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Cytotoxic activity of isolated constituents from leaves of *Premna serratifolia* on MCF-7 and HT-29 cell lines

Mahesh Biradi and Kirankumar Hullatti

Department of Pharmacognosy, KLES College of Pharmacy, Nehru Nagar, Belagavi 590 010, India.

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Abstract

Premna serratifolia (Syn: *Premna integrifolia*) is an important medicinal herb known as "Agnimantha" in Ayurveda and traditionally used for anticancer activity. The objective of present study was to isolate the cytotoxic phyto-constituents from the n-hexane soluble fraction of *P. serratifolia* leaf extract. Unsaponifiable portion of n-hexane soluble fraction was subjected to silica based column chromatography. The major constituents present in all the sub-fractions were identified by TLC and phytochemical tests. Two constituents were isolated and they were purified. Sub-fractions with isolates were tested for cytotoxic effect by BSL bioassay. Two isolates were found to be active and which were tested on cancer cell lines MCF-7 and HT-29 for their cytotoxicity. Among two isolates, one compound has shown significant cytotoxicity. From the results we conclude that the plant isolates showed cytotoxicity against selected human cancer cell lines.

Introduction

Premna serratifolia Linn., commonly known as Arni or Agnimantha, is a large shrub or a small tree common along the Indian peninsular and Andaman coast. Pharmacologically, it has been reported to have activities such as hepatoprotective (Vadivu et al., 2009), immunomodulatory, cardiac stimulant, antibacterial, cytotoxic, antiarthritic, anti-inflammatory (Biradi and Hullatti, 2013), anti-diabetic (Mujumder et al., 2014), anti-oxidant (Mali et al., 2014), anti-obesity and used in treatment of tumors (Cragg and Newman, 2005).

Plant derived cytotoxic constituents have played an important role in the development of clinically useful anticancer agents (Cragg and Newman, 2005). Various cytotoxic constituents have been isolated from plants and which are used as anti-cancer agents.

Among all cancer types breast cancer is the most common disease in women worldwide with high mortality rate (18%) and relative risk factors, including dietary factors (Ahmed et al., 2014). Colorectal cancer is the third most leading cause of cancer death in both

men and women. About 96% of colorectal cancers are adenocarcinomas, which evolve from glandular tissue (Anonymous, 2013).

However, the efficacy and effect of currently available drugs is very limited and are more toxic on normal cells. By increasing the use of existing knowledge with newer and established screening tests the majority of these cancers and deaths could be prevented.

Although various extracts of *P. serratifolia* are reported to have cytotoxic activity in the literature (Selvam et al., 2009; Singh, 2011), there are no reports available on isolation based study. As part of ongoing investigation *P. serratifolia* plant has been selected for bioactivity guided isolation and here cytotoxic activity of two isolated constituents from the n-hexane soluble fraction (unsaponifiable portion) of methanolic extract of *P. serratifolia* leaves on human cancer cell lines.

Materials and Methods

Collection and authentication of plant material



Leaves of *P. serratifolia* were collected from the premises of Regional Medical Research Centre, (ICMR), Belgaum during January 2011 and authenticated by Dr. Harsha V. Hegde, Scientist 'B' RMRC, Belgaum, India. The voucher specimen is deposited in ICMR Herbarium repository (Accession Number RMRC-554).

Extraction, fractionation and cytotoxicity by BSL assay

The extraction and fractionation of *P. serratifolia* leaves has been done according modified Cos et al., 2006. Based on the results of previous study (Biradi and Hullatti, 2013), *n*-hexane soluble fraction was taken up for present study.

Phytochemical investigation

Phytochemical investigation of isolates was carried out according to the standard procedure (Trease and Evans, 2005; Sandjo and Kuete, 2013).

Optimization of TLC system

Different solvents systems were tried for developing a TLC system for identification of constituents in the extracts and the one with good resolution was selected as the mobile phase for the study. Toluene: Ethyl acetate: Glacial acetic acid (7:2:1) was found to be the most suitable solvent system for *n*-hexane fractions as it gave maximum number of bands.

Isolation of the phytoconstituents

Unsaponifiable portion of *n*-hexane soluble fraction was subjected to silica based column chromatography. The different fractions were generated using gradient elution technique using petroleum ether, chloroform and acetone with increasing the polarity. Five sub-fractions (SF) generated from unsaponifiable portion (i.e. PS/F1/USM). The chemical nature of all the sub fractions was established by TLC (Plate No. 1-3) and chemical tests (Table I).

Processing of the isolated precipitates

The sub fractions PS-01 and PS-02 were purified by washing with cold methanol followed by ethanol. The solid mass was weighed and collected in previously labelled eppendorf tubes and stored in a desiccator.

In vitro cytotoxicity of the isolated compounds on selected cell lines

Brine shrimp lethality (BSL) bioassay

BSL assay was performed for all the five sub fractions to confirm the cytotoxic activity and only the isolates which shown the cytotoxicity were subjected for cell line studies.

Cytotoxicity of the active compounds on MCF-7 and HT-29 cell lines

The active fractions from BSL bioassay were subjected

for *in vitro* cytotoxicity studies using human cancer cell lines MCF-7 (breast carcinoma) and HT-29 (adenocarcinoma).

Cell lines and culture medium (Koba et al., 2009)

HT-29 (adenocarcinoma) and MCF-7 (breast carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in 25 cm² culture flasks in Dubelcco's Modified Eagle's Minimum Essential Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 mg/mL) and amphotericin B (5 mg/mL) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS).

Preparation of test solutions

Stock solution of 1 mg/mL concentration of each test drugs were prepared separately by dissolving in distilled DMSO and made volume with DMEM in 2% FBS and filtered. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 × 10⁵ cells/mL using DMEM containing 10% FBS. To each well of the 96-well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, supernatant was flicked off upon formation of partial monolayer. Then washed the monolayer once with medium and 100 mL of different test concentrations (62.5 to 1000 µg/mL) of test drugs were added on to the monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere and observations were noted every 24 hours interval by microscopic examination. After 72 hours, the drug solutions in the wells were discarded and 50 mL of MTT in PBS was added to each well. The plates were gently shaken and incubated again for 3 hours. The supernatant was removed and 100 mL of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line.

%Growth inhibition = (Mean OD of individual test group / Mean OD of control group) × 100

Results and Discussion

The study was taken to identify the constituents

responsible for cytotoxic activity of *P. serratifolia* leaf extract. The *n*-hexane soluble fraction (F1) has shown significant cytotoxicity in the earlier studies (Biradi and Hullatti, 2013). The unsaponifiable portion which was rich in steroids and triterpenoids was subjected for further fractionations using column chromatography. The column chromatography has yielded 5 sub-fractions (PS-01 to PS-05) with gradient elution. Out of these 5 sub-fractions, PS-01 and PS-02 were found single compound fractions as identified by TLC. The chemical tests and TLC has shown that all the 5 sub-fractions are triterpenoids in nature. The BSL bioassay of these sub-fractions has indicated that PS-01 and PS-02 are more cytotoxic than other 3 sub-fractions LC₅₀ values are depicted in Table II. Further after purification these 2 fractions were analysed on MCF-7 and HT-29 cell lines. Result of this study has indicated that the sub-fractions PS-02 has significant cytotoxicity in both the cell lines which are depicted in Figure 1 and 2 (LC₅₀ value 100.0 and 99.9 µg/mL respectively). Various authors have reported cytotoxic activity of number of triterpenoids such as ursolic acid, oleanic acid and sterols from different plants (Da Silva Filho et al., 2009; Awasare et al., 2012). These data indicate that the certain triterpenoids of *P. serratifolia* may be useful as cytotoxic component that needs further characterization to identify the structure.

Fractions	LC ₅₀
PS-01	54.6 ^a
PS-02	30.8 ^a
PS-03	323.3
PS-04	143.6
PS-05	209.6

^aNote: The fractions with LC₅₀ values ≤100 µg/mL were considered as active fractions

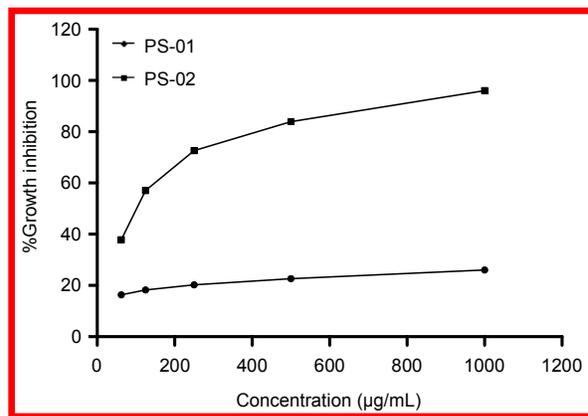
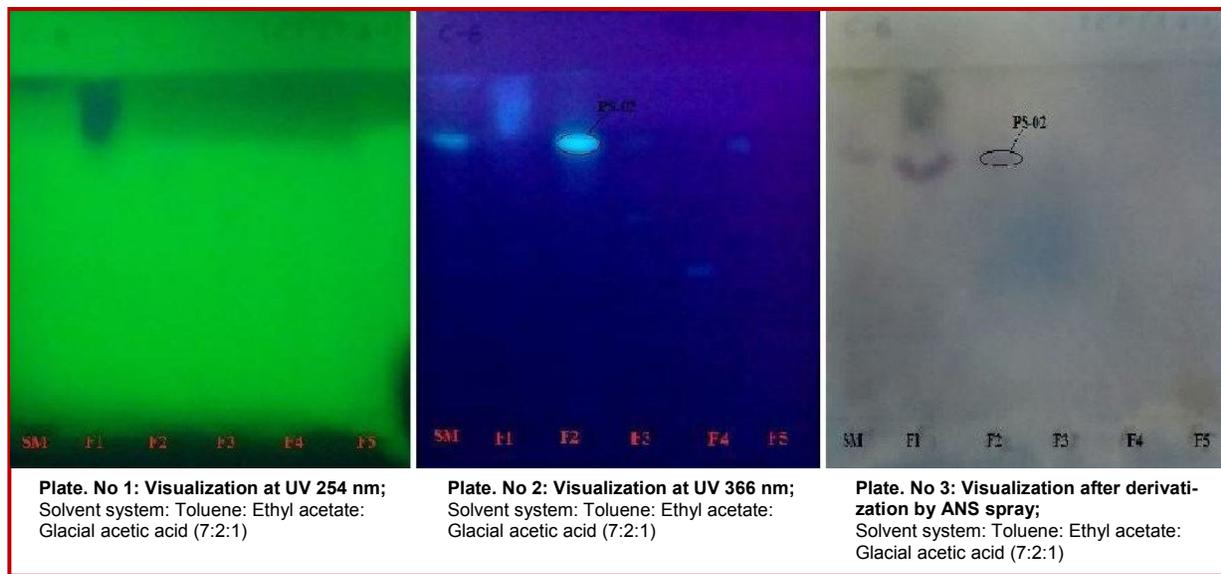


Figure 1: Graphical representation of cytotoxic effect of drugs on MCF-7 cell line



Plates 1-3: TLC of sub-fractions at different pre and post derivatization

Fractions	Gradient program	Physical nature	Number of spots observed	Rf value
PS-01	Petroleum ether	Yellowish and amorphous	2	0.82, 0.62
PS-02	CHCl ₃ :PE (1:4)	White and amorphous	1	0.65
PS-03	CHCl ₃ :PE (1:1)	Yellowish and sticky	3	0.69, 0.49, 0.36
PS-04	CHCl ₃	Yellowish and sticky	2	0.69, 0.36
PS-05	Me ₂ CO	Brownish and sticky	2	0.69, 0.33

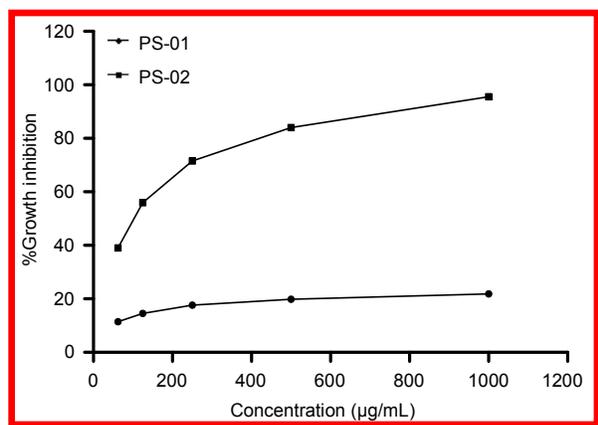


Figure 2: Graphical representation of cytotoxic effect of drugs on HT-29 cell line

From the present study, the triterpenoids of *P. serratifolia* provide a new insight into the anti-cancer approach.

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Author Info

Kirankumar Hullatti (Principal contact)
e-mail: drhullatti@klepharm.edu

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