Introduction (*Xylocarpus granatum*)

*Xylocarpus granatum* Koenig, a marine mangrove plant has different classes of chemical constituents and possess wide range of biological activities, a miniscule number of mangroves plants have been studied when compared to the number of terrestrial plants explored, more than 150 compounds have been isolated from the three species of *Xylocarpus* namely *granatum*, *moluccensis* and *rumphii*. Mangroves in India are concentrated in the eastern and western coast.

**Taxonomical Classification**

- **Kingdom:** Plantae
- **Phylum:** Magnoliophyta
- **Class:** Eudicots
- **Order:** Sapindales
- **Family:** Meliaceae
- **Genus:** *Xylocarpus*
- **Species:** *granatum*

**General description**

Trees are up to 12 m tall, branchlets sparsely lepidote. Trunk surface smooth, pale, blotched greenish or yellowish, bark peeling in patches base often enlarged. Leaves are pinnate, with 2-4 pairs of leaflets, Flowers are white, 8 mm wide with fragrance, Inflorescence axillary thyrses, fruits are capsules, 25 cm wide and when split open, present four valves and release 5-20 seeds.

**Common names**

Mangrove Cannonball (English), Samudraphal, Bhelanda (Hindi) Somuntheri, kadal manga (Tamil), Chenuga (Telugu), Kontalai, mutti kad (Singhalese).

**Distribution range**

It is found in the coastal areas of East Africa, South Asia, Australia and the Pacific Islands[1]. In India it occurs in coastal area of South up to Maharashtra and in Andaman Islands. In Sri Lanka it occurs in southwestern part.
Traditional uses

Woods of this plant are used as fuel, making furniture, boat building, frame of wooden houses etc, bark used for tanning, root contain chemical constituents which are active against cholera and dysentery, oil from its seeds can be used for illumination and grooming hairs, seeds of the plant is known to possess potential to cure breast cancer.

REVIEW OF LITERATURE

A. Phytochemistry

Large number of chemical constituents have been isolated from the three species of *Xylocarpus* which include triterpenoids, alkaloids, phenolic acids, flavanol, steroids, monoterpenes,[1] most of the compounds isolated are Liminoids which are derived from tetracyclic triterpenes, the classes of Liminoids found in *Xylocarpus* are (a). Gedunin group (b). Andirobin group (c). Mexicanolide group (d).Phragmalin group (e).Obacunol group.

Structures of compounds from genus *Xylocarpus*
Xylocarpus granatum

Xylogranatin B

Xylogranatin C

Xylogranatin D

Xylogranatin E

Xylogranatin F

Xylogranatin G

Xylogranatin H

Xylogranatin I

Xylogranatin J
**Xylocarpus granatum**

- **Butyrospermol 3β-O-palmitate**
- **Butyrospermol 3β-O-oleate**
- **Butyrospermol 3β-O-stearate**
- **Butyrospermol 3β-O-linoleate**

- **β-Sitosterol 3β-O-myristate**
- **β-Sitosterol 3β-O-oleate**

- **Abscisic acid**
- **Xylomollin**
- **Aurantiamide**
Xylocarpus granatum

7-Acetoxy dihydronomilene

Augustidenolide

Destigloyl-6-deoxyiswietenine acetate

Hydroxyproline

N-Methyl Flindersine

Glucose

β-D-Fructose

Sucrose

Stigmasterol

β-Sitosterol

β-Sitosterol β-D-glucoside
B. Pharmacology

Biological activity
Most of the biological activities possessed by the genus Xylocarpus are mainly due to Liminoids, the activities include anti-cancer, anti-malarial, anti-filarial activity, Gastroprotective activity, anti-diarrhoeal etc.

Anticancer activity:-
Xylogranatins (A-D) have been isolated from Xylocarpus granatum. Xylogranatins (B-D) have been found moderately active against P-388 murine leukemia cell line with IC₅₀ 8.9, 6.3 and 14.6 µM respectively whereas Xylogranatins (A, B) have exhibited activity against A-549 human lung carcinoma cell line with IC₅₀ 15.7 and 11.3 µM respectively. [2] The AcOEt extracts
of X. granatum showed growth-inhibitory activity against HeLa cells with IC$_{50}$ 23.0 mg/ml.[3] Xylocarpin H and Granaxylocarpin A from Xylocarpus granatum have shown weak cytotoxic activity against the P-388 cell line with IC$_{50}$ 9.3 and 4.9 µM respectively.

**Antifeedant activity:**
Number of compounds from Xylocarpus granatum and moluccensis have exhibited positive response against antifeedent activity, Xylomollin [4] from X. moluccensis and chelerythrine dihydrochelerythrine, Xyloccensin P, Xyloccensin Q, Xylogranatins F, G, and R from X. granatum are good examples of it.

**Antimicrobial Activities:**
N-Methylflindersine and β–catechin are the two compounds from Xylocarpus granatum which have displayed inhibitory effect on the growth of lactic acid bacteria. [3]

**Antimalaria Activities:**
Xylocarpus granatum fruit’s chloroform fraction on evaluation showed anti-malarial activity and chemical constituents responsible for this activity were found to be gedunin and xyloccensin I, antimalarial activity was determined using an in vitro model of Plasmodium falciparum, MIC for gedunin was found to be 10 µg/ml, an additive effect was displayed in combination with chloroquine.[5]

**Antidiarrhoeal activities:**
The MeOH extract of the barks of X. moluccensis has shown antidiarrhoeal activity, the extract was found active at doses of 250 and 500 mg/kg. The AcOEt fraction of the bark also exhibited similar activity at a dose of 250 mg/kg. [6]

**Neuropharmacological properties**
The MeOH extract of the stem bark of X. moluccensis possess CNS-depressant activity. [7]

**Gastroprotective activity**
Gedunin and Photogedunin isolated from Xylocarpus granatum 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. [8]

**Antifilarial activity**

The ethyl acetate fraction of *Xylocarpus grnatum* fruit was found to have antifiliarial activity with IC$_{50}$ of 8.5 and 6.9 µg/ml in adult and microfiliariae respectively while the pure compounds from active fraction, gedunin showed IC$_{50}$= 0.239 µg/ml and photogedunin with IC$_{50}$=0.213 µg/ml against adult human lymphatic filarial parasite B. malayi.[9]

**Aim and design of work**

**Present study**

The fruits of *Xylocarpus granatum* was identified and provided by the Division of Botany, CSIR-Central Drug Research Institute (CDRI), Lucknow (CDRI plant code No. 134). The chapter deals with the isolation of compounds from fruit and determining the mechanism of action of photogedunin at molecular level for its anti-adipogenic / lipid lowering activity *in vitro.*

![Photogedunin](image)

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

The dried and powdered plant material (fruit) 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 compounds designated as XG-1 to XG-4.

**Flowchart-1 Summary of extraction, fractionation and isolation procedure for *Xylocarpus granatum* (fruit).**
Table-1: Compounds isolated from *X.granatum* stem (fruit)

<table>
<thead>
<tr>
<th>Code no.</th>
<th>Molecular formula</th>
<th>Characterized as</th>
<th>Molecular wt.</th>
</tr>
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<tbody>
<tr>
<td>XG-1</td>
<td>C_{28}H_{34}O_{7}</td>
<td>Gedunin</td>
<td>482</td>
</tr>
<tr>
<td>XG-2</td>
<td>C_{28}H_{34}O_{9}</td>
<td>Photogedunin</td>
<td>514</td>
</tr>
<tr>
<td>XG-3</td>
<td>C_{35}H_{42}O_{14}</td>
<td>Xylocensin E</td>
<td>686</td>
</tr>
<tr>
<td>XG-4</td>
<td>C_{33}H_{46}O_{15}</td>
<td>Xylocensin Q</td>
<td>674</td>
</tr>
</tbody>
</table>

Structure of isolated compounds.
**Biological Activity** (Antidyslipidemic)

**Introduction**

Dyslipidemia and associated disorders have been recognized to be gaining the status of epidemic and entered pandemic proportions [10] the elevated levels of lipids in the fat storing cells called adipocytes is considered as an important aspect and is directly or indirectly related to obesity and dyslipidemia, adipocyte are roughly 1/5th of body weight so by this virtue, they are relevant to our study. It is estimated that the cases of dyslipidemia and obesity have increased enormously in the past two decades both in developed and developing countries, data available to this date reveal that more than 25% of the American population is obese and more than 60 million Chinese have become obese in the past ten years whereas India is going to be world capital in the heart related diseases by 2050, all this can be attributed to high levels of lipids, which is the consequences of unhealthy lifestyle, eating behavior and cultural adaptation resulting in metabolic disorders such as coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications and osteoarthritis. In other words homeostatis is disturbed, intake of energy exceeds the expenditure and apart from this new adipocytes are constantly generated and replace the old ones and in this process approximately 50% subcutaneous adipocytes are replaced every 8 years [11] so targeting adipocytes for pharmacological intervention of lipid disorder seems to be a good proposition. Number of natural products like quercetin, resveratrol, genstein are known to affect adipogenic differentiation at various stages of its life cycle, exerting the effect either through induction of apoptosis, inhibition of adipogenesis, stimulating lipolysis or combinations thereof [12] *Xylocarpus granatum* mainly contain liminoids and possess many biological activities but its lipid lowering activity is still remains unexplored in terms of molecular mechanism. As most of the biological activities of this plant are mainly due to gedunin and photogedunin and anti-adipogenic activity of photogedunin is not yet reported, so it would be intriguing to assess its anti-adipogenic potential.

**Parameters considered for assessment**

In present study, we investigate the effect of photogedunin on 3T3-L1 and C3H10T1/2 cells under influence of hormonal inducers, the parameters under scrutiny are (a).effects of
photogedunin in terms of lipid accumulation (b) changes in gene expression for various adipogenesis associated markers of 3T3-L1 cell line and (c). cell cycle analysis

Materials and Methods

Differentiation of 3T3-L1 and C3H10T1/2 adipocytes

3T3-L1 and C3H10T1/2 cell lines were purchased from the American Type Culture Collection. Cells were cultured in a humidified atmosphere at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal bovine serum and Penicillin-streptomycin antibiotics (cDMEM). For adipogenic induction cells were seeded in 24-multi-well plates. Two day post confluence, culture media was replaced with differentiation medium (MDI) (medium containing Insulin 5 µg/ml, IBMX 0.5 mM and Dexamethasone 250 nM). This media was replaced after 48 hr with medium containing 5µg/ml Insulin. This medium was replaced after 48 hours and cells were maintained in cDMEM. Lipid droplet started appearing from day 4th and >90% cells show lipid globules after 6-8 days after induction. To study effect of photogedunin on adipogenic differentiation, cells were differentiated at given concentrations and/or time exposed to photogedunin as indicated in figures.1

Oil Red-O (ORO) staining

Differentiated 3T3-L1 (with or without compound) adipocyte were rinsed in phosphate buffered saline (PBS) (pH 7.4). The adipocyte lipid globules were stained with ORO (0.36% in 60% Isopropanol) for 20 min. Accumulated dye was extracted using 100% Isopropanol and measured absorbance at 492 nm.

Real Time PCR

Total RNA was isolated from 3T3-L1 cells using TRIZOL reagent (Invitrogen CA, USA). First strand cDNA synthesis performed using Megascript reverse transcriptase kit (Applied Biosystems) and subsequently used for quantitative real time PCR analysis on Light Cycler 480 (Roche Diagnostics). Statistical analysis of the quantitative real time PCR obtained using the (2^ΔΔCt) method, which calculates the relative changes in gene expression of the target, normalized to an endogenous reference (GAPDH) and relative to a calibrator that serves as the control group.
Cell cycle analysis using flow Cytometry

Two day post confluent 3T3-L1 preadipocytes were incubated in adipogenesis MDI with or without photogedunin at different concentrations. The cells were harvested after 24 hours, washed and re-suspended in PBS. Then, cells were fixed in 70% ice-cold ethanol. Pelleted cells were suspended in propidium iodide for 30 minutes at room temperature. At least 10000 events were acquired per sample on flow cytometer (BD, FACS Calibur). Analysis was performed using Modfit software to determine the relative amount of cells in G1, S and G2/M software.

Statistical analysis

Data were expressed as mean+s.d. Students t-test was used for comparisons of measured parameters. A probability value of P<0.05 and/or P<0.01 was used as measure of statistical significance. Data was analyzed on Graph Pad Prism (Version3.00, Graph pad Software Inc. San Diego, CA, USA).

Results

Photogedunin inhibits adipogenesis

Varying concentrations of photogedunin (5, 10, 20µM) was added to MDI during differentiation. Microscopic observation showed photogedunin decreased lipid droplet accumulation in differentiated cells in concentration dependent manner (Figure 1A). Absorbance of extracted ORO accumulated in lipid droplets confirms that photogedunin inhibits adipogenesis significantly at 5µM concentration and more than 50% lipid accumulation is inhibited at 20µM concentration (Figure 1C) in 3T3-L1 cell line. Anti-adipogenic effect of photogedunin was also evaluated in C3H10T1/2 cells, a mouse mesenchymal stromal cell line. Photogedunin addition to MDI inhibits adipogenesis at different concentrations (5, 10, 20µM). This was confirmed qualitatively by microscopy (Figure 1B) and quantitatively by ORO absorbance (Figure 1D). Collectively these results indicate that, photogedunin possesses an anti-adipogenic potential in-vitro. The same experiments were carried out for both cell lines (3T3-L1 and C3H10T1/2) in a time dependent manner at 20 µM concentration, the molecule on addition to MDI inhibits adipogenesis in both cell lines confirmed by microscopy (Figure 2A and 2B) as well as by ORO
absorbance (Figure 2C and 2D). The preliminary results clearly establishes photogedunin as a potent anti adipogenesis agent and encourages us to further explore other aspects of it.

**Figure 1**

**Figure. 1.** Effects of Photogedunin on MDI-induced adipogenesis in 3T3-L1 and C3H10T1/T2 preadipocytes in concentration dependent manner. Photogedunin inhibited differentiation of 3T3-L1 and C3H10T1/T2 preadipocytes as indicated by staining with Oil red-O (ORO) staining solution (C, D). Data are presented as of control and are expressed as means±s.d. Significant differences between the group treated with MDI only and the group treated with MDI and Photogedunin (***P<0.01 and ****P<0.001, respectively).
Figure. 2. Photogedunin inhibit MDI-induced Mitotic clonal expansion in 3T3-L1 and C3H10T1/T2 preadipocytes in a time schedule days. Photogedunin reduced lipid accumulation when introduced during days 0–2, 0-4, 0-6 significantly in 3T3-L1 and C3H10T1/T2 (C,D). Data are presented as of control and are expressed as means±s.d. Significant differences between the group treated with MDI only and the group treated with MDI and Photogedunin (**P<0.01 and ***P<0.001, respectively).
RT data analysis

Photogedunin at 20µM brings about significant reduction in gene level expression of adipogenesis associated genes viz. PPARγ, C/EBPα and ap-2 in 3T3-L1 cell line (Figure 3).

Figure. 3. Photogedunin suppressed the expression of PPARγ, C/EBPα and ap-2 in 3T3-L1 adipocytes. Data are representative of three independent experiments that showed the same tendency after 4 and 6 days of differentiation in comparison control and Photogedunin-treated cells. The results were verified by three of the experiments, each of which were conducted in triplicate and expressed as means±s.d. Significant difference between the control and MDI-treated groups(**P<0.01). Significant differences between the group treated with MDI only and the group treated with MDI and Photogedunin (**P<0.0001).
Photogedunin arrests the cell cycle during preadipocyte differentiation in 3T3L-1 cell line.

When MDI is added to growth arrested confluent cells, it forces cells to enter into 2-3 rounds of cell cycles ie: MCE which is pre-requisite for adipogenesis, addition of Photogedunin to MDI induced MCE at concentrations resulted in inhibition of MCE and cell differentiation by arresting cell cycle progression in preadipocytes. FACS analysis demonstrated that photogedunin arrests the cells in G1/S phase of cell cycle and brings about S phase delay after 16 and 24 hr at 10 and 20 uM concentration (figure-4). All undifferentiated MdI-ve cells did not undergo cell cycle progression. The cells treated with both MDI and photogedunin also did not undergo cell cycle progression, staying in G1/S phase until 24 hr. These results exhibit that photogedunin arrest cell cycle during cellular differentiation, which may contribute to inhibition of MDI induced adipogenesis.
**Figure. 4.** Effects of photogedunin on MDI-induced cell cycle progression in 3T3-L1 preadipocytes. (a) photogedunin inhibited MDI-induced cell cycle progression in G2/M phase. The data are representative of more than three independent experiments that give similar results (b) The population of cells in each stage of the cell cycle was quantified and showing in dose dependent arrest in G2/M phase.

**Discussion**

Adipocytes are central to lipid metabolism. 3T3L-1 fibroblast cell line is considered an appropriate model to identify molecules effective in the process of inhibiting adipogenesis and lipid accumulation, number of molecules are already known with proven potential for being anti-adipogenic in nature (in vitro and in vivo)[13,14]. In present study, primary screening of photogedunin exhibited concentration as well as time dependent decrease in ORO accumulation during 3T3-L1 adipogenic differentiation, its anti-adipogenic potential was also confirmed by mouse mesenchymal stromal cell line C3H10T1/2. Once establishing the authenticity of molecule as anti-adipogenic, exploring the mechanism of action, genes involved in orchestrating it were emphasized on.

PPARγ, in general is considered as a master regulator of adipogenesis. PPARγ along-with C/EBPα leads to increased expressions of protein related to adipogenesis and activate downstream target genes such as fatty acid binding protein-4( aP2), Lipoprotein lipase (LPL), Sterol regulatory element binding protein (SREBP-1c) etc. When estimated by real time PCR after 8 days of adipogenesis protocol with 20µM concentration, photogedunin showed decreased expression levels of PPAR-γ, C/EBPα and aP2 (Figure-3), this results further confirms anti-adipogenic property of the molecule. It is worth noting that maximum inhibition was achieved at concentration 20 µM where total lipid accumulation was little less than that of undifferentiated cells,

Another aspect of the molecule which comprises of cell cycle analysis was also determined and the stage in which cell cycle progression was inhibited, data at two time points (16 and 24hr) and two concentration (10 and 20 µM) reveals inhibition of progression at G1/S phase.
Summing up the above results, it can be clearly stated that photogedunin is antiadipogenic in nature so indirectly antiobesity, antidyslipidemic and lowers the risks of metabolic disorders such as type 2 diabetes, hypertension and arteriosclerosis.
References


human lymphatic filarial parasite *Brugia malayi* in experimental rodent host,’ Parasitology Research, 109, pp1351–1360


