In vitro Anticancer Effect of Acanthus ilicifolius on Hepatocellular Carcinoma Cells

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ABSTRACT
The leading cause of liver cancer is viral infection with hepatitis B and C virus. Liver cancer usually forms inflammation and cirrhosis caused by these viruses. The aim of the study was to investigate anti-liver cancer potential of Acanthus ilicifolius. Powdered leave material was extracted with ethanol and evaluated their anticancer potential on hepatocellular carcinoma cells (HepG2). Treatment with ethanolic extract of A. ilicifolius (AIEE) posses significant changes in cytotoxicity, apoptosis and caspase 3 expressions in HepG2 cells. The results indicated 100 μg mL⁻¹ of AIEE reduced the viability of HepG2 cells in a dose-dependent manner, elevated lactase dehydrogenase and observed condensed chromatin, crooked vesicle shaped membrane in apoptotic nuclei and dead cells appeared bright orange. The expression of caspase 3 was decreased. This study demonstrates the A. ilicifolius have a potent anticancer source. Further analytical studies will be exploring the active constituents in holy mangrove.

Key words: Caspase 3, ethanol, holy mangrove

INTRODUCTION
Hepatocellular carcinoma (HCC) is the fifth most common cancer in worldwide representing 83%. It represents the third cause of cancer-related deaths and the first cause of death among cirrhotic patients (Farazi et al., 2006). The curative treatment such as tumour resection and liver transplantation are not feasible in advanced stages of HCC (Herold et al., 2002). Furthermore, HCC is well known for its multidrug resistance poor response to current chemotherapeutic agents (Geng et al., 2003). Formation of free radicals leads to oxidative stress which is basic for all disorders, organ dysfunction, including cancer development (Kasai, 1997). Flavonoids are a group of common phenolic plant pigments, they are considered as dietary anticarcinogens and anti-oxidants. Several studies showed the naturally-occurring plant phenols possess substantial anti-mutagenic effects against variety of chemicals (Sreepriya et al., 2001). Researchers focused their interest towards evaluation of new chemotherapeutic agents from ethno medicinally plant bioactive to overcome the side effects caused by the commercial available drugs and improve the survival rate of cancer patients. Acanthus ilicifolius is commonly known as holy mangrove and used as a folklore medicinal sub-shrub belongs to acanthaceae family, which found in wet lands along tidal streams of Southeast coast of India. Historically used in the indigenous system of medicine to treat pain and inflammation, rheumatism, paralysis, neuralgia, asthma, snake bite, leucorrhoea and debility effects (Nayar et al., 1996). The aerial parts of holy mangrove contain acanthicifoline,
lupeol, oleanolic acid, quercetin and glycopyranoside (Rajamanickam, 2010). Furthermore, analgesic, anti-inflammatory, hepatoprotective and antimicrobial activities were validated (Babu et al., 2001). Hence, the present study aimed to evaluate the anticancer potential of *A. ilicifolius* in HepG2 cells.

**EXPERIMENTAL STUDIES**

**Collection and extraction:** Leaves of *A. ilicifolius* were collected from Agaram area of Parangipettai coast, Tamil Nadu, India during January 2008. The voucher specimen (AUCASMB 01/2008) was deposited in the herbarium of C.A.S. in Marine Biology, Annamalai University, Parangipettai, India.

**Preparation of plant extract:** The leaves were dried under shade and then powdered with a mechanical grinder and stored in an airtight with a mechanical grinder and stored in an airtight container. The dried powder material of the leaves (1 kg) was extracted with ethanol by Soxhlet method. The solvent was completely removed by drying and ethanolic extract of *A. ilicifolius* (AIEE) was prepared freshly in distilled water and used for studies.

**Effect of AIEE on HepG2 cells:** HepG2 cell line represents one of the most widely used experimental models for *in vitro* studies on Hepatocellular carcinoma (Emanuele et al., 2002). Therefore, it is very interest to examine the efficacy of AIEE on HepG2 cells.

**Cell culture:** Human hepatoma cell line (HepG2) was obtained from National Center for Cell Sciences, Department of Biotechnology, Pune, India. Cells were routinely grown as monolayer cultures at 37°C in a humidified atmosphere of 5% CO₂ in air Dulbecco's Modified Eagle Medium containing 10% (v/v) fetal calf serum, penicillin (50 IU mL⁻¹) and streptomycin (50 μg mL⁻¹). The medium was changed every 3 days.

**Preparation of drug:** AIEE was dissolved in 0.1% (v/v) DMSO (final concentration of the DMSO not exceeded 0.1% (v/v)) prepared in Dulbecco's Modified Eagle Medium (DMEM) and filtered by 0.3 mm syringe filter and stored at 4°C.

**Cytotoxicity:** The cytotoxicity of AIEE was assessed by cell viability study using Tryphan Blue Exclusion method (Morita et al., 2003). For the determination of cell viability, monolayer of HepG2 cell was trypsinized and seeded at a density of 1×10⁶ cells well⁻¹. After 24 h, the medium was replaced with the serum-free medium (DMEM medium, supplemented with antibiotics penicillin 100 U mL⁻¹, streptomycin 10 U mL⁻¹, 1 mM sodium pyruvate) and the cells were cultured for 24 h to arrest the cell growth. The monolayer of HepG2 was treated with different concentration of AIEE for 48 h and cells incubated with 1% DMSO as solvent control. Both attached and floating cells were collected by trypsinization and an aliquot of the cells was mixed with an equal volume of trypan blue dye. The cells excluding to dye (viable cells) and those taking up dead cells were counted in duplicate using a hemocytometer and the numbers of these cells were expressed as the percent of the total number.

**Lactate dehydrogenase (LDH) leakage assay:** Lactate dehydrogenase leakage assay was performed by the method of Grivell and Berry (1996). 100 mL of sample from the growth medium
of experimental cultures was added to a 1 mL cuvette containing 0.9 mL of a reaction mixture to yield a final concentration of 1 mM pyruvate, 0.15 mM NADH and 104 mM di-sodium hydrogen phosphate after mixing thoroughly, the absorbance of the solution was measured at 340 nm for 45 sec. LDH activity was expressed as moles of NADH used per minute per well. Based on the above studies, 60 and 100 μL mL\(^{-1}\) of AIEE were selected for further experiments.

**Experimental design:** The treatment has been divided into three groups. Group I: Normal HepG2 cells; Group II: HepG2 cells+AIEE (60 μL mL\(^{-1}\)); Group III: HepG2 cells+AIEE (100 μL mL\(^{-1}\)).

**Fluorescent microscopic studies:** Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA binding dye. After treatment with AIEE, the cells were collected, washed and suspended in PBS. After staining with the equal mixture of acridine orange and ethidium bromide (dissolved in 100 g mL\(^{-1}\) of phosphate buffer saline) and the cells were examined under the fluorescent microscope (Di Felice et al., 1998). The differential uptake of these two dyes allows the identification of viable and non-viable cells and the results were recorded.

**DNA fragmentation:** The HepG2 cells were plated in 60 mm culture dish at a density of 1×10⁶ cells and treated with AIEE for 48 h at 37°C. The cells attached at the bottom were scraped off and collected together with unattached cells by centrifuging at 1500×g for 5 min at 4°C. The DNA was prepared from the pelleted cells. The cells were lysed with lysis buffer and extracted with two ml of phenol (TE buffer, pH 7.5) followed by extraction with 1 mL of chloroform and isooamyl alcohol in the ratio of 24:1. The aqueous supernatant was precipitated with 2:5 volumes of ice cold ethanol with 10% volume of sodium acetate at 20°C overnight. After centrifugation at 13,000 rpm for 10 min the pellets were air dried and resuspended with 50 μL of TE buffer containing 0.5 μL of ethidium bromide. After electrophoresis, the gel was photographed under UV light (Chen et al., 1997).

**Expression of p53 and Caspase 3 proteins:** HepG2 cells (1×10⁵ mL) were treated with the AIEE at the concentration of 60 and 100 μg mL\(^{-1}\) for 48 h at 37°C. Cells were lysed with 10 μL of lysis buffer. Cell proteins were separated in a Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of (Laemmli, 1970). 4% stacking gel and 10% resolving gel were used to separate the proteins. After electrophoresis, gel was placed over a nitrocellulose membrane, separate blotting was done for each protein. The gel was packed by three cut-pieces of whatmann filter paper (No. 3). This set up was covered on both sides with absorbers (provided with the system) and clamped. The whole set up was immersed in a tank containing blotting buffer. A current of 25 mA was passed through overnight. Then, the membrane was removed from the system and immersed in methanol for a minute. The membranes were blocked by treating with the blocking solution for 1 h at 37°C. After washing, the membranes were incubated with anti mouse p53 (1:100) and anti-mouse caspase-3 (1:1000) for 1 h at 37°C. After three washes in PBS/0.1% and Tween 20, the membrane was incubated with Horse Raddish Peroxidase (HRP)-conjugated anti-mouse IgG antibody for 1 h at 37°C. Diaminobenzidine reagent was used to develop the immuno blots.

**RESULTS**

The total yield from the extraction was approximately 39.5%. Table 1 represented the viability of control and AIEE treated (20, 40, 60, 80 and 100 μg mL\(^{-1}\)) HepG2 cells after 48 h. The results
showed that treatment with AIEE markedly reduced the viability of HepG2 cells in a dose-dependent manner. It was observed that LDH activities found to be significantly (0.57±0.02) elevated after 48 h of exposure in the medium containing 100 µg mL⁻¹ AIEE when compared to the control. The morphological changes of control and 60 and 100 g m⁻¹ of AIEE treated HepG2 cells shown in Fig. 1. Progressive structural alterations and reduction of HepG2 cell populations were observed in both the concentrations but compared to group 2, group 1 indicated highly significant destruction of the monolayer, swelling and rounded morphology of HepG2 cells with condensed chromatin, membrane also became crooked and vesicle shaped was observed by the light microscope which was not seen in control. The fluorescent microscopic picture of control and treated group were represented in Fig. 2. The above-normal live cells were appeared bright green whereas drug treated group of dead cells appeared luminous orange. In addition to this, ordinary nuclei showed chromatin with an organized structure, while drug treated group showed highly condensed chromatin in HepG2 cells.

An electrophoresis pattern of DNA of normal and treated (60 and 100 µg mL⁻¹) group was represented in Fig. 3. There was no fluorescence signal observed in control compared to the treated group. The signal expressed fragmented laddering pattern of DNA and indicated characteristics of apoptosis. The protein expression in control and treated (60 and 100 µg mL⁻¹) groups were studied by western blotting assay Fig. 4. The band intensity was 53 kDa of protein in the treated group. The accumulation of p53 protein indicated expression of tumour suppressor protein- induced apoptosis in AIEE treated group. The expression of caspase-3 was observed that the treatment of HepG2 cells with AIEE reduced the intensity of 32 kDa. These results strongly suggest that AIEE treatment stimulated proteolytic cleavage of caspase-3 protein and initiate the apoptosis.
Fig. 2(a-c): Fluorescence microscopic observation of control and AIEE treated HepG2 cells, (a) Control, (b) AIEE (60 µg mL⁻¹) and (c) AIEE (100 µg mL⁻¹)

Fig. 3: DNA fragmentation, M: DNA marker (100 bp DNA ladder), Lane 1: Control, Lane 2: AIEE (60 µg mL⁻¹) and Lane 3: AIEE (100 µg mL⁻¹)

DISCUSSION

The curative treatment such as tumour resection and liver transplantation are not feasible in progressive stages of human liver cancer. Therefore, searching for effective chemotherapeutic agents is important to improve the survival rate of patients with foremost or recurrent human liver cancer. Recently, the cytotoxic effects of various chemicals and natural substances on malignant tumour cells in culture have been extensively studied as a primary screening for anti-tumour activities. In the present study, AIEE markedly reduces the cell viability by induction of cell death
rather than the inhibition of cell proliferation in a concentration dependent manner. LDH is a more reliable and more accurate marker to study the cytotoxicity of the extract because damaged cells are fragmented completely during prolonged incubation with toxic substances (Satyavani et al., 2011). In the present study, the LDH was increased significantly in 100 \( \mu \text{g mL}^{-1} \) of AIEE treated HepG2 cells when compared with the control cells.

Apoptotic induction has been a new target for anticancer drug discovery. In this connection, light and fluorescence microscopic observation, DNA fragmentation and protein expressions in AIEE treated, HepG2 cells can be characterized by morphological and molecular changes such as cell volume, cell shrinkage, reduction in chromatin condensation and formation of cytoplasmic blebs. Apoptotic cells often produce a unique ladder composed of nucleotide fragments at an interval of 180-200 base pairs, which can be visualized by DNA-agarose gel electrophoresis. In this context, Bai and Cederbaum (2003) reported the Vp16 and mitomicin C chemotherapeutic drugs induced cancer cells to undergo apoptosis through damage of nuclear DNA. In the present study, DNA ladders appeared in AIEE treated HepG2 cells. From this observation, it is inferred that A. ilicifolius may exert an anticancer effect through DNA damage in HepG2 cells and promote apoptosis. Tumour suppressor gene p53 is one of the crucial genes that regulate the onset of DNA replication around G1/S boundary. Furthermore, p53 mediated tumour suppression appears to be necessary for therapeutic potential in the treatment of tumour (Ryan et al., 2001). Therefore, normal p53 expression and function are crucial to prevent the propagation of genetically damaged cells and to prevent proliferation of cells under stress conditions. It is well known that after initiation of the apoptotic program, the release of cytochrome C from mitochondria triggers the activation of caspase-3 and the consequent rapid cleavage of PARP, a substrate of caspase-3 proteins and initiates the apoptosis (Emanuele et al., 2002).

Earlier researchers have indeed confirmed that HepG2 cells express a wild-type p53 protein that can be activated to elicit normal p53 function (Muller et al., 1997). In the present study, AIEE treated HepG2 cells shows upstream regulation of p53 protein expression at the concentration of 100 \( \mu \text{g mL}^{-1} \) after 48 h of incubations. Hence, A. ilicifolius may enhance the susceptibility of HepG2 cells to undergo apoptosis by attenuating the tumour suppressor protein. Generally, Caspase is present as inactive proenzymes, most of which are activated by proteolytic cleavage. Caspase-3 may then cleave vital cellular proteins or activate
additional caspase by proteolytic cleavage (You et al., 2001). AIEE treated HepG2 cells showed low intensity of 32 kDa protein which is correlated with Budihardjo et al. (1999) report.

The present study clearly demonstrates anticancer potential of A. ilicifolius evidenced with morphological and molecular changes in HepG2 cells.

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REFERENCES


