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The apoptosis inducing effect of *Glycosmis pentaphylla* (Retz.) Correa and its influence on gene expression in hepatocellular carcinoma cell line, Hep3 B

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ABSTRACT

Ethnopharmacological relevance: Glycosmis pentaphylla (Retz.) Correa is used in Indian traditional medicine against jaundice and other liver disorders.
Aim of the study: The study aims to determine the *in vitro* anticancer and apoptosis inducing activity of *Glycosmis pentaphylla* in hepatocellular carcinoma cell line, Hep3 B.
Materials and methods: The cytotoxic and apoptosis inducing activity of the crude extract and active fractions were estimated on Hep3 B and RAW264.7 cell lines by MTT assay, Hoechst staining, DNA fragmentation, morphological studies, reverse transcription polymerase chain reaction and *anti-poly-(ADP-ribose)-polymerase assays*. The phytochemical profiling of active extract was done by TLC and HPTLC methods.
Results: Ethanol extract of *Glycosmis pentaphylla* was more effective than other extracts in reducing the proliferation of Hep3 B cells. As revealed by the results from DNA fragmentation, Hoechst staining, morphological studies, RT-PCR, PARP cleavage and gene expression studies, active extract induced apoptosis on Hep3 B cell line in concentration and time dependent manner with increase in the Bax/Bcl2 gene expression ratio. Chemo profiling data revealed the presence of flavonoid in the active fraction.
Conclusions: The study showed that major active component in the ethanol extract of *Glycosmis pentaphylla* is a flavonoid which induces apoptosis on cancer cell line, Hep3 B, by increasing the expression ratio of Bax/Bcl2 genes in a time and dose dependent manner.
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1. Introduction

Hepatocellular carcinoma (HCC) has gained major clinical interest because of its worldwide increasing incidence (El-Serag and Mason, 1999). Liver cancer is the fifth most common cancer in the world (564,000 cases/year) and the third cause of cancer related death (Parkin et al., 2001). A complete cure for this disease is not available. But today chemotherapy is considered as one of the important treatment options for prolonging the patient's life. It has been found that most of the cancer chemotherapy drugs exert cytotoxicity on malignant cells by inducing apoptosis (Kaufmann and Earnshaw, 2000). Apoptosis is a well-identified biological response exhibited by cells when subjected to DNA damage. It is a useful marker for screening compounds for subsequent development as possible anticancer agents (Srivastava and Gupta, 2007).

Glycosmis pentaphylla (GP) belongs to Rutaceae family. It is commonly known as Ashvashakota, Vananimbuka (Sanskrit), Bannimbu (Hindi) and Paanal (Malayalam). The plant is used in indigenous medicine for fever, cough, rheumatism, anaemia and liver disorders (Sastri, 1956; Kirthikar and Basu, 1991). The antioxidant and hepatoprotective activity of GP is already reported by different groups (Trease and Evans, 1972; Mitra and Sur, 1997; Gupta et al., 2011; Nayak et al., 2011). The present study was conducted to determine the anti-HCC activity and molecular mechanism behind the activity of GP in Hep3 B cell line.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) and N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) were purchased from Gibco-BRL, USA. Trypsin, *Hoechst* 33342 DNA stain, ethidium bromide, methyl thiazolyl blue tetrazolium bromide (MTT), silymarin and sodium dodecyl sulphate (SDS),

Abbreviations: GP, *Glycosmis pentaphylla* (Retz.) Correa; DMEM, Dulbecco's Modified Eagle Medium; SQ-RTPCR, semi quantitative reverse transcriptase polymerase chain reaction; dNTP, di nucleotide triphosphates; G3PDH, glyceraldehyde 3 phosphate dehydrogenase; BCL2, B-cell lymphoma 2; BAX, Bcl2 associated X protein; PARP, poly ADP-ribose polymerase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

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TLC plates were purchased from Sigma, USA. Tris and low melting point agarose (LMPA) were purchased from Sisco, Bombay. $NaHCO_3$ and KH_2PO_4 were purchased from Hi Media, Bombay. All other chemicals and reagents used were of analytical grade.

2.2. Cell lines

Human hepatocellular carcinoma (HCC) cell line, Hep3 B and murine macrophage cell line RAW264.7 were purchased from American Type Culture Collection (ATCC), Manassas, USA. Cells were maintained in DMEM containing HEPES and sodium bicarbonate supplemented with 10% foetal bovine serum (FBS) and 1× antibiotic–antimycotic mix solution. Cells were incubated at 37 °C in a humidified, 5% CO_2 atmosphere (Hera cell 150, Heraeus, Langenese, Germany).

2.3. Preparation of plant extract

GP was collected from Palghat, Kerala, India and authenticated by experts of Ayurveda Research Institute, Thiruvananthapuram, India. A voucher specimen was kept in the Institute herbarium (Ethno 41). The shade dried whole plant was powdered, sieved and extracted with alcohol. Ten grams of dried powder was Soxhlet extracted with 400 mL of alcohol for 24 h. The percentage yield of alcohol extract in our study was approximately 5.2%. The Soxhlet extraction was continued until a drop of the solvent from the siphon tube when evaporated does not leave a residue. Then the extract was collected and the solvent evaporated under vacuum in a rotary evaporator (VV 2000, Heidolph, Schwabach, Germany). A stock solution of silymarin and solvent extract (100 mg/mL) were prepared in DMSO and stored at 4 °C. Test solutions were prepared on the day of experiment by diluting the stock solution with DMEM to get the desired concentration. Maximum concentration of DMSO was maintained as 0.1% (v/v).

2.4. Cytotoxicity screening

2.4.1. Cell culture

Hep3 B and RAW264.7 cells were used for cytotoxicity screening. Cells were cultured in DMEM supplemented with 10% heat inactivated FBS and antibiotics. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air (Sheeba and Asha, 2009).

2.4.2. MTT assay

The MTT colorimetric assay (developed by Mosmann, 1983; Popiolkiewicz et al., 2005) was used with minor modifications to screen the cytotoxic activity of the alcohol extracts (Wills and Asha, 2009). Briefly, the cells were seeded in 96-well plates (Greiner, Frickenhausen, Germany) with a seeding density of 5×10^3 cells/well. The cells were treated with different concentrations of the extract (25, 50 and 100 μg/mL), silymarin (50 μg/mL), DMSO 0.1% (v/v) and incubated for different time periods (24 and 48 h). After incubation, 50 μL of 5 mg/mL MTT in phosphate buffered saline (PBS) was added. After incubation for 4 h, 150 μL of 10% SDS in DMSO was added to stop the reaction and incubated overnight at 37 °C. Absorbance was read at 570 nm using a 96-well microplate reader (Bio-Rad, Hercules, CA, USA).

The percentage growth inhibition and percentage viability of the culture were calculated according to the following equation:

$$\text{Percentage of growth inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group} \times 100}{\text{Mean OD of the control}}\right)$$

2.5. Cell morphology studies

Cells were seeded in 6-well plate with a seeding density of 1×10^5 cells/well, and treated with 25, 50 and 100 μg/mL concentrations of extracts for 24, 48 and 72 h intervals. The morphological variations were observed and photographed with CCD camera attached with phase-contrast microscope ((Leica DFC 280) Leica Microsystems Digital Imaging, Cambridge, UK).

2.6. Detection of apoptosis

2.6.1. Chromatin condensation and apoptosis measurement/Hoechst staining

Chromatin condensation is one of the hallmarks of apoptosis. The changes in chromatin morphological features were detected by Hoechst 33342 staining (Diaz-Ruiz et al., 2001). Briefly, cells were seeded in 6 well plates. After treatment with different concentrations of extract for desired time periods, the cells were incubated with Hoechst stain (1 μg/mL) for 30 min. Condensed chromatins were observed by fluorescent microscope (Leica DFC 280 CCD camera).

2.6.2. DNA fragmentation analysis

The cells were seeded in 6 well plates and treated with different concentrations of GP extract (25, 50 and 100 μg/mL), silymarin (50 μg/mL), vehicle control (DMSO, 0.1%) for 24, 48 and 72 h. After treatment, the DNA fragmentation was assessed by method as described earlier (Radhika et al., 2010). Briefly, after extract treatment the cells were harvested, pelletized and treated with lysis buffer containing 1 M Tris, 5 M NaCl, 0.2 M EDTA and distilled water. DNA was extracted using phenol/chloroform/isopropanol (25:24:1, v/v/v) and re-extracted with chloroform/isopropanol (24:1, v/v). The DNA was precipitated with 2.5 M ammonium acetate and ethanol at −20 °C for 1 h and was centrifuged at 13,000 rpm for 10 min. Precipitated DNA pellete was dissolved in Tris EDTA buffer. After RNase treatment total DNA was run on a 2% TBE-agarose gel containing 0.05 mg/mL ethidium bromide. DNA ladder on the gel was visualised using UV trans-illuminator and the image was recorded by Quantity One Gel documentation system (Bio-Rad).

2.7. Differential gene expression studies by SQ-RTPCR

2.7.1. RNA isolation and RT-PCR

Hep3 B cells were seeded in 6 well plates and treated with GP alcohol extract and DMSO. The untreated cells were maintained as the control. After treatment the total RNA was isolated by Trizol Reagent method (Suresh and Asha, 2008). The RNA was quantified using spectrophotometer at 260 nm. cDNA was prepared from the total RNA (2 μg) by using an Oligo (dT) 18 mer as a primer in a 20 μL reaction mixture containing 200 units of reverse transcriptase, 10 mM dNTPs at 42 °C for 1 h. Two microliters of the cDNA was used for the PCR reaction as template. The PCR was performed in buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 mM of each primer and 5 units Taq DNA polymerase in a thermal cycler. The expression levels of Bax, Bcl2, p53 and G3PDH (Internal Control) were analysed by PCR amplification of these genes using specific primers at specific annealing temperatures (Table 1). The resulting PCR products were analysed by 1.5% agarose gel electrophoresis. The bands on gel were measured by densitometric analysis with Gel documentation system using Quantity One Software (Bio-Rad, USA). The values were normalised by calculating the densitometric ratio with that of internal control (G3PDH) as base line.

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