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Glycopentalone, a novel compound from *Glycosmis pentaphylla* (Retz.) Correa with potent anti-hepatocellular carcinoma activity

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ABSTRACT

Ethnopharmacological relevance: *Glycosmis pentaphylla* (Retz.) Correa is used in Indian traditional medicine against various liver ailments, including cancer.
Aim of the study: Isolation and characterization of the most active anti-hepatocellular carcinoma (HCC) compound from the alcohol extract of *G. pentaphylla.*
Materials and methods: Different chromatographic (HPLC, TLC and column chromatography) and methods like IR, LCMS and NMR were used for the isolation and structural identification of the active anti-HCC compound from *G. pentaphylla.* Cytotoxic and apoptosis inducing effect of the active compound were assessed in Hep3 B, RAW264.7 and HEK293 cell lines by MTT assay, morphological studies, Hoechst staining and Annexin V FITC assay.
Results: The most active compound was isolated as yellow needle shaped crystals. The structure of the compound was identified by IR, LCMS and NMR methods. The structural details show that the isolated compound is a novel chemical and have structural similarity with chalcone. MTT assay, physiological and FACS analysis proved the anti-HCC efficacy of the isolated compound in vitro.
Conclusion: The study confirmed that the most active anti-HCC compound present in the alcohol extract of *G. pentaphylla* is a chalcone derivative. This compound showed specific cytotoxicity against Hep3 B with minor cytotoxicity against non HCC cell lines, RAW264.7 and HEK293. The present study, therefore, supports the folklore knowledge for the utility of *G. pentaphylla* and provides a scientific basis for their traditional usage against liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) or primary liver cancer is one of the most common malignancies in the world and the second leading cause of cancer related death. It is estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total) (Llovet et al., 2003; Bosch et al., 2004; Ferlay et al., 2012; Globocan, 2012 and Stewart and Wild, 2014). Among the different "treatment strategies" chemotherapy is the most dependable option against HCC. More than 60% of the current anti-cancer drugs have their origin from natural sources including plants. Natural products were one of the main sources of pharmaceuticals for thousands of years, and have made enormous contributions towards maintaining human health.

G. pentaphylla belongs to Rutaceae family. It is commonly known as Vananimbuka in Sanskrit and Paanal in Malayalam. In India, Ayurvedic and other traditional medicinal practitioners have used *G. pentaphylla* for the treatment of various ailments like cough, rheumatism, anemia, arthritis, jaundice and facial inflammation (Sastri, 1956; Gopi, 2000 and Mohammed et al., 2010). The traditional healers in Gazipur district of Bangladesh utilize *G. pentaphylla* for prevention of all forms of cancer (Ariful et al., 2010). Hepatoprotective, anti-cancer, anti-inflammatory, anti-bacterial, anti-oxidant and anti-arthritic activities of Glycosmis have been reported previously by many researchers (Abbas et al., 2011; Amran et al., 2011; Gupta et al., 2011; Nayak et al., 2011; Sreejith et al., 2012a; Sivakumar et al., 2014; Yang et al., 2014 and Prawej et al., 2015). Recent studies from our lab showed that the alcohol extract of *G. pentaphylla* effectively induced apoptosis in hepatocellular carcinoma cell line (Sreejith et al., 2012b). The present study focuses on the active principle in the *G. pentaphylla* alcohol extract responsible for apoptosis induction in HCC cell lines. The study intended to isolate and characterize the most active anti-HCC compound from the active extract by different chromatographic and spectroscopic methods and the most active anti-HCC compound present in the

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IR, Infra red; LCMS, Liquid chromatography mass spectrometry; NMR, Nuclear magnetic resonance

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alcohol extract of *G. pentaphylla* is a novel chalcone derivative and named Glycopentalone. The results therefore support the folklore knowledge for the utility of *G. pentaphylla* and provide a scientific basis for their traditional usage against liver cancer.

2. Materials and methods

2.1. Cell lines

Human hepatocellular carcinoma cell lines–Hep3 B, Murine macrophage cell line–RAW264.7 were purchased from American Type Culture Collection (ATCC), Manassas, USA. Cells were maintained in complete DMEM containing 10% FBS and antibiotics. Activated hepatic stellate cell line (LX2) was a kind gift from Dr. L. S. Friedman of Mount Sinai School of Medicine, USA. LX2 cells were maintained in 2% FBS and 1X antibiotic–antimycotic mix solution in DMEM. Cells were incubated at 37° C in a humidified, 5% CO_2 atmosphere (Hera cell 150, Heraeus, Langenese, Germany).

2.2. Plant collection and extract preparation

G. pentaphylla was collected from Palghat, Kerala, India during March and April to ensure maximum dry weight per unit volume of the plant, and a voucher specimen was maintained at the institute herbarium (Ethno 41). The collected plants were authenticated by D. Padmaja, Regional Ayurvedic Research Center, Thiruvananthapuram. The plant was shade dried, powdered, sieved and extracted with solvents having different polarity. Ten grams of *G. pentaphylla* whole plant powder was soxhlet extracted with 400 mL of solvents like hexane, ethyl alcohol and chloroform. The extract was collected and the solvent evaporated under vacuum in a rotary evaporator (VV 2000, Heidolph, Schwabach, Germany). For water extract preparation, the plant powder was extracted with distilled water (10 g in 400 mL). Then it was freeze dried in a lyophilizer. The percentage yield of each extracts is as follows – hexane 4.1%, chloroform 7%, ethyl alcohol 5.2% and water 9.3%. Equal concentration of all the four extracts (water, ethyl alcohol, chloroform and hexane) was tested for their anti-HCC activity in Hep3 B, HepG2, HEK 293, LX2 and RAW 264.7 cell lines. Preliminary studies showed that among the four different extracts alcohol extract showed maximum anti-HCC activity in HCC cell lines. Therefore, alcohol extract was used for further studies to isolate and characterize the active compound (Supplementary Fig 1 and 2).

2.3. Column chromatography

The most active fraction of the alcohol extract of *G. pentaphylla* has been identified in our previous research (Sreejith et al., 2012b). Therefore, the alcohol extract was subjected to column chromatography to isolate the fraction containing the most active compound. Silica gel (60–120 mesh size) was used as the stationary phase while differerent solvent combinations of increasing polarity were used as the mobile phase. The silica column was prepared by mixing 150 g of silica powder and 350 ml of hexane. The sample was prepared in a ceramic mortar by adsorbing 10 g of the extract in methanol to 20 g of silica, followed by drying. The dried powder was then gently layered on top of the packed silica column. The following ratios of solvent combinations were sequentially used in the elution process; Hexane: ethyl acetate 100:0, 80:20, 70:30, 60:40 and 50:50. Purity of the eluted fractions were checked using analytical TLC. Contaminated fractions were pooled together and elution was repeated. 260 mg pure compound in the fraction was collected for further studies.

2.4. High performance liquid chromatography (HPLC)

5 μL of the isolated compound in acetonitrile (1 mg/ml) was analyzed using Gilson analytical HPLC with 321 Binary gradient pump and 156 UV/VIS detector (Gilson, U.S.A) to confirm the purity of the isolated compound. Fractions were analyzed using a Kromasil 100C18 column (250 nm × 4.6 nm) with particle size of 5 μm. Acetonitrile: water at different ratios (70:30 and 50:50) were used as the mobile phase with a flow rate of 1 ml/min. Eluted compound in the column was detected at 274 nm.

2.5. Identification of the percentage content of active compound in the alcohol extract

UV/VIS spectrophotometeric method was used for identifying the percentage content of the active compound in the alcohol extract. Different concentrations of the active compound ranging from 1, 3, 5, 7 to 9 μg/ml was prepared. The serially diluted compound was then scanned with UV/VIS spectrometer and a standard graph was plotted based on the absorption values. With reference to the standard graph, concentration of the active compound in the alcohol extract was identified (Wilson and Hagerman, 1990; Venkatesh et al., 2004; Singh and Kalaiselvan, 2011; Suresh et al., 2015).

2.6. Structural identification of the most active compound

2.6.1. Fourier transform infra-red spectrometer (FT-IR)

FT-IR spectra of the active compound was recorded in the range 4000–μ400 cm^{-1} in FT-IR spectroscopy, Thermo Nicolet, Avatar 370.

2.6.2. Elemental analysis of the compound

The percentage content of different elements like carbon, hydrogen and nitrogen in the isolated compound was analyzed with Elementar Vario EL III instrument.

2.6.3. Liquid chromatography mass spectrometry (LC/MS)

Total mass of the isolated compound was identified by LC/MS. Analytical LC/MS was carried out using Waters equipment (Waters 3100 Mass Detector with ESI unit): an Xbridge C18 column (4.6 mm × 150 mm; 5 μm particle) for separation and a Waters 2996 photodiode array as detector. Mass spectra were scanned in ESI negative mode in the range between 50 and 1000 m/z for five minutes. Empower2 software was used for data analysis.

2.6.4. Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra of the isolated compound were documented on a Bruker Avance III, 400 MHz NMR spectrometer. The chemical shifts were reported as parts per million (ppm). Tetramethyl silane (TMS) was used as internal standard.

2.7. Detection of anti- HCC activity of the active compound

2.7.1. MTT assay

MTT assay was used with minor modifications to screen the cytotoxic activity of the isolated active compound (Mosmann, 1983; Radhika et al., 2010). Briefly, Hep3 B, HEK293 and RAW264.7 cell lines were seeded in 96 well culture plates (Greiner, Frickenhausen, Germany) with a seeding density of 5×10^3 cells/well. The cells were treated with different concentrations of the active compound (1, 3 and 5 μg/ml), silymarin (50 μg/ml) and DMSO (0.1% v/v). After incubation for 48 h, 50 μl of 5 mg/ml MTT in phosphate buffered saline was added. After 4 h, 150 μl of 10% SDS in DMSO was added to stop the reaction and incubated overnight at 37° C. Absorbance was

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