Isolation and characterization of dengue protease inhibitory prenylated chalcone from Polyalthia cerasoides Stem bark

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Abstract

Dengue infection is one of the leading causes of death globally. Still today there are no therapeutic agents to treat the disease. Plants are rich source of therapeutic phytochemicals. In the present study, we have explored Polyalthia cerasoides for possible anti-dengue phytoconstituents. Dried powdered Polyalthia cerasoides stem bark was extracted with methanol. Then, the methanol extract was fractionated with petroleum ether. The petroleum ether fraction was chromatographed over silica gel column chromatography and eluted with petroleum ether and ethyl acetate. The compound isolated from fractions 8-14 was characterized as 6, 4-dihydroxy-3-propane chalcone: a prenylated flavanone using mass spectroscopic data. The isolated prenylated flavanone was investigated for the inhibition of dengue viral protease using molecular docking studies and in vitro protease assay.

Results indicated that the prenylated flavanone binds to the active site of dengue viral protease with binding energy -10.2 Kcal/mol and -12.8 Kcal/mol of Serotype 1 and Serotype 4 respectively. Further, the prenylated flavanone was investigated for the inhibition of DENV protease using in vitro high throughput protease assay. Results indicated that prenylated flavanone inhibits the DENV protease in a dose-dependent manner. Prenylated flavanone reported a less toxic effect against HepG2 cells in the MTT cytotoxicity test. Further investigations may lead to developing the isolated compound as a potential dengue viral therapeutic agent.

Keywords: Isolation, Chalcone, Antiviral, Polyalthia cerasoides, Dengue.

Introduction

Polyalthia cerasoides (Roxb.) Bedd. belongs to the family Annonaceae and is also known as Guatteria cerasoides or Uvaria cerasoides. Polyalthia cerasoides is a medium-sized tree, growing to a height of about 10–20 m with 20–50 cm in diameter and is found mostly in Asian countries. The pharmacological properties of the plant are used in the traditional system of medicine extensively across Asian and African countries in the treatment of toothaches, fever and to combat stress. African tribes use the decoction made from roots, leaves and fruits of the plant. It is also used traditionally as a tonic and febrifuge by the native people in Thailand. In India, especially in the States of Andhra Pradesh and Tamil Nadu, tribal people use the fruits and stem bark of the plant in folklore medicine.

Traditional medicinal practitioners use the stem bark of this plant as a tonic to combat stress and pain. It is also known to exhibit its normalizing activity on brain neurotransmitters, moderate cytotoxicity and antioxidant activity through in vitro experimental studies. Studies have also revealed the antiproliferative, apoptotic and antimutagenic activity from the seed extract of the plant. Even though a large number of compounds have been isolated from root and stem bark, pharmacological studies on these isolated compounds are limited. Plant secondary metabolites have been the most successful source of potential drug leads. Flavonoids are secondary metabolites produced by plants exhibiting a variety of potential biological benefits such as antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal and antiviral activity.

Dengue is a mosquito-borne viral disease prevalent mostly in tropical and subtropical regions of the world. Aedes aegypti is one of the most efficient vectors for the disease. Dengue virus (DENV) is an enveloped virus belonging to the Flaviviridae family with four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4 that genetically share ~65% similarities. This positive-sense flavivirus RNA genome codes for a single polyprotein precursor of 3391 amino acid residues arranged in order NH2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. The N-terminal region of the non-structural protein (NS3) is a serine protease that binds to an NS2B cofactor which is required to cleave the polyprotein. This NS2B-NS3 protease complex is required for viral replication.

Thus, it serves as a promising target for dengue virus antiviral drug development. Disruption of NS2B-NS3 protease inhibits the viral replication. This poses NS2B-NS3 protease of DENV as a promising target for antiviral drug design.

In the current work, we have established a protocol to isolate prenylated flavanone from the stem bark of Polyalthia cerasoides. Further in-silico study of the isolated compound...
was performed with the active site of the dengue protease enzyme. Finally, the isolated molecule was investigated for its possible protease inhibitory activity using a protease assay.

Material and Methods
The stem bark of *Polyalthia cerasoides* was collected from the forest and authenticated through the Department of Biotechnology, Kuvempu University.²² The collected material was dried in an oven at 40 °C for 10 days and powdered using a mixer grinder. 1000 g of powdered stem bark material was extracted with methanol (5 L× 3 times) at room temperature for 24 hrs. The extract was concentrated to dryness under reduced pressure at low temperature yielding 62 g residue, equivalent to 6.2% of the dried plant powder. 40 g of dried extract was dissolved in 500 ml of methanol and partitioned with petroleum ether three times. Petroleum ether fraction (28 g) was subjected to column separation.

Isolation and characterization: Petroleum ether fraction (20 g) was separated by column chromatography (2.5 cm × 80 cm) using silica gel (170 g) (Merck) with a gradient of hexane (Merck): ethyl acetate (Merck). Initially, 100% hexane was reduced to 70% hexane in 5% increments, finally, 100% ethyl acetate and 100% methanol (100 mL of each) were used. In the fractions 8-14 saffron-colored precipitate was observed. Fractions 8-14 were subjected to centrifugation at 10000 rpm for 10 minutes to obtain 180 mg red colored precipitate. The saffron-colored precipitate was further purified using preparative TLC using chloroform (Merck) and methanol (Merck) (96:4) to yield 54 mg of PCSR-1.

Gas Chromatography-Mass Spectrometry (GCMS) analysis: GC-MS analysis of isolated compound (methanolic) was performed at the analytical instrument facility (Shimadzu GC2010 plus instrument) of the organic chemistry department of IISc Bangalore. 1.0 μl of the sample in methanol was injected using split method injection. The MS Spectrum was taken at 70 eV. After the separation in the column, the components were identified and further analyzed.

Docking study: Molecular docking is an important bioinformatics tool to understand protein-ligand interactions and facilitate the design of potent drugs. Docking is a computational technique to predict the binding of the ligand in the active site of the protein and to estimate its binding affinity. In this method, thousands of possible orientations between the ligand and the protein are carried out. Then the best matching mode is evaluated using an energy scoring function, the orientation with the lowest energy score is predicted as the “best match” i.e. the binding mode.¹¹

The structure of the isolated compound, PCSR-1 (ligand) was drawn and optimized using ChemDraw Ultra and Chem3D Ultra respectively. The structure of Dengue virus NS2B/NS3 protease of Serotypes 1 and 4 were obtained from Protein Data Bank (PDB) using PDB ID: 3L6P and 2VBC respectively (http://www.rcsb.org/pdb). The docking of the ligand i.e. inhibitor with the receptor’s structure was performed using AutoDock Vina.¹⁶ The grid box within the dimension was set using AutoGrid to cover the entire receptor molecule so that the ligand can easily lodge with it making the binding pocket.

The grid box having conformation (x = -23.5270, y = -19.2167, z = 27.1656) and (x = -9.8210, y = 3.5911, z = -0.7172) was chosen for the Serotype 1 and Serotype 4 respectively covering the active sites.

Dengue viral protease assay: Purified DENV protease and increased concentrations (10, 20, 30 and 40 µM) of the isolated compound was pre-incubated in a black 96-well plate for 15 min at 37 °C in reaction buffer (50mM Tris HCl (Merck) at pH 7.5, 150mM NaCl (Merck), 0.05% Tween (Sigma-Aldrich), 20% Glycerol (Merck), 1mM DTT (Sigma-Aldrich) 0.25-1.0mM). The synthetic substrate containing DENV protease cleavage site with quencher and fluorophore was added to the above reaction mix and further incubated for 1 hr at 37°C.

Fluorescence was measured using excitation filter 485nm and emission filter 538nm in a fluorometer (Modulus TM Microplate Multimode reader). The difference between the fluorescence obtained in the presence and absence of the compound was taken as the measure of the inhibitory activity.²¹ Quercetin was used as standard. Results represent the average of three independent experiments.

MTT assay- Cytotoxicity assay: HepG2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich) and 10% fetal calf serum (Sigma-Aldrich) at 37 °C and humidified with 5% CO₂. After 24 hrs, the medium was replaced with fresh media containing isolated compound (5, 10, 15, 20 and 25µM) dissolved in Dimethyl sulfoxide (DMSO) (Merck) (1%) and was added to each well and incubated for 72 hrs. At the end of 72 hrs incubation, the medium in each well was again replaced with fresh medium containing 0.5 mg/ml of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) and further incubated for 4 hrs. MTT containing media was replaced with 100 µl of DMSO and optical density (OD) was recorded at 570 nm. Cells without compound treatment were taken as blank and cell viability was calculated using the following formula:

% Viable cell = (OD of Test/OD of Control) × 100

Results and Discussion
Isolation and characterization of flavonoid: 1000 g of *Polyalthia cerasoides* stem bark powder yielded 62 g of a concentrated extract. The obtained methanol extract of stem bark was partitioned with petroleum ether and the obtained petroleum ether fraction was subjected to column
chromatographic separation as described above. Fractions (8-14) containing saffron-colored precipitate were pooled together and dried using a rotary evaporator to obtain brown oil containing saffron precipitate. Saffron precipitate was sedimented by centrifugation at 10000 RPM for 1 minute. From the sediment, 54 mg of compound 1 (PCSR) was isolated using preparative TLC (chloroform-methanol 96:4 solvent system).

PCSR-1 was subjected to GC-MS analysis; chromatogram showed the major peak at 26th minutes with a purity of 70.83% (Fig.1). The EIMS spectral analysis of PCSR showed molecular [m+] ion peak at m/z 280 and after proton migration m/z 279.1588 (Fig. 2) indicating the molecular formula C_{18}H_{16}O_{3} (cal. m/z 280) and C_{18}H_{16}O_{3} (cal. m/z 280). The m/z value and fragmentation pattern of the isolated compound PCSR are in agreement with previously reported compound 6, 4-dihydroxy-3-propane chalcone: a prenylated flavanone.

Fig. 1: GC-MS analysis chromatogram of the isolated compound

Fig. 2: Mass spectrum of Isolated compound PCSR-I

Fig. 3: Structure of 6, 4l dihydroxy 3l propane chalcone (PCSR-I).
Docking study: The docking of the inhibitor (PCSR-1) extracted from the plant *Polyalthia cerasoides* with the DENV NS2B/NS3 protease of Serotype 1 and Serotype 4 revealed the following results.

**Interaction of PCSR-1 with DENV Serotype 1:** PCSR-1 was found to interact with the residues Lys-124 and Asn-202 via hydrophilic bonding and hydrophobic interactions with Ala-214, Ala-216, Lys-123, Asp-125 and Gly-201 with binding energy -10.2 Kcal/mol (Fig. 4 and Fig. 5).

![Fig. 4: Interaction of PCSR-1 with DENV Serotype 1 showing hydrophilic and hydrophobic interactions.](image)

**Interaction of PCSR-1 with DENV Serotype 4:** PCSR-1 was found to interact with the residues via Arg-24, Ile-25, Phe-46, Ile-58, Tyr-23, Leu-65, His-60 and His-41 hydrophobic interactions with binding energy -12.8 Kcal/mol (Fig. 6 and Fig. 7).

![Fig. 6: Interaction of PCSR-1 with DENV Serotype 4 showing hydrophobic interactions.](image)

**Protease assay:** Viral proteases are excellent antiviral targets as evidenced by the several protease inhibitors of the human immunodeficiency virus (HIV) currently in clinical use and the numerous protease inhibitors of the hepatitis C virus (HCV) in clinical trials. In addition, several plant origin inhibitors of viral protease have shown potent antiviral activity in pre-clinical studies.

By analogy with the success of HIV and HCV protease inhibitors, efforts have been made to screen inhibitors against DENV using dengue virus NS2B/NS3 protease as a molecular target. Although to date there are no dengue virus protease inhibitors in clinical trials a simple yet efficient fluorescent high-throughput assay using a fluorogenic substrate is employed for the identification of leads targeting the various viral proteases including dengue protease. We have used a synthetic fluorogenic substrate consisting of three linked modules: a fluorophore, peptide-containing dengue protease site and a quencher. The cleavage of the fluorogenic substrate by dengue protease would separate fluorophore and quencher, the resulting fluorescence is measured by fluorometry. Recent studies have reported the inhibitory effect of natural compounds against viral enzymes including protease. In our study, the inhibitory effect of proteases was tested at a concentration of 8 - 40 μm. The isolated compound showed a significant inhibitory effect against DENV protease as depicted in fig. 8. Standard drug quercetin also reported protease inhibition in a dose-dependent manner.
Fig. 8: Inhibitory effect of PCSR against DENV NS3 protease

Fig. 9: Cytotoxic effect of PCSR against HepG2 cells

**MTT assay:** A comparison of the antiviral effect and toxicity is an important criterion in anti-viral drug discovery. Direct-acting antiviral drugs are expected to inhibit viral replication inside the host cell without causing toxic effects on the host cells. Hence, we have investigated the cytotoxic effect of the isolated compound using MTT cell viability assay. As depicted in fig. 9, 92% of HepG2 cells are viable after treatment with isolated compound at 40 µg/ml concentration.

However, the standard drug quercetin reported only 40% viability of HepG2 cells after treating with 40 µg/ml concentration for 72 hrs. These results indicate that the isolated compound is less toxic than the quercetin. Hence an isolated compound is a better choice to treat dengue infection than that of quercetin.

**Conclusion**

In the present study, we have established a new method to isolate prenylated flavanone 6, 4-dihydroxy-3-propane chalcone. Biological studies indicate that the isolated compound inhibits dengue viral protease in a dose-dependent manner and also the nontoxic effect of the compound against HepG2 cells. Further studies using virus culture and animal models may lead to developing 6, 4-dihydroxy-3-propane chalcone as potent dengue inhibitor.

**References**


