Research Article

Susceptibility of Human Hepatocellular Carcinoma Cell Lines (HepG-2) in *Linderina madayiparense* Extracts

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ABSTRACT

Cancer is a complex disease and the second major cause of death in human. Generally it is associated with escalating effects both at the molecular and cellular levels. A combination of surgery, radiation therapy, and chemotherapeutic agents are involved for the treatment of cancer but still cancer remains associated with high mortality. Many medicinal plants have been reported for the management and treatment of various cancer and cancer-related conditions by the local herbalists. *Lindernia madayiparense* (Family: Linderniaceae) is widely distributed in the laterite hills, Kerala, India. Petroleum ether, ethyl acetate and ethanol extracts of *L. madayiparense* were prepared by continuous hot extraction method using Soxhlet apparatus. All the extracts were assessed for their *in-vitro* cytotoxic effect on Human hepatocellular carcinoma cell lines (HepG-2 cell line) by MTT assay. Pet ether extract exhibited the maximum cytotoxic effect (92.47%) at a concentration of 1000 µg/ml against the HepG-2 cell lines followed by ethyl acetate extract (92.22%) and ethanol extract (84.35%). The effective CTC_{50} value for pet ether extract of *L. madayiparense* was found to be 149 µg/ml.

Keywords: Human hepatocellular carcinoma cell line, *Linderina madayiparense*, Cytotoxicity MTT assay.

INTRODUCTION

Cancer is a disease caused by uncontrolled cell division of abnormal cells. Cancer is the second leading cause of death after cardiovascular disease. The most frequent types of cancer worldwide in order of the number of global deaths are due to lung, stomach, liver, colorectal, esophagus and prostate cancers among men and breast, lung, stomach colorectal and cervical cancers among women. Smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation are the major causes of cancer.[1] Cancer related deaths are predicted to increase to over 11 million in 2030.[2] Hepatocellular carcinoma (HCC), a primary liver cancer, is one of the most frequent tumors representing the fifth commonest malignancy worldwide and the third cause of mortality from cancer. Still the treatment of liver cancer is unsatisfactory because liver cancer cells are present p53 gene mutations and tend to be more aggressive and extremely resist to chemotherapy.[3] Moreover due to the late diagnosis and poor treatment efficacy with the available chemotherapeutic agents associated with other standard cancer therapies such as surgery to remove the major cancer cells and subsequent radiotherapy and metastasis to other organs affect the prognosis and survival of cancer patient.[4,5] Liver cancer is largely intractable to chemotherapy because of tumor heterogeneity and the development of multidrug resistance phenotypes.[6]

Medicinal plants have an important key role in the discovery and development of new therapeutic agents. In developing countries, the practice of medicine still relies heavily on plant and herbal extracts for the treatment of various human ailments.[7] Traditional medicine as an alternative therapy used for maintaining health, boosting immune system function, prevention, therapy and remission of cancer.[8] According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products.[9] India is one of the countries blessed with rich biodiversity and massive treasure of herbal plants. Many indigenous systems such as Ayurveda, Yoga and Siddha are more prominent and prevailing in India from decades.[10] Lot of researches demonstrate the epidemiological evidence of some plants as a whole, their parts or identified ingredients with specific biological properties which have substantial protective effects on human carcinogenesis. The use of medicinal herbs is still a tradition adopt by ethnic communities who are living in undulating plains and at foothills of dense forest and some of the traditional medical practitioner.[11] Therefore, the search for new phytochemical(s) extracted from plant as potential cytotoxic agents for hepatoblastoma is an important line of research in the discovery of novel natural anticancer drugs.

Lindernia madayiparense (Family: Linderniaceae) is a plant discovered first from the laterite hills, Madayipara, Kannur District, Northern Kerala, India. The Plant is used by the tribal inhabitants and local villagers for therapeutic purposes since long time.[12] But there is no scientific evidence for the pharmacological effects of this plant. Therefore, a detailed phytopharmacological study is warranted to explore the major active compounds and related bioactivities of this plant. Hence, the present study was undertaken to explore the *in-vitro* cytotoxic

effect of *L. madayiparense* plant extracts against human hepatocellular carcinoma cell lines (HepG-2 cell line).

MATERIALS AND METHODS

Collection and identification of Plant Materials

Wild crafted plant, *Linderina madayiparense* was collected during its flowering season in the month of October to December, 2013 in Kannur District, Kerala, India. The plant material was identified and authenticated by botanist Mr. P. Biju, Assistant Professor, Government College, Kasaragod, Kerala, India.

Preparation of Plant Extracts

Various extracts of *L. madayiparense* were prepared by continuous hot extraction method using Soxhlet apparatus.[13] The whole plant, *L. madayiparense* was washed thoroughly with distilled water and dried under air-shade and pulverized mechanically into coarse powder. The coarse powder (1000gm) has been successively extracted with three different solvents by changing the solvent polarity from non polar to polar solvents such as petroleum ether, ethyl acetate and ethanol respectively. At the end of each successive extraction, the mixture was collected and concentrated using rotary vacuum evaporator under reduced pressure to remove the excessive solvents. All the extracts obtained were freeze dried to get an absolute extracts and stored properly and preserved in 8^0C until further use. The percentage yield of all the extracts was calculated.

Preliminary phytochemical studies

The various extracts of *L. madayiparense* were subjected for preliminary qualitative analysis to identify the presence of phytochemical constituents such as alkaloids, carbohydrates, glycosides, flavonoids, steroids, triterpenoids, phenols, proteins, tannins etc. as per the standard methods.[14] The presence and absence of the secondary metabolites in the extracts was noted in table 1.

Evaluation for cytotoxic activity

Chemicals

All the chemicals used were analytical grade. 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle Medium (DMEM) and Trypsin (Sigma Aldrich); EDTA, Glucose and Antibiotics (Hi-Media); Dimethyl sulfoxide (DMSO) and Propanol (E. Merck) were used.

Cell lines

Human hepatocellular carcinoma cell lines (HepG-2 cell line) were used in this study and procured from National Centre for Cell Sciences (NCCS), Pune, India.

Culture medium

Stock cells of HepG-2 cell line were cultured in DMEM and supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). 25 cm^2 culture flasks were used to grow the stock cultures and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of test Solutions

Each weighed pet ether, ethyl acetate and ethanol extracts were separately dissolved in distilled DMSO and the volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1000 µg/ml concentration and sterilized by filtration. From this stock solution, four different lower dilutions (500, 250, 125 and 62.5 µg/ml) were prepared. [15, 16]

Determination of cell viability by MTT Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added to each well of the 96 well microtitre plates. After 24 h, when a partial monolayer was formed, the supernatant was flicked off and washed the monolayer once with medium. To this, 100 µl of different test concentrations of pet ether, ethyl acetate and ethanol extracts of *L. madayiparense* were added on to the partial monolayer in the respective microtitre plates. The plates were then incubated at 37^0C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the test substance in the wells was discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^0C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added. All the plates were gently shaken to solubilize the formed formazan. Then the absorbance was measured using a micro plate reader at a wavelength of 540 nm using UV-visible spectrophotometer. The percentage growth inhibition was calculated using the following formula and recorded (table 2). The concentration of the extract needed to inhibit the cell growth by 50% (CTC_{50} value) was determined from the dose-response curve (Figure 1) for the cell line. [15, 16, 17]

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

RESULTS

The percentage yield of pet ether, ethyl acetate and ethanol extracts of *L. madayiparense* were ranged from 4.78% - 26.40%. Among these three extracts obtained ethanol extract had the highest percentage yield (26.40%)

followed by ethyl acetate extract (15.46%) whereas pet ether extract had the lowest percentage yield (4.78%).

Phytochemical tests revealed that the presence of coumarins and saponins in all the three extracts while the absence of proteins and amino acids were found in these all extracts. Oils and fats were oresent only in pet ether extracts. Alkaloids, carbohydrates and glycosides, flavonoids, steroids, terpenoids, tannins, and phytosterols are present in both ethyl acetate and ethanol extracts of L. madayiparense (Table 1).

Table 1: Preliminary phytochemical analysis of various extracts *L. madayiparense*

Phytoconstituents	Pet ether extract	Ethyl acetate extract	Ethanol extract
Alkaloids	-	+	+
Carbohydrates	-	+	+
Glycosides	-	+	+
Flavanoids	-	+	+
Proteins and Amino acids	-	-	-
Steroids	-	+	+
Coumarins	+	+	+
Terpenoids	-	+	+
Tannins	-	+	+
Phytosterols	-	+	+
Saponins	+	+	+
Oils/ Resins	+	-	-
Poly phenols	-	+	+
Fats	+	-	-

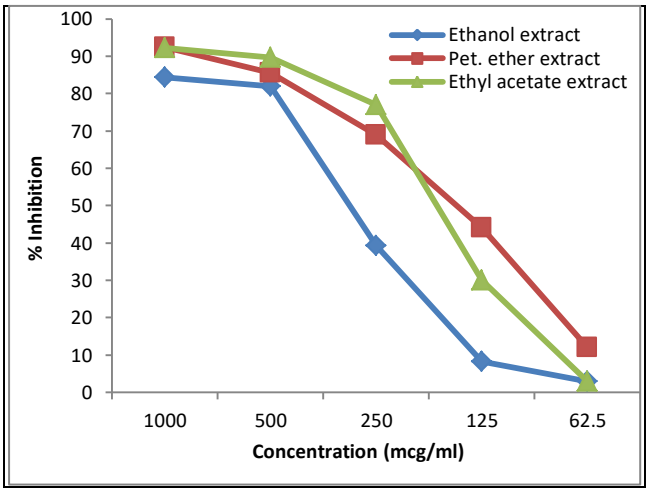
+ = Presence; - = Absence

Table 2: Cytotoxic effect of various extracts of *L. madayiparense* against HepG-2 cell line

S. No	Extract tested	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	Ethanol extract	1000 500 250 125 62.5	84.35±3.2 82.03±3.0 39.34±1.8 8.15±2.3 2.98±1.5	313.33 ± 5.8
2	Pet ether extract	1000 500 250 125 62.5	92.47±0.1 85.58±0.6 68.98±1.8 44.09±3.4 11.93±5.3	149.00± 11.5
3	Ethyl acetate extract	1000 500 250 125 62.5	92.22±0.2 89.67±0.9 77.02±2.8 30.01±3.2 2.98±0.7	178.07± 7.6

The *in-vitro* cytotoxic effect of all the extracts of *L. madayiparense* was studied on Human hepatocellular carcinoma cell lines (HepG-2) by MTT assay method. The cytotoxic effect of pet ether, ethyl acetate and ethanol extracts of *L. madayiparense* was shown in table 2 and figure 1. Among the tested extracts, pet ether extract exhibited the maximum cytotoxic effect (92.47%) at a concentration of 1000 µg/ml against HepG-2 cell lines followed by ethyl acetate extract (92.22%) and ethanol extract (84.35%). Both pet ether extract and ethyl acetate extract exhibited similar degree of range of cytotoxic effect (table 2). The most effective CTC_{50} value was found to be 149 µg/ml for pet ether extract of *L. madayiparense*.

Figure 1: Cytotoxic effect of various extracts of *L. madayiparense* against HepG-2 cell line



DISCUSSION

Pet ether, ethyl acetate and ethanol extracts of *L. madayiparense* were obtained by continuous hot extraction method using Soxhlet extractor. The type of solvents used for the extraction procedure plays a key role to obtain a maximum quantity of extract because every phytochemical constituent has its own solubility competency in different solvent.[18] Therefore the order of increasing polarity of solvents used in the present study is pet ether, ethyl acetate and ethanol.

The *in-vitro* cytotoxic effect of the various extracts of *L. madayiparense* was determined by 3-(4,5-dimethyl thiazol-2-yl)- 2-4-diphenyl tetrazolium bromide (MTT) assay. This method is a colorimetric assay and based on the capacity of the enzyme, mitochondria succinate dehydrogenase in living cells to reduce the yellow water soluble substrate, 3- (4, 5-dimethyl thiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. The ability of the cells to survive a toxic insult is the basis of most cytotoxic assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends on both the mitochondrial activity per cell and number of cells present. The cleavage of 3-(4, 5 dimethyl thiazole-

2yl)-2, 5-diphenyl tetrazolium bromide (MTT) to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based. The reduction of MTT can only take place in metabolically active cells and the level of activity is a measure of the viability of the cells. The amount of cells was found to be proportional to the extent of formazan production by the cells used. MTT reduction as a cell viability measurement is now extensively chosen as the most advantageous end point to assess the cytotoxicity.[19, 20]

In this study, both pet ether extract and ethyl acetate extract exhibited maximum cytotoxic effect in similar degree of range (table 2). It reveals that the secondary metabolites present in both pet ether extract and ethyl acetate extract of the plant, *L. madayiparense* inhibits the HepG-2 cell line at same degree of potency. It might be due to the cytotoxic action of the phytoconstituent (s) present in both pet ether extract and ethyl acetate extract of *L. madayiparense*. The cytotoxic selective activity of the phytoconstituent might depend on the type of chemical composition and their concentration as well as the type and developmental stage of the cancer.[21] The findings suggest that HepG-2 cell line is susceptible due to the presence of phytoconstituent(s) in the plant, *L. madayiparense*. The present study demonstrates the cytotoxic potential of the plant, *L. madayiparense* extract against human hepatocellular carcinoma cell lines (HepG-2 cell line) for the first time. The findings were thought worthwhile for the further investigations to explore the presence of responsible biologically active phytoconstituents in the extracts of *L. madayiparense*.

CONCLUSION

The study may be concluded that the plant, *L. madayiparense* showed cytotoxic effect against Human hepatocellular carcinoma cell lines (HepG-2 cell line). Further investigations to explore the mechanism of action on animal models and to isolate the possible cytotoxic phytoconsistuents are under process to validate the goodness of consumption of this plant, *L. madayiparense*.

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