**Xylocarpus moluccensis**

**Introduction**

The genus *Xylocarpus* belongs to the family meliaceae. It is a large genus distributed all over the world. Different species are found in the coastal regions of India, Ceylon, Burma and Malaya. It is called as ‘dabi’ (or legi legi) by indigenous people [1, 2]. Three species are found around littoral of the tropical Indian Ocean, these include *Xylocarpus moluccensis* (Lamk) M. Roem., *X.granatum* (Koenig) and *X. mekongensis* [3-5]. These species also grow in the Pacific Islands. *X. moluccensis* is a medium size branched mangrove tree, with pointed leaves, deeply serrated bark and mandarin orange size spherical fruits. It is also found in Australia where it is known as *X. australiacus* [6], *X. mekongensis* is found in the coast region of East Africa. It is a large size branched mangrove, with rounded coriaceous leaves, thin smooth stem bark, tough red color heartwood and small size fruits typical of *X. moluccensis*.

**Taxonomic Classification**

<table>
<thead>
<tr>
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<th>Plantae</th>
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<tr>
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<tr>
<td>Supervision</td>
<td>Spermatophyta</td>
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<tr>
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<td>Species</td>
<td><em>moluccensis</em></td>
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<tr>
<td>Binomial name</td>
<td><em>Xylocarpus moluccensis</em> (lamk.)</td>
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</table>

**Traditional uses**

Wood obtained from this plant is used for different purposes including making the furniture, boat and frame of wooden houses, bark is used for tanning, different parts of *Xylocarpus moluccensis* are used in different traditional systems of medicine in India, Australia, China and other parts of the world. All parts of the plant are useful in treatment of a number of diseases by traditional healers but stem bark and root are used more frequently. This plant has been found to possess antibacterial, antiviral, anti-inflammatory, antioxidant, lipid lowering and cytotoxic activities.
Phytochemistry

Phytochemical investigations have showed the presence of wide range of chemical constituents including fatty acids, triterpenes, tetranortriterpenoids and limonoids. All the species of *Xylocarpus* are reported to have a special class of compounds referred to as ‘Limonoids’ [9]. These constitute a group of triterpene derivatives with structural similarity which is found abundantly in different species belonging to families Meliaceae and Rutaceae. The term “limonoids” is originated from the compound limonin which was first of all isolated from the bitter fraction of lemon and other citrus fruits [10]. Structurally, limonoids are highly oxygenated nortriterpenoids formed by loss of four terminal carbons of the side chain in the apotirucallane or apoeuphane skeleton which then cyclized and give rise to 17 β-furan ring hence also termed as tetranortriterpenoids. These are polyhydroxylated compounds that occur either as free hydroxyl derivatives or as esters. The species belonging to genus *Xylocarpus* characteristically produce a special class of limonoids termed as xyloccensins [4, 11-15]. These are derivatives of mexicanolide containing a hemiketal bridge, exclusively found in genus *Xylocarpus*. These have the structural complexity, intermediate in between the phragmalin groups of limonoids and common mexicanolides [16]. Limonoids without furan ring are called as protolimonoids. Up to now, altogether 23 phragmalins and 42 mexicanolides have been isolated from different parts of *X. moluccensis* and *X. granatum*.
Xylocarpus moluccensis

Angustidienolide

Destigloyl-6-deoxyswietenine acetate

Methyl ester of phenyl keto acid

Xylococcisin A

Xylococcisin B

Xylococcisin C

Xylococcisin D

Xylococcisin E

Xylococcisin F

7-Oxo-7-Deacetoxy Gedunin Phragmalin-3, 30-diacetate

2-Hydroxy destigloyl-6-Deoxy swietenine acetate
Pharmacology

A broad range of biological activities have been reported in different extracts and compounds isolated from Xylocarpus species [17]. Limonoids are shown to exhibit significant insect growth regulatory as well as antifeedant activity [18-19].

Several limonoids have shown to exhibit antifungal activity against Drechslera oryzae, Alternaria tenuis, and Fusarium oxysporum [20]. Other studies have shown that the extracts and compounds have prominent antimalarial potential [21]. Gedunine as well as 7-methoxygedunin showed significant antimalarial activity against Plasmodium falciparum (clone D6 and W2) with IC$_{50}$ values in nM range [22, 23]. Chloroform fraction of Xylocarpus granatum fruits and its chemical constituents gedunin and xylocensins I showed significant anti-malarial activity. MIC for gedunin was found to be 10 µg/ml, it also showed an additive effect when applied in combination with chloroquine [24]. Some of the limonoids also showed prominent insecticidal effect against plasmodium vector Anopheles stephensi [25].

Some of the limonoids isolated from Xylocarpus have also been found to exhibit significant activity against different types of cancer [26]. Isoazadironolide and turrapubesin E showed antiproliferative potential against P388 cell line, with IC$_{50}$ values in micromolar concentration.
Some other extracts and compounds have also exhibited potent anticancer activity against HeLa cells and A-549 human lung carcinoma cell line [27].

Some of the Limonoids exhibited prominent antibacterial effect against *Mycobacterium tuberculosis*, showing MIC in micromolar range [28]. Moluccensin I exhibited significant antibacterial activity against different strains including *Staphylococcus hominis*, and *Enterococcus faecali* [29]. 7-Deacetylgedunin and 7-oxo 7-deacetoxy-gedunin showed potent antiprotozoal activity against *Trypanosoma cruzi*, *T. brucei rhodesiense*, *Leishmania donovani* and *Plasmodium falciparum* [30]. Gedunin, 6α-acetyoxygedunin, 7-deacetoxy-7-oxogedunin, and methyl angolensate showed significant antiallergic response in mice through modulating the NF-κB signaling pathway. The methanolic extract of the stem bark of *X. moluccensis* exhibited CNS-depressant activity [31]. Semisynthetic analogs of gedunin have shown to exhibit significant effect against different neurological disorders through acting as TrkB agonists, and modulating the activity of BDNF (brain derived neurotrophic factor) subsequently stimulating the growth and survival of neurons.

The methanolic extract of the bark of *X. moluccensis* has shown antidiarrhoeal activity, the extract was found active at doses of 250 and 500 mg/kg. The AcOEt extract of the bark also exhibited similar activity at a dose of 250 mg/kg [13]. Gedunin and photogedunin displayed significant anti secretory and gastro protective effect against cold restraint (CRU), aspirin (AS), alcohol (AL) and pyloric ligation (PL) induced gastric ulcer models in rats and histamine (HA) induced duodenal ulcer model in guinea pigs [32]. The ethyl acetate fraction of *Xylocarpus grnatum* fruit as well as its active constituents, gedunin showed IC₅₀ = 0.239 μg/ml and photogedunin showed antifilarial activity against adult human lymphatic filarial parasite *Brugia malayi* [33].

**Aim and design of work**

The fruits of *Xylocarpus moluccensis* were collected and authenticated by the Botany Division of CSIR- Central Drug Research Institute (CDRI), Lucknow (CDRI plant code No. 276). The chapter deals with the isolation of medicinally active compounds from fruits. The compounds isolated from fruits have been evaluated for anti-parkinson’s effects.
Extraction, Fractionation, Isolation and characterization of compounds

The dried and powdered fruits were extracted with 95% ethanol by cold percolation method. Solvent was evaporated under reduced pressure at 50°C. The crude ethanolic extract was fractionated into n-hexane soluble (F001), chloroform soluble (F002), n-butanol soluble (F003) and n-butanol insoluble (F004) fractions. The most active chloroform soluble fraction (F002) was taken for detailed chemical investigation. Repeated column chromatography of fraction F002 over a silica gel column afforded six molecules designated as XM-1 to XM-6. These compounds were evaluated for anticancer activity but found inactive. A summary of isolation procedure is given in flow sheet 3.1 and the compounds isolated are given in table 3.1

Flow sheet 3.1: Summary of extraction, fractionation and isolation procedure for Xylocarpus moluccensis (fruits).
Table 3.1: Compounds isolated from *Xylocarpus moluccensis* (fruits)

<table>
<thead>
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<th>Compound code</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Characterized as</th>
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<tr>
<td>XM-1</td>
<td>$\text{C}<em>{35}\text{H}</em>{42}\text{O}_{14}$</td>
<td>686</td>
<td>Xyloccensin-E</td>
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<tr>
<td>XM-2</td>
<td>$\text{C}<em>{36}\text{H}</em>{48}\text{O}_{12}$</td>
<td>672</td>
<td>Xyloccensin-X</td>
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<td>XM-3</td>
<td>$\text{C}<em>{36}\text{H}</em>{48}\text{O}_{12}$</td>
<td>672</td>
<td>Xyloccensin-Y</td>
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<td>XM-4</td>
<td>$\text{C}<em>{33}\text{H}</em>{46}\text{O}_{12}$</td>
<td>646</td>
<td>Xyloccensin-I</td>
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<td>XM-5</td>
<td>$\text{C}<em>{33}\text{H}</em>{44}\text{O}_{12}$</td>
<td>632</td>
<td>Xyloccensin-J</td>
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<tr>
<td>XM-6</td>
<td>$\text{C}<em>{27}\text{H}</em>{34}\text{O}_{7}$</td>
<td>470</td>
<td>Methyl Angolensate</td>
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</tbody>
</table>

Structure of isolated compounds

![Structure of isolated compounds](image-url)
All the above compounds are known and were compared with the extensive literature [2, 34, 35] available for their IR, mass and NMR data (\(^1\)H and \(^{13}\)C) as well as with their authentic samples on TLC.

**BIOLOGICAL ACTIVITY: ANTI-PARKINSONIAN**

Parkinson’s disease is the most common progressive neurodegenerative disease characterized by the death of nerve cells. It affects brainstem extrapyramidal neurons (e.g., dopaminergic substantia nigra neurons) of middle-age individuals affecting approximately 2% of the world population [36]. An estimated 4 million individuals above 50 had Parkinson’s disease in 2005 [37, 38], and this number is likely to reach up to 9 million by 2030. Epidemiologically, Parkinson’s disease is classified into familial and infamilial, depending on whether it is hereditary or from unknown origin, possibly due to exposure to environmental neurotoxicants. Several environmental contaminants have been identified as possible factors causing infamilial Parkinson’s disease; these include pesticides and heavy metals. The disease is diagnosed by the presence of protein aggregates known as Levy bodies (LBs) in perikarya of degenerating brainstem neurons and is composed mainly of \(\alpha\)-synuclein, a soluble neuronal protein that evolves into insoluble oligomers during the occurrence of disease [39]. Ubiquitin (Ub) and neurofilament subunits [40] are also found in LBs. Levy bodies are also found in a subset of cortical neurons in a late-onset neurodegenerative disorder known as dementia with Lewy bodies (DLB), a most frequent type of dementia followed by Alzheimer's disease [41]. Parkinson’s disease commences with a decline in cognitive function, particularly short-term memory and consequently progresses in severe symptoms including problems in speaking, reading, logical thinking, and long-term memory. Clinical symptoms of Parkinson’s disease include bradykinesia, tremor, rigidity, dystonia and occasionally akinesia. These symptoms arise due to progressive loss of motor function caused by degeneration of the dopaminergic neurons in substantia nigra, pars compacta.

In order to find a new safe and effective drug, that can inhibit the progression of Parkinson’s disease at the advanced stage, without incurring side effects, efforts are being made to search for new molecules from various medicinal plants. Several Indian medicinal plant species have been reported to possess anti-neurodegenerative activities. In view of the above facts we have evaluated the anti Parkinson effect of ethanolic extract of *Xylocarpus moluccensis* fruits and its active constituents. Oxidative stress and mitochondrial impairment leading to cell death naturally

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*Xylocarpus moluccensis*
occur with aging regardless of neurodegenerative diseases. Aging is associated with oxidative damage caused by reactive oxygen species generated in different pathologic conditions as well as in mitochondria during respiratory chain. It is also caused by mitochondrial iron overload, decrease in antioxidant response and DNA-repairing capabilities as well as increase in oxidation of biomolecules including proteins, lipids and nucleic acids. This sensitive state of the aging brain renders it more vulnerable to oxidative stress or other injuries. It explains the possible cause of parkinson’s disease in elderly people.

*Caenorhabditis elegans, α-Synuclein and parkinson’s disease*

α-Synuclein is a soluble neuronal protein associates with synaptic vesicles and is known to be involved in excitation-secretion coupling. It plays an important role in the regulation of biosynthesis of DA and also influences the function of DA transporter (DAT). Mutations in α-synuclein lead to the alteration in its structure resulting in formation of insoluble oligomers and subsequent formation and deposition of Lewy body (LBs), reducing the viability of DAergic neuron. In normal physiological conditions, α-Synuclein is degraded by a proteosomal pathway mainly involving cathepsin D, casein kinase 2. Recent studies have postulated that there is a positive correlation between oxidative damage and α-synuclein aggregation that may results in parkinson’s disease. The nematode *Caenorhabditis elegans* (*C. elegans*) does not normally express α-synuclein but overexpression of wild type human α-synuclein in *C. elegans* increases the vulnerability to mitochondrial complex-I inhibitors like rotenone and fenperoximate, which is reversed by treatment with free radical scavangers. Overexpression of mutant human α-synuclein in DAergic neurons show accumulation of α-synuclein in the cell bodies and neurites along with reduced neuronal DA content leading to alteration in DAergic neuron function and worm behavior, which is restored with the administration of dopamine. Recent 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 parkinson’s disease, but different studies have proved its utility and relevance as a model organism to identify the pathways involved in pathogenesis as well as to develop new therapeutic agents for parkinson’s disease. Compounds isolated from chloroform fraction have been evaluated for their effect on Parkinson’s disease.
Materials and Methods

C. elegans culture and maintenance

Worms were cultured and maintained as described previously [42, 43]. In brief, worms were cultivated on Nematode growth medium (NGM) agar plates seeded with E. coli - OP50 bacteria at 22°C using standard techniques. NGM media was prepared by adding 50 mM Sodium chloride (Merck), 2.5g L⁻¹ Peptone (Sigma), 17 g L⁻¹ Agar (Hi-media) in 975ml double distilled water and autoclaved for 30 to 40 minutes at 15 lb/inch². After the cooling of media to 50-60 °C, 5μg/ml cholesterol solution (Sigma) prepared in ethanol, 1mM Calcium chloride (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 (Punc-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 Caenorhabditis Genetics Center (University of Minnesota, USA).

Obtaining synchronous nematode population by embryo isolation

To isolate the embryos from various strains of C. elegans, worms were synchronized by alkaline sodium hypochlorite bleaching method. Worms were washed with M-9 buffer and treated with axenizing solution (2mL of sodium hypochlorite and 5mL of 1M sodium hydroxide solution) and then subjected to intermittent vortexing [44]. Thereafter, worms were washed with M-9 buffer twice, so as to obtain worms in their synchronized embryo stage.

Treatment of worms with compounds

2 mg of each compound was dissolved into 100μl ethanol and further diluted in equal volume in OP50 (25 μl compound + 25 μl OP50) before seeding onto 12 well NGM plates. The plates were incubated at 37°C overnight 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 [45] Worms were grown on control and treated with compounds for 48 hrs, washed with M9 buffer to remove adhering...
bacteria and immobilized with 100 mM 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 fluorescence 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.

**Estimation of Reactive Oxygen Species (ROS)**

To investigate the anti-oxidant properties of compounds, we conducted 2, 7-dichloro dihydrofluorescein-diacetate (H2-DCFDA) (Cat. No – D399, Invitrogen) assay using standard protocol [46]. In this assay, control as well as plant samples 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 H2-DCFDA 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.

**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**

**Compounds isolated from ethanolic extract of Xylocarpus moluccensis fruits decreased aggregation of alpha synuclein protein in transgenic NL5901 strain of C. elegans**

We used the transgenic *C. elegans* models - NL5901 expressing ‘human’ alpha synuclein protein tagged with yellow fluorescent protein [NL5901 (Punc- 54:: alphasyuclein:: YFP+unc-119) in the body wall muscle. We explored the role of compounds isolated from active fraction of *Xylocarpus moluccensis* fruits on alpha synuclein aggregation. Worms treated with compounds
were analyzed using confocal microscopy. We further quantified the images for fluorescence intensity of α synuclein aggregation using Image J software. Out of the six compounds tested, compounds XM-1, XM-2 and XM-3 significantly reduced the aggregation of alpha synuclein protein. Worms treated with compounds XM-1 and XM-2 and 3 displayed significant reduction by 53.8 and 57.6% respectively, in comparison to control worms (Figure 3.1 and 3.2).

**Figure 3.1:** Graphical representation for fluorescence intensity of alpha synuclein aggregation in NL5901 strain of *C. elegans* treated with compounds XM-1, XM-2 and 3. ***p < 0.001.

**Effect of most active compounds XM-2 and 3 on antioxidant properties in *C. elegans***

To further evaluate the role of the two active compounds, showing more than 50% reduced aggregation of alpha synuclein protein, anti-oxidative properties were determined employing H2-DCFDA assay using wild type N2 strain of *C. elegans*. Worms treated with compounds XM-2 and 3 showed slight reduction in oxidative stress as compared to worms fed onto control diet indicating the fact that these compounds possesses free radical scavenging property (Figure 3.3).

**Treatment with compounds increased Acetyl Cholin (Ach) availability in N2 strain of *C. elegans***

Out of six compounds isolated from active fraction, compound XM-2 and 3 demonstrated significant increases in Acetyl Cholin (Ach) availability in Aldicarb assay (Figure 3.4).
Figure 3.2: Effect of compounds XM-1, XM-2 and 3 on alpha synuclein protein aggregation in NL5901 strain of *C. elegans*.

Figure 3.3: Effect of compounds XM-2 and 3 on formation of reactive oxygen species (ROS) measured by H$_2$DCFDA assay in N2 strain of *C. elegans*. p>0.05 (non-significant).
Discussion

Parkinson’s disease is a neurodegenerative disease affecting the central nervous system in old age. Several factors have been identified which induce the death of brain cells, one such factor is abnormal accumulation of alpha synuclein, forming inclusions known as Lewy bodies (LBs). α-Synuclein possesses structural flexibility, in aqueous solutions, it occurs in unstructured form, whereas on binding to the lipid molecules adopts α-helical structure [45, 47]. Misfolding of this protein leads to the formation of ordered fibrils with a rigid β-sheet-like structure. Lewy bodies first appear in the medulla oblongata, olfactory bulb and pontine tegmentum, at this stage, individuals do not show any clinical symptom. Subsequently, with the progression of disease, these appear in the substantia nigra, areas of the midbrain and basal forebrain, and in the neocortex. Treatments include the use of levodopa, dopamine agonists and MAO-B inhibitor. In

Figure 3.4: Aldicarb assay performed in Wild type strain of *C. elegans* treated with compounds XM-1 to XM-6. *P < 0.05, **P < 0.01.
the advanced stage of disease when dopaminergic neurons continue to be lost, these drugs eventually become ineffective.

Levodopa has been the most widely used drug for the past so many years. It is converted into dopamine in the dopaminergic neurons by dopa decarboxylase. The administration of L-DOPA temporarily diminishes the motor symptoms produced due to lack of dopamine in the substantia nigra. Several dopamine agonists that bind to dopaminergic post-synaptic receptors in the brain have similar effects as levodopa.

MAO-B inhibitors increase the level of dopamine in the basal ganglia by blocking its metabolism. 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 are less effective than levodopa. Acetylcholine (Ach) is indirectly associated with dopamine and there exists a balance between dopamine and Ach. The imbalance in this natural ratio results in parkinson’s like symptom. There is an urgent need to develop new drugs with significant effect in progressive stage of the disease.

The active constituents isolated from the ethanolic extract of *X. moluccensis* have shown promising results against Parkinson's disease, Aggregation of misfolded α-synuclein results in neurodegeneration, both compounds significantly reduced the aggregation of α-synuclein protein. Recent studies suggested that environmental toxins or genetic defects lead to mitochondrial dysfunction which causes excessive free radical generation and oxidative stress resulting in proteosomal dysfunction, protein aggregation and ultimately cell death.

The *C. elegans* is an ideal model to study neuronal disorders as it has a simple nervous system, comprising of precisely 302 neurons wired by 7000 synapses, it 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.

Compounds XM-2 and 3 have displayed anti-oxidant activity. Free radical scavenging property of these compounds might have contributed in decreasing α-synuclein protein aggregation by reversing the effect of ROS and ultimately increasing in the life span of *C. elegans*. 
Among the all molecules evaluated XM-2 and 3 (Xylocensin-X and Y) were found most effective and its better modification or hybridization, could yield a potential drug for the treatment of parkinson’s disease.

REFERENCES


