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RESEARCH ARTICLE

In Vitro and *In Vivo* Anticancer Activity of *E. littorale* Extract on Hepatocellular Carcinoma

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Abstract

Enicostemma littorale Blume is a perennial herb of the Gentianaceae family. It has been noted that different parts of the plant have hepatoprotective, anti-ulcer, antioxidant, antibacterial, and hypoglycemic properties. The aim of the current study was to assess the anticancer potential of the plant *in vitro* as well as *in vivo* animal studies. First, we evaluated the cytotoxic effect of the extracts in HepG2 cells. The alcoholic extract exhibited concentration-dependent cytotoxicity. In HepG2 cells, the extract's anticancer activity was observed with an IC_{50} value of 373 ± 3.0 µg/mL. Following *in vitro* studies, the *in vivo* anticancer effectiveness of the extract was evaluated using a xenograft model. Vehicle-treated mice showed a time-dependent increase in tumor volume. Mice treated with the extract showed a decrease in tumor growth as compared to vehicle treated group, indicating the anticancer activity of the extract in tumor xenografts study. On day 21, a marked reduction in tumor volume was noted. The findings of the study suggest that the alcoholic extract of *E. littorale* is effective against hepatocellular carcinoma. To fully understand the anticancer potential of *E. littorale*, further research with specific phytoconstituents is required.

Keywords: Anticancer, Cytotoxicity, *E. littorale,* Hepatocellular Carcinoma

1. Introduction

Globally, Hepatocellular Carcinoma (HCC) is the primary liver cancer and has the third highest cancer-related death rate[1]. HCC is the seventh most frequently occurring cancer in the world[2]. Metastasis, recurrence, and the development of a new primary tumor with a poor prognosis are the three main causes of death for individuals with HCC[3]. The only proven possibly curative treatments for this cancer are percutaneous ablation, surgical resection, and liver transplantation in the early stages of the disease[4].

Targeted therapy is now restricted to sorafenib, lenvatinib, regorafenib, ramucirumab, and cabozantinib. Though these drugs have potential to improve patient survival, they face challenges of drug resistance and severe side effects. Among these, sorafenib is the first medication to receive FDA approval and is a vital treatment option for patients with advanced HCC[1,5]. However, hepatotoxicity with aberrant increase of aspartate transaminase and alanine transaminase has been recorded in 22–34 % of sorafenib-treated patients, which can result in medication discontinuation and treatment failure. Alternative and complementary medicine offer strategies to lessen side effects of the sorafenib[6]. So, there is a need to have an anticancer agent with hepatoprotective activity as an adjunct to enhance efficacy of sorafenib.

Enicostemma littorale belonging to Gentianaceae family has been utilized in traditional medicine to treat a number of diseases. In India, since many years it is been widely used to treat variety of diseases including filariasis, rheumatism, dropsy, swellings, and itching[7]. The plant is commonly called *Nagajihva* in *Ayurveda* and it has a strong bitter taste[8]. The tribal population of Gujarat uses the hot aqueous extract of *E. littorale*

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to treat diabetes, fever, dyspepsia, stomach pain, and malaria. In Western and Southern India, the herb is also utilized as a traditional diabetic remedy[9]. *E. littorale* significantly improves renal function, lipid profile, blood pressure, and blood sugar levels[10]. Moreover, it has been demonstrated that swertiamarin, an active ingredient in *E. littorale*, can increase insulin sensitivity in rats by acting on PPAR-γ[11]. Additionally, the anthelmintic, anti-inflammatory, and antidiabetic properties of the extract have also been documented in various literature[12-16].

Ethanolic extract of *E. littorale* leaves delayed the formation of chemically induced buccal pouch carcinogensis in hamster[7]. Debnath *et al.*, reported that methanolic extract of *E. littorale* at 500 mg/kg increased the mean survival time in mice from 19 to 31 day in Ehrlich's Ascetics Carcinoma[17]. According to Wang *et al.*, the main bioactive ingredient of *E. littorale* is swertiamarin, which also inhibits cell migration and triggers apoptosis in Hela cells, human cervical cancer cells, and targets the MEK-ERK pathway[8]. A significant component of many traditional remedies sold in Japan is swertiamarin, a secoiridoid glucoside that was first identified from the leaves of *E. littorale*[18].

The utility of *E. littorale* extracts in the treatment of liver cancer is still unknown. However, it has been reported that, *E. littorale* has traditionally been used to treat the liver problems in India. Furthermore, a hepatoprotective effect of *E. littorale* extract was demonstrated in CCl_4-induced liver damage model in rats as a result of its antioxidant and free radicals scavenging activity[19-23]. In our knowledge, no academic research has been done to support the chemopreventive effects of *E. littorale* extracts on hepatocellular cancer cell lines. Thus, the objective of this study was to explore the anticancer effects of *E. littorale* extract using HepG2 cell lines.

2. Materials and Methods

2.1 Reagents and Antibodies

Sorafenib was received from Sun Pharma Industries Limited. Sodium pyruvate, EMEM, penicillin (100 I.U./mL), streptomycin (0.1 mg/mL), 0.05% trypsin/EDTA, and L-glutamine were purchased from Gibco, dimethyl sulfoxide (DMSO) from Sigma Life Science and fetal bovine serum from Himedia. The HepG2 cell line was procured from ATCC and was supplied by the *in vitro* Biology Department, Sun Pharma Advanced Research Company Limited.

2.2 Plant Collection and Authentication

E. littorale (whole plant) was procured from local Ayurvedic Medicine Practioner, Vadodara, Gujarat, India. Specimen was authenticated by Dr P. Nagar, Associate Professor, Department of Botany, The Maharaja Sayajirao University of Baroda with Voucher Specimen number K000438312 which is deposited at the Herbarium of Botany Department.

2.3 Preparation of Plant Extract

The whole plant was cleaned with distilled water, diced into small pieces, and shade dried for two to three days at room temperature before being crushed into powder and utilized for extraction. The fine powder of the plant was placed in a Soxhlet equipment unit and extracted with different solvents-petroleum ether, ethyl acetate, alcohol, hydro-alcohol and water. After concentrating using a rotary vacuum evaporator, the extract was lyophilized and stored for later use at -20°C in an airtight container[19]. In some cases, the lyophilized powder of alcoholic extract was also procured externally from a supplier (Amsar Private Limited, Indore, India). Both, the extract as well as lyophilized powder was used in the studies described in this report.

2.4 Phytochemical Screening

Standard phytochemical tests were used to perform the qualitative preliminary screening of extracts for alkaloids, tannins, phenolic compounds, proteins, carbohydrates, amino acids, steroids, glycosides, saponins, and flavonoids[24].

2.5 Drug Solutions and Cell Culture

Alcoholic extract stock solution was prepared in water. The remaining all other extracts and sorafenib stock solution was prepared in DMSO. The solutions were sterilized by Millipore 0.22 µ membrane filter. To get the final concentrations, stock solutions were serially diluted in culture medium. Working solutions were kept at -20°C and stock solutions at -80°C for storage. Prior to usage, all solutions were thawed. HepG2 cell was cultured at 37°C in a humidified environment with 5% CO_2 in EMEM medium. The medium was

supplemented with 10% (v/v) fetal bovine serum, penicillin (100 IU/mL), and streptomycin (0.1 mg/mL). Cells were passaged using 0.05% trypsin/EDTA. The cell culture media was replaced every two to three days unless otherwise specified. Before adding the stock solutions to the cells, they were diluted at the proper concentrations in cell culture medium.

2.6 Cytotoxicity Assay

The cytotoxicity of extract on HepG2 cells was determined with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (MTT) assay. This colorimetric assay quantifies the amount of mitochondrial succinate dehydrogenase that converted to the yellow tetrazolium salt of MTT into an insoluble formazan product with a dark purple color. In brief, 96-well plates were seeded with 5×10^3 HepG2 cells for 24 hours. The cells were then incubated with extracts (5 to 640 µg/mL) and the reference standard sorafenib (0.156 to 20 µM) for duration of 48 hours. Following the completion of the treatment, the wells were refilled with 15 µL of MTT (5 mg/mL) and incubated for a further 4 hours. After removing the supernatant, 100 µL per well DMSO was added to dissolve the formazan crystals. The absorbance of each sample at 570 nm was measured using a microplate reader. The following equation was used to compute the percentage cell viability[25].

Cell viability percentage = [1-(ODt/ODc) x 100%]

Where, ODc and ODt represent the mean optical density of untreated and treated cells respectively with the test compounds. A dose-response curve was plotted for IC_{50} value calculation. All the assays were performed in triplicate.

2.7 *In Vivo* Antitumor Activity

The Institutional Animal Ethics Committee (IAEC), Sun Pharma Advanced Research Company Ltd., approved the study protocol with IAEC no. 908. The Ministry of Environment, Forests and Climate Change, Government of India, issued guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and these rules were followed when maintaining all the mice and carrying out the experimental procedures. Female 6-8 weeks old athymic nude mice were used in the xenograft study. Autoclaved Reverse Osmosis (RO) purified water and Harlan rodent diet was provided *ad libitum* to the mice.

The efficacy study of the extract was performed in female athymic nude mice carrying subcutaneous tumor of HepG2 cells. For tumor xenograft, mice were inoculated with 5×10^6 HepG2 cells. As the tumor was palpable, the long (a) and short (b) diameters of tumor were measured in millimeters using a vernier caliper, and the tumor volume was computed by the formula - Tumor volume = $(a \times b^2)/2$.

Once tumors were around 200 and 300 mm^3 in volume, mice were randomized and divided in two three group (n = 6 for each group). Mice were dosed orally once daily with vehicle or alcoholic extract of *E. littorale* (500 mg/kg) or sorafenib (30 mg/kg) for 21 days. During the course of study, mice were closely monitored for changes in body weight, any clinical symptoms, and tumor volume[26].

Tumor Growth Inhibition (TGI) is calculated for each group using the formula: TGI (%) = [1−(Ti−T0)/(Vi−V0)] × 100; Ti and T0 is the average tumor volume of a treatment group on a given day and first day of treatment respectively. Vi and V0 is the average tumor volume of the vehicle control group on the same day with Ti, and on the first day of treatment respectively[27].

2.8 Statistical Analysis

The experimental data were expressed as mean ± Standard Deviation (SD). For the statistical study, GraphPad Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA) was used. One- or two-way analysis of variance (ANOVA) was employed to evaluate statistical differences between treatment groups, and for multiple comparisons, a Bonferroni post hoc test was used. A p-value of less than 0.05 was considered as an indicator of statistical significance.

3. Results

3.1 Phytochemical Analysis

A preliminary analysis of the extract for phytochemical composition revealed that they consist of a variety of secondary metabolites. Positive results for tannins and phenolic compounds were identified in all of the extracts. Except petroleum ether extract, all other extracts showed presence of amino acid, steroids, saponin, flavonoids and alkaloids. Glycoside was

present in alcoholic, hydroalcoholic and aqueous extract. The alcoholic extract of *E. littorale* has the highest extraction yield of 12% w/w. All other extracts of the *E. littorale* have extraction yield from 4 to 7 % w/w. All the extracts had characteristic odour with semisolid consistency. The organoleptic characters (Table 1) and result of preliminary phytochemical screening of the extracts (Table 2) are summarized in given table.

Table 1. Organoleptic properties of *E. littorale* whole plant extracts

Specification	Petroleum ether extract	Ethyl acetate extract	Alcoholic extract	Hydroalcoholic extract	Aqueous extract
Color	Green	Blackish green	Dark green	Brown	Blackish green
Consistency	Semisolid	Semisolid	Semisolid	Semisolid	Semisolid
Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
Yield (% w/w)	4.0	8.0	12.0	7.0	6.8

Table 2. Preliminary phytochemical screening of *E. littorale* whole plant extracts

Class	Test/ Reagents	Petroleum ether extract	Ethyl acetate extract	Alcoholic extract	Hydro alcoholic extract	Aqueous extract
Carbohydrate	Molisch Test	-	-	+	+	+
	Fehling's Test	-	-	+	+	+
	Benedict's Test	-	-	+	+	+
Protein	Biuret Test	+	-	+	+	+
	Millon's Test	+	-	+	+	+
Amino acid	Ninhydrin's Test	-	+	+	+	+
Steroids	Salkowski Test	-	+	+	+	+
Glycoside	Keller-kiliani Test	-	-	+	+	+
	Liebermann's Test	-	-	+	+	+
Saponins	Foam Test	-	+	+	+	+
Flavonoids	Alkaline Reagent Test	-	+	+	+	+
Alkaloids	Dragendorff's Test	-	+	+	+	+
	Mayer's Test	-	+	+	+	+
	Wagner's Test	-	+	+	+	-
Tannins and phenolic compounds	Ferric chloride Test	+	+	+	+	+

+ present, - absent

3.2 *E. littorale* Showed the Cytotoxic Effect on HepG2 Cells

The MTT assay was used to determine the anticancer activity of the extracts. The petroleum ether extract showed minimum cytotoxicity while the alcoholic extract showed the highest activity. The HepG2 cells were found sensitive to cytotoxicity above 160 µg/mL concentration with petroleum ether, hydro-alcoholic, and aqueous extract. The alcoholic extracts and sorafenib showed dose-dependent cytotoxicity with IC_{50} value of >370 µg/mL and 6.47 µM (Table 3) respectively. All other extracts except alcoholic extract had IC_{50} greater than 500 µg/mL. Since the alcoholic extract showed acceptable cytotoxicity and was found to have all the major phytoconstituents with greater yield, further *in vivo* animal studies were carried out with it. Figures 1 and 2 show the specific anticancer activity of various extracts and sorafenib against the HepG2 cell line.

Table 3. IC_{50} value of extract and sorafenib

Extract/reference compound	IC_{50}
Alcoholic extract of *E. littorale*	373 ± 3.0 µg/mL
Sorafenib	6.47 ± 0.1 µM

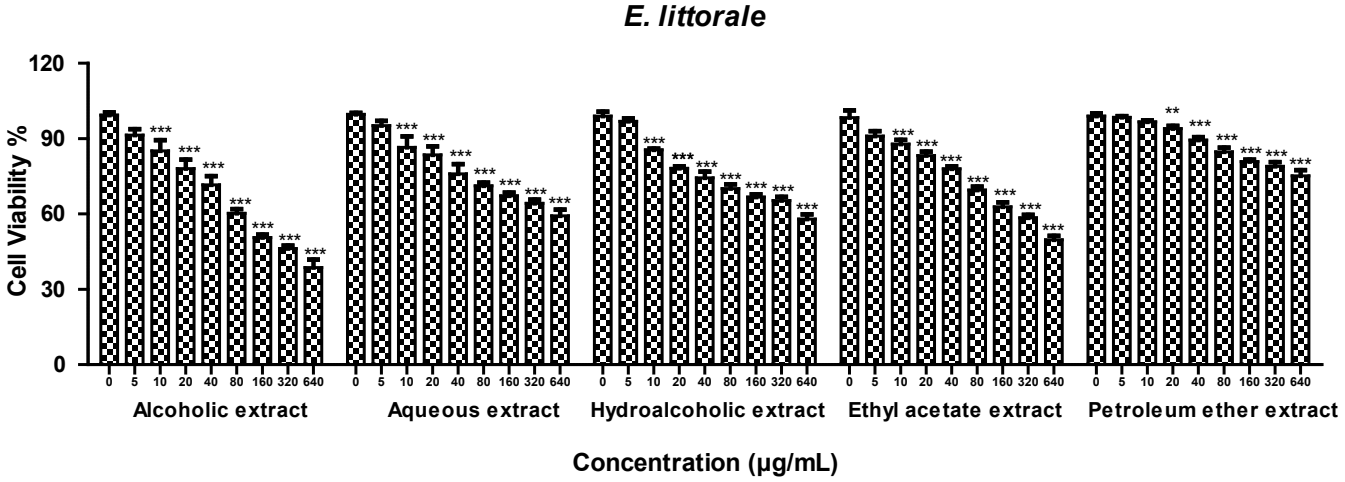

Figure 1. Cell viability (%) of *E. littorale* extracts in MTT assay. All values described as mean (n = 3) ± SD. One-way ANOVA was used to compare treated and untreated cells followed by Bonferroni post hoc test. ** P < 0.01, and *** P < 0.001 denote significant difference as compared to untreated cells.

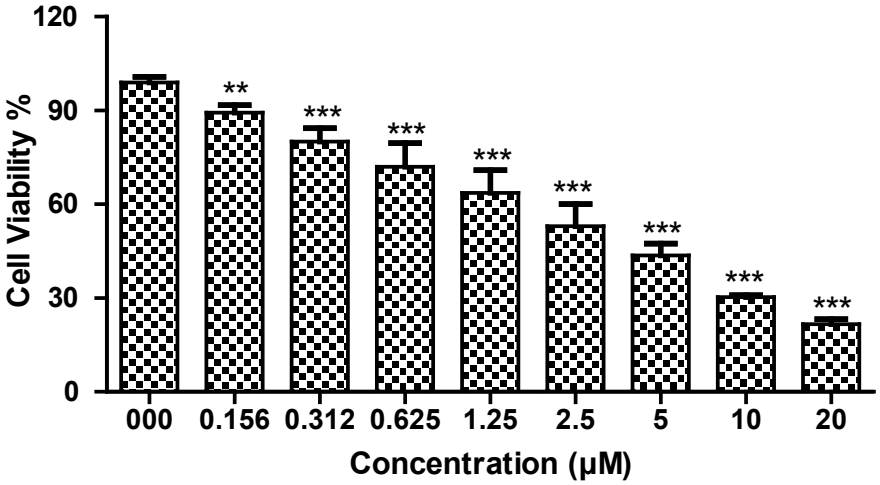

Figure 2. Cell viability (%) of sorafenib in MTT assay. All values described as mean (n=3) ± SD. One-way ANOVA was used to compare treated and untreated cells followed by Bonferroni post hoc test. ** P < 0.01, and *** P < 0.001 denote significant difference as compared to untreated cells.

3.3 *E. littorale* Extract Inhibited the Tumor Progression in HepG2 Cells Tumor Bearing Mice

Time-dependent increase in tumor growth was noted in vehicle treated group mice. Compared to the vehicle-treated group, mice administered with alcoholic extract exhibited a decrease in tumor growth. Effect was significantly ($p < 0.01$) lower on day 21. The percentage tumor growth inhibition (% TGI) for alcoholic extract was found to be 29% on day 21. As expected, reference drug sorafenib ($p < 0.001$) treated group also showed noteworthy decrease in tumor growth in comparison to mice in vehicle treated group (Figure 3). The percentage tumor growth inhibition (% TGI) of sorafenib was found to be 47%. The extract and sorafenib were well tolerated and it did not impact on animal body weights (Table 4).

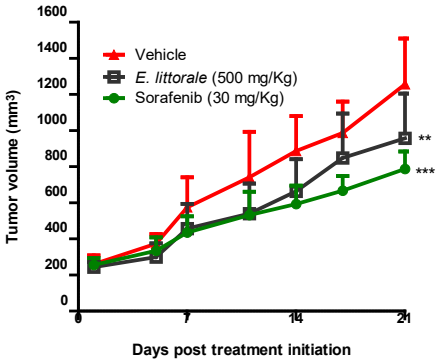

Figure 3. Effect of *E. littorale* extract on tumor volume in HepG2 tumor bearing in mice. All the values described as mean (n = 6) ± SD. Two-way ANOVA was used to compare treated and control group mice followed by Bonferroni post hoc test. ** $P < 0.01$, and *** $P < 0.001$ denote significant difference as compared to vehicle group mice.

Table 4. Effect of extract on body weight in HepG2 tumor bearing mice

Extract/reference compound	Body weight (gm)	
	Day 1	Day 21
Vehicle	25 ± 5	26 ± 4
Alcoholic extract of *E. littorale*	26 ± 3	27 ± 5
Sorafenib	27 ± 5	26 ± 4

Data represented as mean ± SD, n = 6

4. Discussion

Historically, HCC has been associated with a dismal prognosis and low treatment efficacy. Resistance to the majority of therapeutically available anticancer drugs was the primary barrier to the systemic treatment of HCC. Consequently, there is a need for new drugs or treatment strategies for HCC[28]. Natural products being relatively safe; they are investigated as possible anticancer agents for a variety of maligenices[29]. Natural products appear to be rich source for the development of anticancer drugs, as evidenced by the fact that 83% (113 out of 136) of approved small molecule anticancer compounds are either natural product or product derived from them[30].

As a result, preclinical research on *E. littorale* has demonstrated its enormous pharmacological potential, including its anticancer effectiveness against a wide range of cancer types[7,17,31]. Nevertheless, the anticancer potential of the extract has not been investigated in relation to HCC. In this study plant extract was prepared using five different solvents, and its *in vitro* cytotoxicity was assessed. When compared to other extracts, the alcoholic extract exhibits the highest yield and cytotoxicity. Alcoholic extract contains a wide range of phytoconstituents which are not limited to alkaloids, sterols, saponins, triterpenoids, flavonoids, and phenolic acids as confirmed by preliminary phytochemical test which are as per the literature[32].

Results from phytochemical analysis and cytotoxicity studies showed that the alcoholic extract was the most potent among the five extracts of the *E. littorale*. Hence, the alcoholic extract was selected for the *in vivo* studies to confirm the translation of its cytotoxicity effect (*in vitro* data) in animal model.

In the present study, oral treatment of 500 mg/kg body weight of *E. littorale* to HepG2 tumor-bearing mice markedly suppressed the growth of the tumor in comparison to mice treated with a vehicle. The body weights of the mice were maintained in both groups, which is indicative of the high tolerability of extract and sorafenib. Numerous reports also suggest that *E. littorale* is safe and tolerated well in rats and humans[16,33,34]. Also the extract complies for heavy metal and microbial count as per the regulatory guideline[35,36]. The methanolic extract was reported to reduce the oxidative stress, induce apoptosis of cells

and prolong survival of the tumor bearing mice[37,38]. The present study suggests that the alcoholic extract might be responsible for the anticancer activity in HCC in a similar way as reported in the literature.

5. Conclusion

In conclusion, the *in vitro* and *in vivo* studies showed that the *E. littorale* extract inhibited the growth of HCC. The *in vivo* study also improved the antitumor effect by increasing the tumor growth inhibition and potentially no adverse effects in our limited study. The results of this investigation provide valuable information about the use of plants in HCC. Detailed mechanistic and molecular studies are required to further explore the efficacy and safety of the plant extract. Our findings point to more studies to explore potential use of *E. littorale* as a supplementary dietary substance or as a complementary medicine along with the currently available treatment options.

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