elderly people.

C. elegans, α-Synuclein and PD

α-Synuclein is a presynaptic protein that is associated with synaptic vesicles as well as involved in excitation-secretion coupling. α-synuclein participates in the regulation of both DA biosynthesis and DA transporter (DAT) function. PD-associated mutations (A30P and A53T) in α-synuclein alter its structure and results in formation and deposition of Lewy body, reducing DAergic neuron viability. α-Synuclein is normally degraded by a proteosomal pathway and by a lysosomal pathway mainly involving cathepsin D, casein kinase 2. Several studies have proved that a link persist between oxidative damage and formation of α-synuclein aggregates, a known feature of PD. The nematode Caenorhabditis elegans (C. elegans) does not normally express α-synuclein but overexpression of wild type (wt) human α-synuclein in C. elegans increases the vulnerability to mitochondrial complex-I inhibitors like rotenone, fenperoximate, which is reversed by treatment with free radical scavangers. Transgenic worms overexpressing mutant A30P or A53T human α-synuclein in DAergic neurons show accumulation of α-synuclein in the cell bodies and neurites of the neurons along with reduced neuronal DA content, altered DAergic neuron function and worm behavior, which is restored with the administration of dopamine. Recently, C. elegans study confirmed that α-synuclein is involved in synaptic vesicle recycling and that the endocytic pathway plays a key role in α-synuclein neurotoxicity. As observed in mammalian models, neuroprotectants, such as acetaminophen, were found to be effective in dealing with DAergic neuronal loss in nematodes. Though C. elegans does not exhibit PD, but the findings emphasize its utility and relevance as a model organism to gain insights into the genetic pathways involved in PD and its application to search for new anti-PD drugs through high-throughput screening method.
Four known compounds have been isolated from the chloroform fraction of the stem bark of the plant, B.C-1 was isolated in large amount (1gm), which paved the way for its derivatization.

![Chemical structures of compounds isolated from Bruguiera cylindrica](image)

**Figure.10** Structure of compounds isolated from *Bruguiera cylindrica*.

**Representative procedure for the Synthesis of target compounds (K002-K006)**

To a magnetically stirred solution of lupenone (B.C-1) (0.424 g, 1.0 mmol) in ethanol (20 ml), different substituted benzaldehydes (1.0 mmol) and NaOH (1.0 mmol) were added. Reaction mixture was stirred for 24 hr at room temperature. After completion of reaction (TLC monitoring) ethanol was evaporated under reduced pressure. The compound was extracted with chloroform. The combined organic extract was washed with water, brine solution, dried (Na$_2$SO$_4$) and solvent was removed under reduced pressure. The crude product obtained was column chromatographed (60-120 mesh, SiO2) using hexane and chloroform in different concentration (ranging from 10% to 80% chloroform in hexane) to give the target compounds. (Scheme 2). These chalcone derivatives were synthesized in moderate to good yield (Table 3).
**Table-3: Differently substituted chalcone derivatives of lupenone.**

<table>
<thead>
<tr>
<th>Compound No:</th>
<th>R(^1)</th>
<th>R(^2)</th>
<th>R(^3)</th>
<th>R(^4)</th>
<th>R(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K002</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>K003</td>
<td>H</td>
<td>O CH(_3)</td>
<td>O CH(_3)</td>
<td>O CH(_3)</td>
<td>H</td>
</tr>
<tr>
<td>K004</td>
<td>H</td>
<td>OCH(_3)</td>
<td>OCH(_3)</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>K005</td>
<td>H</td>
<td>OCH(_3)</td>
<td>C(_6)H(_5)OCH(_2)</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>K006</td>
<td>H</td>
<td>H</td>
<td>NO(_2)</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

**Biological Materials and Methods**

*C. elegans* culture and maintenance

Worm culture and maintenance were the same as previously described. [46-47] In brief, worms were cultivated on Nematode growth medium (NGM) agar plates seeded with *E.coli* - OP50 bacteria by use of standard techniques at 22°C. NGM media was prepared by adding 50mM Sodium chloride (Merck), 2.5gL\(^{-1}\) Peptone (Sigma), 17 gL\(^{-1}\) Agar (Hi-media) in 975ml double distilled water and autoclaved for 30 to 40 minutes at 15lb/inch\(^2\). After the cooling of media to 50- 60 °C, 5\(\mu\)gml\(^{-1}\) cholesterol solution (Sigma) prepared in ethanol, 1mM Calcium chloride...
Bruguiera cylindrica

(Sigma), 1mM Magnesium Sulphate (Sigma) and 25mM Potassium dihydrogen phosphate (SRL) additives were added. The wild-type strain was N2 Bristol. The following transgenic strains were used: NL5901 (P\text{unc-54}::alphasynuclein::YFP+unc-119; expressing human alpha synuclein protein with YFP expression in the muscles) and TJ356 (integrated DAF-16:: GFP roller, expressing GFP with DAF-16 were used. These strains were obtained from the \textit{Caenorhabditis} Genetics Center (University of Minnesota, USA).

**Obtaining synchronous nematode population by embryo isolation**

For the isolation of embryos from various strains of \textit{C. elegans}, worms were synchronized by alkaline sodium hypochlorite bleaching method. In this method, worms after being washed with M-9 buffer were treated with axenizing solution (2mL of sodium hypochlorite and 5mL of 1M sodium hydroxide solution)[48] and then subjected to intermittent vortexing. Thereafter, the worms were washed with M-9 buffer, twice, so as to obtain worms in their synchronized embryo stage.

**Treatment of worms with natural product compounds**

In the experiment, 2mg natural product compound was dissolved into 100µl ethanol. This compound was further diluted in equal volume in OP50 (25 µl compound + 25 µl OP50) before seeding onto 12 well NGM plates. The plates were incubated overnight at 37°C for optimum growth of bacteria OP50. Age synchronized worms were grown on the plates, for further studies.

**Assay for analysis of alpha synuclein aggregation**

Analysis of alpha synuclein was carried out as described previously[49] In brief, worms were grown on control and treated with different natural compounds for 48 hrs, washed with M9 buffer to remove adhering bacteria and immobilized with 100mM sodium azide (Sigma, cat no. 71289) onto 2 % agar padded slides and sealed with a cover slip. Imaging of live (immobilised) worms using confocal microscopy (Carl Zeiss) was carried out to monitor the aggregation of alpha synuclein protein which was further quantified by measuring flourescence intensity using image J Software (Image J, National Institutes of Health, Bethesda, MD). At least 20-30 animals were analyzed on at least 3 separate days, and results were consistent between experiments.
**Assay for analysis of dopamine signaling using nonanol repulsion assay.**
Dopamine signaling plays an important role in the regulation of motor function. Therefore, impaired dopamine signaling reduces responses to any volatile attractants or repellents. To evaluate the effect of compounds on the DA-related functions, we employed the odor based repellent assay using 1-nonanol following a method described previously [50]. In this assay, we studied the response of control and compound treated worms against 1-nonanol. In case of worms with altered DA function, have a delayed repulsion response against the nonanol whereas healthy worms have a quick repulsion response against this repellent. After treatment of 48 hrs, worms were washed off the culture plates and cleaned thrice with M9 buffer. A drop of 1-nonanol was placed near the head of a worm and the response time of worms against the repellent was recorded for ten worms per treatment condition. The experiment was repeated twice and the mean response time ± SE for each group was calculated and the statistical significance was evaluated by Student’s t-test.

**Estimation of Reactive Oxygen Species (ROS)**
To investigate the anti-oxidant properties of compounds, we conducted 2, 7-dichlorodihydrofluorescein-diacetate (H2-DCF-DA) (Cat. No – D399, Invitrogen) assay using standard protocol.[51] In this assay, control and natural product compound treated worms of N2 strain were washed twice using PBS after washing with M-9 buffer. 100 µl volume of worm suspension having an approximately 100 worms were transferred into the wells of a 96-well plate replica of three. After this, 100µl of 100µM H2DCFDA dye was added to each well and basal fluorescence was quantified immediately using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Plate was incubated for 1 hr and then second measurement was quantified. We further calculated fluorescence per worm by dividing the delta by number of worms after subtracting initial fluorescence from the final reading. Experiments were conducted two times with three samples each time.

**Analysis of daf-16 nuclear localization**
For analysis of daf-16 expression, worms were treated with different compounds, fluorescent microscopy was performed using transgenic strain TJ356 which expresses GFP with DAF-16. In this assay, the embryos of TJ356 strain were obtained by alkaline sodium hypochlorite method of
Bruguiera cylindrica

embryo isolation. These embryos were then placed on control and treated plates. After 48hrs incubation, these worms were washed off with M-9 buffer and then mounted on to the slides after immobilizing them with sodium azide. The slides were then analyzed using a 40x objective of the fluorescence microscope (Carl Zeiss) equipped with a digital camera.

Data Analysis

All graphs show the mean ± standard error of the mean of at least two independent experiments. Statistical analyses were carried out employing Student’s t test using Graph Pad prism 5 software packages. Statistical significance was accepted only when *P < 0.05.

Results:

Test compounds decreased aggregation of alpha synuclein protein in transgenic NL5901 strain of C.elegans

In the present study we employed transgenic C. elegans models - NL5901 expressing ‘human’ alpha synuclein protein tagged with yellow fluorescent protein [NL5901 (Punc-54::alphasynuclein::YFP+unc-119)] in the body wall muscle. In subsequent studies, we explored the role of different test compounds on alpha synuclein aggregation. Worms treated with different compounds were analyzed using confocal microscopy. We further quantified the images for fluorescence intensity of α synuclein aggregation using Image J software. Out of five compounds tested from B.C, compound (B.C-1) significantly reduced aggregation of alpha synuclein protein, B.C -1 was further derivatized to give K002, K003, K004, K005, and K006. In case of worms treated with test compound, B.C-1 and K002 showed mean fluorescence intensity 10.70 ± 1.090 and 10.77 ± 0.7774 arbitrary units, showing maximum reduction of 2.1 (p<0.01), and 2.1 (p<0.001), folds when compared to untreated worms (Fig. 11). On the other hand, worms fed with compound K003, K004, K005, and K006 displayed moderate reduction by 1.4, 1.4, 1.9, and 1.6 folds showing mean fluorescent intensity 16.12 ± 0.6226,16.28 ± 1.019,12.26 ± 0.3508 and 14.39 ± 2.616 arbitrary units, respectively, in comparison to control worms (Fig. 12).
Graphical representation for fluorescence intensity of alpha synuclein aggregation as quantified using Image J software, in the NL5901 strain of *C. elegans* treated with different test compounds.

**Figure. 11** Alpha synuclein aggregation in NL5901 strain of *C. elegans* treated with different test compounds. *p < 0.05, **p < 0.01, ***p < 0.001.

Effect of the test compounds on alpha synuclein protein aggregation in NL5901 strain of *C. elegans*. 
**Bruguiera cylindrica**

**Figure. 12** Graphical representation for fluorescence intensity of aggregation of the nematodes as quantified using Image J software. *p < 0.05, **p < 0.01, ***p < 0.001.

**B.C-1 and K002 compound treated worms exhibited increased dopamine Signalling**

Among the various compounds evaluated, two compounds showed beneficial effects in reducing the aggregation of alpha synuclein by more than 2 folds, therefore B.C-1 and K002 compound were further checked for their effect on dopamine signaling. The nonanol assay was carried out in NL5901 strain in order to determine this signaling. It is an indirect assay, in which repulsion time for the worm towards nanonol was monitored. In this assay, worms with normal dopamine content showed early response but the worm with altered dopamine content exhibited delayed response towards nonanol. In our studies, worms treated with B.C-1 and K002, the response time was significantly increased by 3 folds (p<0.01) and 1.5 folds (p<0.05) respectively, as compared to untreated worms; displaying increased dopamine content. (Fig 13).

**K002 and B.C-1 compound treated worms exhibited increased dopamine content**

![Graphical representation for fluorescence intensity](image)

**Figure. 13** Effect of different compounds on dopamine content as estimated by nonanol repulsion assay in NL5901 strain of C.elegans. *p < 0.05 , ** p < 0.01, NS - Not significant

**Compound B.C-1 increased localization of DAF-16:: GFP in C. elegans**

To further find out the mechanistic role of identified compounds on Parkinson’s disease, we focused on daf-16/FOXO pathway via evaluating the expression of daf-16 protein using a
transgenic strain TJ356 of *C. elegans*. Since, PD is an age related disorder and the cytoplasmic retention of DAF-16 is a major determinant of longevity. Here, we studied the translocation of *DAF-16*/Forkhead transcription factor from cytoplasm to the nucleus. In the present studies, we observed increased GFP intensity for *DAF-16* expression in the case of worms treated with B.C-1 as compared to the worm raised on normal *E. Coli* OP50 diet. Figure 5 depicts the representative images for control (fig. 14A) and B.C-1 treatment (fig. 14C). But, no significant effect was observed in K002 (fig. 14B) treated worms, indicating that this compound might not be acting through this pathway. Furthermore, evidence also implicated that neuroprotective effects of B.C-1 might be associated with daf-16 pathway.

**Compound B.C-1 showed increased expression of DAF-16 protein**

![Image](image_url)

**Figure. 5** Daf-16 expression in TJ356 strain of *C. elegans* fed with OP50 (A), treated with compound K002 (B) and B.C-1 (C). Scale bar, 50 μm.

**Antioxidant properties were displayed in worms treated with B.C-1 compound.**

To further evaluate the role of the two compounds, which showed more than 2 folds reduced aggregation of alpha synuclein protein; anti-oxidative properties were determined employing H2DCFDA assay using wild type N2 strain of *C. elegans*. Worms treated with compounds B.C-1 showed 1.8 folds (p<0.01) reduction in oxidative stress as compared to worms fed onto control diet. (Fig6). The results demonstrated the non-significant levels of ROS with respect to control but in case of B.C-1 treated worms in wild type N2 strain of *C. elegans*, results were significant, clearly indicating the fact that B.C-1 possesses free radical scavenging property.
**B.C-1 treated worms showed reduced oxidative stress**

![Graph showing reduced oxidative stress](image)

**Figure 15** Effect of various compounds on Relative formation of reactive oxygen species (ROS) measured by H2DCFDA assay in N2 strain of C. elegans. **p < 0.01, NS - Not significant.**

None of the compounds treated worms increased ACh availability in N2 strain of *C. elegans*

No test compound demonstrated increase in ACh availability.

![Graph showing ACh availability](image)

**Figure 16** Effect of the test compounds on ACh availability using aldicarb assay in N2 strain of *C. elegans*. *p < 0.05, NS - Not significant
Physicochemical data of compounds K002 to K006

Melting points were recorded on Buchi-530 capillary melting point apparatus and are uncorrected. IR spectra were recorded on Perkin-Elmer AC-1 spectrometer. $^1$H NMR spectra were run on Bruker Advance DPX 300 MHz and 200 MHz in CDCl$_3$. $^{13}$C NMR spectra were recorded at 75 MHz and 50 MHz in CDCl$_3$. Chemical shifts are reported as values in ppm relative to CHCl$_3$ (7.26) in CDCl$_3$ and TMS was used as internal standard. ESI mass spectra were recorded on JEOL SX 102/DA-6000. Chromatography was executed with silica gel (60-120 mesh) using mixtures of chloroform and hexane as eluents in varying ratio.

**K002**

![Structure diagram](image)

(Yield: 65%; mp: 164-166$^\circ$C; IR (KBr cm$^{-1}$) 3288, 2942, 2363, 1669, 1457.$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.43 (d, $J = 6$ Hz, 2H), 7.38 (d, $J = 6$ Hz, 2H), 7.32 (s, 1H), 4.73 (s, 1H), 4.63 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H). HRMS (El) calcd for [M+H]$^+$: C$_{37}$H$_{52}$ClO : 547.3707, Found 547.3738

**K003**

![Structure diagram](image)

(E)-3a,5a,5b,8,8,11a-hexamethyl-1-(prop-1-en-2-yl)-10-(3,4,5-trimethoxybenzyldiene)octadecahydro-1H-cyclopenta[a]chrysene-9(5bH)-one
**Bruguiera cylindrica**

Yield: 68%; mp: 140-1420C; IR (KBr cm⁻¹): 3452, 2938, 2367, 1659, 1440, 1128, 1027.\(^1\)H NMR: (300 MHz, CDCl₃) δ 7.4 (s, 1H), 6.64 (s, 2H), 4.69 (s, 1H), 4.58 (s, 1H), 3.88 (s, 3H), 3.85 (s, 6H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H). HRMS (EI) calcd for [M+H]\(^+\): C\(_{40}\)H\(_{59}\)O\(_4\) : 603.4413, found 603.4369

\[\text{K004}\]

\begin{center}
\includegraphics[width=0.5\textwidth]{image1.png}
\end{center}

Yield: 66%; mp: 160-1620C; IR (KBr cm⁻¹): 3397, 2947, 2368, 1668, 1456, 1254, 1143, 1026. \(^1\)H NMR: (300 MHz, CDCl₃) δ 7.5 (s, 1H), 7.08 (d, \(J = 8.49\) Hz, 2H), 6.95 (s, 1H), 4.72 (s, 1H), 4.62 (s, 1H), 3.93 (s, 3H), 3.87 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.82 (s, 3H). HRMS (EI) calcd for [M+H]\(^+\): C\(_{39}\)H\(_{57}\)O\(_3\): 573.4308, Found 573.4293.

\[\text{K005}\]

\begin{center}
\includegraphics[width=0.5\textwidth]{image2.png}
\end{center}
**Bruguiera cylindrica**

Yield: 59%; mp: 190-192 °C; IR (KBr cm\(^{-1}\) ):3239, 2944, 2365, 1665, 1586, 1456, 1142, 1025.

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.40-7.45 (m, 6H), 6.98 (m,3H), 4.73 (s, 1H), 4.63 (s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10(s, 3H), 1.02 (s, 3H),0.82 (s, 3H). HRMS (EI) calced for [M+H]\(^+\):C\(_{45}\)H\(_{61}\)O\(_3\) : 649.4621, Found 649.4600

**K006**

![Structure](image)

\((E)\)-3a,5a,5b,8,8,11α-hexamethyl-10-(4-nitrobenzylidene)-1-(prop-1-en-2-yl)octadecahydro-1H-cyclopenta[α]chrysene-9(5bH)-one

Yield: 60%; mp: 186-188 °C; IR (KBr cm\(^{-1}\) ):3260, 2957, 2369, 1660, 1462, 1115 \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 8.28 (d, \(J = 9\) Hz, 2H),7.5(d, \(J = 9\) Hz, 2H),7.49 (s, 1H), 4.73 (s, 1H), 4.63(s, 1H), 3.91 (s, 3H), 2.48-2.37 (m, 1H), 1.90-2.02 (m, 1H), 1.75 (s, 3H), 1.49-1.25 (m, 24H), 1.17 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H),1.02 (s, 3H), 0.82 (s, 3H). HRMS (EI) calced for [M+H]\(^+\): C\(_{37}\)H\(_{52}\)NO\(_3\) : 558.3947, Found 558.3928

**Discussion**

Parkinson's is a degenerative disorder of the central nervous system. Lewy bodies are the pathological hallmark of this idiopathic disorder. There are several factors which could be held responsible for the death of brain cells, one such factor is abnormal accumulation of alpha-synuclein, 140 -amino-acid protein in the neurons forming inclusions called Lewy bodies. \(\alpha\)-Synuclein possesses tructural flexibility, In aqueous solutions, the protein is unstructured, whereas on binding to the lipids adopts \(\alpha\)-helical structure [52-55]. In contrast, its misfolding leads to formation of ordered fibrils with a rigid \(\beta\)-sheet-like structure both invtro and invivo in Lewy bodies. Lewy bodies first appear in the olfactory bulb, medulla oblongata and pontine
tegmentum, at this stage, individuals do not show any symptoms. With progression of the disease, Lewy bodies appear in the substantia nigra, areas of the midbrain and basal forebrain, and at last in the neocortex. Other cell-death factors could be proteosomal dysfunction, reduced mitochondrial activity etc.

Treatments at our disposal are effective in managing the disease at an early stage, mainly through the use of levodopa, dopamine agonists and MAO-B inhibitor. As the disease progresses and dopaminergic neurons continue to be lost, these drugs eventually become ineffective at treating the symptoms and at the same time start to produce complications.

Levodopa has been the most widely used drug for the past so many years. L-DOPA is converted into dopamine in the dopaminergic neurons by dopa decarboxylase. Since motor symptoms are produced by a lack of dopamine in the substantia nigra, the administration of L-DOPA temporarily diminishes the motor symptoms. Only 5–10% of L-DOPA crosses the blood–brain barrier and the remaining is often metabolized to dopamine elsewhere, causing a variety of side effects including nausea, dyskinesias and joint stiffness. Carbidopa and benserazide are peripheral dopa decarboxylase inhibitors, which help to prevent the metabolism of L-DOPA until it reaches the dopaminergic neurons, therefore reducing side effects and increasing bioavailability. Several dopamine agonists that bind to dopaminergic post-synaptic receptors in the brain have similar effects as levodopa. These were initially used as a complementary therapy to levodopa; they are now mainly used on their own as an initial therapy for motor symptoms with the aim of delaying motor complications. Dopamine agonists include pergolide, pramipexole, piribedil, rotigotine, cabergoline, apomorphine and lisuride.

MAO-B inhibitors (selegiline and rasagiline) increase the level of dopamine in the basal ganglia by blocking its metabolism. They inhibit monoamine oxidase-B (MAO-B) which breaks down dopamine secreted by the dopaminergic neurons. The reduction in MAO-B activity results in increased L-DOPA in the striatum. Like dopamine agonists, MAO-B inhibitors used as monotherapy improve motor symptoms and delay the need for levodopa in early disease, but produce more adverse effects and are less effective than levodopa.
Acetylcholine (Ach) is a neurotransmitter and in PD, there is a marked drop in the level of dopamine. Ach is indirectly associated with dopamine and there exists a balance between dopamine and Ach. When this balance is disturbed and dopamine level falls resulting in parkinsonism like symptom.

From the above discussion, it is amply clear that not many drugs are available in the market for curing PD and available drugs too have side effects, so there is an urgent need for battery of drugs which are effective even at progressive stage of the disease.

B.C-1 and K002 are the two compounds which have shown promising results against PD. Aggregation of misfolded \( \alpha \)-synuclein results in neurodegeneration, both compounds reduced \( \alpha \)-synuclein protein aggregation by 2.1 folds when compared to untreated worms.

The mechanisms by which aggregated \( \alpha \)-synuclein might promote neurodegeneration are still under scanner though recent hypothesis suggest that environmental toxins or genetic defects lead to mitochondrial dysfunction which causes free radical generation and oxidative stress leading to protosomal dysfunction, protein aggregation and ultimately cell death. Although, it is not completely clear how \( \alpha \)-synuclein might promote neuronal degeneration, most studies suggest that it is probably the intermediate oligomers.

Both the compounds have shown positive response in terms of response time to nonanol experiment indirectly indicating increased dopamine content in presence of compounds, presenting the fact that compounds may be acting as Dopamine agonist.

The significance of using C. elegans as a model to study neuronal disorders lies in the fact that its nervous system is simple, comprising of precisely 302 neurons wired by 7000 synapses, its importance to the present study lies in the fact that C. elegans contains precisely eight dopaminergic neurons, ablation of these cells or mutations that block synthesis or release of dopamine cause defects in the animal's ability to sense or respond to changes in its environment. Dopamine signaling has established roles in the modulation of locomotion behavior and in learning. Dopamine signaling allows C. elegans to respond to changes in its environment by modulating locomotion behavior. Dopamine is also required for another form of learning, state-dependent olfactory adaptation.
DAF-16 is known to regulate life span of Caenorhabditis elegans and its mammalian homologue is forkhead box transcription factor, class O (FOXO). In mammals, the FOXO class of transcription factors are key players in the regulation of cell-fate decisions, such as cell death, cell proliferation, and cell metabolism, their phosphorylation/dephosphorylation results in the translocation between cytoplasm and nucleus, daf-16 is negatively regulated by daf-2 signaling and is the major downstream effector which translocates into the nucleus and modulates transcription when daf-2 signaling is abrogated. Knockout of the FOXO transcription factor/DAF-16 in C. elegans leads to a substantially decreased lifespan. [56-57]

Knockout of the insulin/IGF-1 receptor /DAF-2 leads to a 2-fold extension of lifespan in C. elegans. [58] In presence of B.C-1, increased GFP intensity for DAF-16 expression was observed in comparison to the worm raised on normal E. Coli OP50 diet, inference that can be drawn from the above facts is B.C-1 is acting through this pathway and positively regulating the pathway, hence increasing the viability period, lifespan of the worm.

B.C-1 has displayed anti-oxidant activity [59-61] as it has brought down the stress level of worms fed with compound by 1.8 folds when compared to worms fed onto control diet. Free radical scavenging property of B.C-1 might have contributed in decreasing α-synuclein protein aggregation and improving dopamine signaling by reversing the effect of ROS and as the molecule is upregulating DAF-16 and decreasing the oxidative stress so compounding the positive effect on the life span of C. elegans. No compound was found active in aldicarp assay carried out for ACh availability.

B.C-1(Lupenone) is the only molecule among all the test molecules which was found effective in most aspects and its better modification or hybridization, could yield a potential drug.
References


45. Boonyapraphat, N.; Chockchaicharaenphorn, C,(1998), Thai Medicinal Plants,’ Prachachon Publisher: Bangkok, 2, pp 151.


