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Selective Anticancer Activity of Pure Licamichauxiioic-B Acid in Cultured Cell Lines

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Abstract

The cytotoxic activities of crude extract and pure licamichauxiioic-B compound from stem and root parts of *Licania michauxii* Prance (LMP) plant were evaluated against brine shrimp larvae and cell cultures. Under *in vivo* shrimp assay, both crude extract and the purified compound were active with ED_{50} values of 122.5 and 32.1 μg/ml, respectively. When tested in four human cancer cell lines, namely, CL-187 (colon adenocarcinoma), MCF-7 (hormone-dependent breast carcinoma), MDA-MB-231 (hormone-independent breast carcinoma), and CRL-2547 (pancreatic carcinoma), the pure compound exhibited 1.5- to about 3-times greater cytotoxic activity than the crude extract. In all cancer cell lines, the LD_{50} values of crude extract ranged from 33.8 ± 0.9 to 88.1 ± 3.6 μg/ml, while with pure compound the values ranged from 21.6 ± 1.2 to 31.8 ± 4.2 μg/ml. In a noncancerous normal mouse adipose areolar (CCL-1 NCTC clone 929) cell line, the pure compound was found to have an LD_{50} value greater than 100 μg/ml. Comparison of cytotoxic results of cell cultures revealed that the pure licamichauxiioic-B compound was relatively inactive in noncancerous cell line. The selectivity index for the pure licamichauxiioic-B compound is greater than 3.14 in all cancer cell lines tested.

Keywords: Anticancer activity; brine shrimp; gopher-apple; licamichauxiioic-B acid; *Licania michauxii* Prance; selectivity index.

Introduction

The use of natural products for medical benefits is found in nearly every culture in the world. The majority of people in developing countries depend on natural products for their medical needs. Secondary metabolites of plants are the major source of cure for many types of diseases like malaria. Certain plants have also been used for treatment of different types of cancers (Hartwall, 1971; Huang, 1999). The national Cancer Institute (NCI) and others have recognized the vast potential source of natural products for various types of anticancer agents. In the course of our continuous search for potent and selective anticancer compounds from natural products, we have screened various plant and marine extracts for cytotoxic activities against brine shrimp larvae and human cancer cell lines (Badisa et al., 2000, 2003, 2004a,b,c; Chaudhuri et al., 2002; Couladis et al., 2002). Previous investigations with the crude extract of *Licania michauxii* Prance (LMP) plant showed the inducing effects on heat shock protein (hsp) 70 mRNA in cultured cells (Badisa et al., 2000). Subsequent bioactivity-guided fractionation of this plant extract resulted in two novel compounds (Chaudhuri et al., 2002). In the current communication, the purified compound, licamichauxiioic-B, from the LMP plant was evaluated for anticancer activities.

LMP belongs to the Chrysobalanaceae family, which has only two genera in the United States, each with a single species. LMP, which is found throughout the coastal plain of South Carolina and South Florida is one of them. It grows to a height of 30 to 40 cm and spreads under the soil partly by means of long, horizontal, subterranean stems (Bell & Taylor, 1982). To the best of our knowledge, the influence of licamichauxiioic-B compound of LMP has not yet been evaluated thoroughly against various human cancer cell lines like pancreatic carcinoma, hormone dependent and independent breast carcinoma cells, and normal cell lines. It is generally

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accepted that plant crude extracts with IC_{50} values of 20 μg/ml or less under *in vitro* conditions are considered for isolation of active principles (Geran et al., 1972; Hou et al., 1995). Similarly for pure compounds, if the IC_{50} value is 4 μg/ml or less under *in vitro* conditions, then they are accepted for further evaluation as chemotherapeutic agents for animal models (Likhitwitayawuid et al., 1993). Screening of thousands of natural products by NCI and many other groups over many years has resulted in only a handful of plant and marine extracts with high activities. This clearly demonstrates the difficulty to find many active plant materials in nature. Moreover, the successful pure compounds from such active extracts most often have shown severe side affects under *in vivo* conditions, as is evidenced in the case of Taxol (Markman, 1993). We, therefore, believe that it is highly reasonable to pursue investigations of natural products even if the IC_{50} values are >20 μg/ml if they could exhibit the ability to kill selectively the malignant cells, leaving the normal cells unharmed.

Thus, based on these observations, we have undertaken the current study with the aim to investigate the differential cytotoxic activity of a pure compound, isolated from the crude extract of LMP, in cancerous and noncancerous cell lines under *in vitro* conditions and under *in vivo* in brine shrimp larvae.

Materials and Methods

Chemicals

Trypsin, minimum essential medium (α modification), antibiotic-antimycotic solution, (Ab/Am), bovine insulin, and human recombinant insulin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum, RPMI-1640 (modified), penicillin and streptomycin (pen-strep), and L-glutamine were purchased from Media Tech (Herndon, VA, USA). Cosmic calf serum and equine serum were purchased from Hyclone Lab Inc. (Logan, UT, USA) and gentamicin reagent solution was purchased from Life Technologies (Grand Island, NY, USA). All other routine chemicals were of analytical grade.

Plant materials

Root, stem, and leaf parts of LMP were collected in the vicinity of St. Joe Timberland Company (Hosford, FL) with prior permission in the month of July 2002. The identity of this plant was verified by Mr. Angus K. Gholson, Jr., field botanist, and a voucher specimen (no. 8419) was deposited at AKG Herbarium (Chattahoochee, FL).

Sample preparation

The plant materials were washed thoroughly with regular tap water in order to wash out soil and dust materials and then air-dried in shade at room temperature (22–28°C). The dried root and stem parts (~300 g) were macerated and steeped in 100% methanol for 2 weeks under dark at room temperature. The methanol extract was then concentrated by roto-evaporation under vacuum at 40°C. A portion of concentrated material (~15 g), was diluted in ethyl acetate (1:1, v/v) under constant stirring. The resulting mixture was vacuum filtered and loaded on a Combi Flash Chromatograph with silica gel column (Redi Sep, Isco Company, Lincoln, NE, USA), which was pre-equilibrated with *n*-hexanes. A continuous gradient system of ethyl acetate in *n*-hexanes was used with increasing polarity for elution of fractions (50 ml). The fractions were pooled according to the peaks, concentrated, and stored at 4°C. After about 10 days, white crystals were seen at the bottoms of the flask. A portion of it was used for identification purpose. For the sake of cytotoxic studies, DMSO was added to the known amount of pure compound to make 25 mg/ml and stored at 4°C until used.

In vivo lethality test by brine shrimp assay

In this technique, the *in vivo* lethality of compounds was tested against brine shrimp larvae. For this purpose, the brine shrimp eggs (*Artemia salina*) were hatched in artificial seawater at room temperature and were used after 48 h. The assay was done as described by Meyer et al. (1982) and modified by McLaughlin (1991). In brief, an equal volume (5 ml) of seawater was added in each glass vial. Then different volumes (2 to 20 μl) of test materials, present in dimethyl sulfoxide (DMSO), were added to the triplicate vials to achieve the required concentrations and mixed well. Ten shrimps were added to each vial with a minimum volume of artificial seawater. The shrimps in artificial seawater or DMSO (maximum final concentration = 0.24%, v/v) in artificial seawater served as controls. After 24 h, the number of live shrimps per dose was recorded, and the data were utilized for analysis.

Cell lines

The following human cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and used in the current investigation: Human colonic adenocarcinoma (CL-187), hormone-dependent human breast adenocarcinoma (MCF-7), hormone-independent human breast adenocarcinoma (MDA-MB-231), and human pancreatic carcinoma (CRL-2547). A noncancerous mouse CCL-1 NCTC clone 929 (strain L) was used as a normal control cell line for comparison. This cell line was derived from normal subcutaneous areolar and adipose tissue of a mouse.

Maintenance of cultures

CL-187 was maintained as described earlier (Badisa et al., 2000). In brief, it was maintained in minimum essential

medium (α modification) supplemented with 10% cosmic calf serum, L-glutamine (2 mM), gentamicin (0.5 μg/ml), and antibiotic-antimycotic solution, which contains penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (0.25 μg/ml). MCF-7 cell line was maintained in RPMI-1640 (modified) medium, supplemented with 10% fetal calf serum (FCS), bovine pancreas insulin (10 μg/ml), L-glutamine (2 mM), and pen-strep (100 units and 100 μg/ml, respectively). MDA-MB-231 was maintained in RPMI-1640 (modified) medium, supplemented with FCS 10%, L-glutamine (2 mM), and pen-strep (100 units and 100 μg/ml respectively). CRL-2547 was maintained in RPMI-1640 (modified) medium, supplemented with 10% FCS, human recombinant insulin (10 μg/ml), L-glutamine (2 mM), and pen-strep (100 units and 100 μg/ml, respectively). NCTC clone 929 was maintained in RPMI-1640 (modified) medium, supplemented with 10% equine serum, L-glutamine (2 mM), and pen-strep (100 units and 100 μg/ml, respectively). All cell lines were grown as monolayer cultures in T-75 cm^2 culture flasks in a humidified chamber at 37°C, 5% CO_2 in air in an incubator. For cytotoxic studies, the cells were removed from T-75 flasks, typically at about 85% confluence by treating with trypsin (0.25%) solution. Cell counts and cell viability were assessed by using 0.4% Trypan blue stain and a hemocytometer.

Treatment of cell lines with samples

The cell cultures were plated at 1×10^4 cells per well in polystyrene, flat-bottom, 96-well microtiter plates (Corning Costar, Corning, NY, USA) in a total volume of 196 μl. Then the cells were allowed to stabilize overnight in the incubator. The following day, the samples were diluted in the culture media in order to achieve required working stocks and added to various cultures in a total volume of 4 μl under sterile conditions. Initially, the crude extract and pure compound were tested at 0.1 mg/ml for 48 h. The materials that killed more than 80% cells at this concentration were further tested at five more concentrations (10, 25, 50, 75, and 100 μg/ml) in order to determine the lethal dose where 50% cells were killed (LD_{50}). All tests were done in quadruplicate wells. In all cell lines, untreated and DMSO (0.4%) treated cells served as controls. The culture plates were incubated for 48 h continuously in a 5% CO_2 incubator at 37°C with the plates capped in the normal fashion. All studies were conducted at least two times independently under identical conditions ($n = 8$).

Evaluation of cytotoxicity of samples

After 48 h of treatment, the cytotoxicity of crude extract and pure compound was evaluated by dye uptake assay using crystal violet (Badisa et al., 2003), which was shown to be more sensitive than neutral red (Shahan et al., 1994). In brief, at the end of incubation, 100 μl of 0.25% aqueous glutaraldehyde was added to each microtiter well, and the cultures were incubated at room temperature under the culture hood for 30 min. The supernatant was then discarded, and the plates were washed five times with tap water and dried under the hood. The cells were then stained with 100 μl of 0.1% aqueous crystal violet for 15 min. The excess dye was removed by washing the plates five times with tap water and dried. Finally, 100 μl of 50 mM unbuffered sodium phosphate solution (monobasic), containing 50% ethyl alcohol, was added to each well. The plates were vortexed gently and the optical density measurements were made at 540 nm by using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The average absorbance value of controls was taken as 100% cell viability. From the treated and control absorbance values, the percent cells killed at all treatments were determined by the following equation:

$$\left[1 - \left(\frac{T}{C}\right)\right] \times 100$$

where T is the average absorbance values of treated cells, and C is average absorbance values of control cells.

ED_{50} and LD_{50} determinations

The live shrimp larvae at different concentrations were counted, and different effective dose values, where 50% shrimp larvae were killed (ED_{50}), were determined with 95% confidence intervals using a Finney computer program. In case of cell culture studies, the lethal dose values were obtained by plotting the graphs between the amounts of extract tested on the *x*-axis versus the percent cell population (both viability and dead) on the *y*-axis. The graphs were plotted in GraphPad Prism Software, version 3.00. The lethal dose of extract that killed 50% cells (LD_{50}) was calculated from the graphs where both curves crossed (Ipsen & Feigl, 1970).

Statistical analysis

The results of cell culture studies are presented as mean ± SD. The data were analyzed for significance by one-way ANOVA and then compared by Dunnett's multiple comparison tests using GraphPad Prism Software, version 3.00. The test values of $p < 0.05$ and $p < 0.01$ were considered significant and highly significant, respectively.

Results and Discussion

The *in vivo* brine shrimp assay is an inexpensive benchtop assay mainly used for screening of crude plant extracts for cytotoxic activities (Meyer et al., 1982; Badisa

Table 1. Effect of crude extract and pure licamichauxiioic-B compound of LMP plant on brine shrimp larvae.

Sample	Concentrations tested (μg/ml)	Mortality, (%)	ED_{50} (μg/ml)	95% confidence interval
Crude extract	10	0		
	50	0		
	100	43.3 ± 5.7	122.5	94.1–371.1
	1000	100 ± 0		
Pure compound	10	0		
	30	53.3 ± 1.1	32.1	25.8–39.8
	60	80 ± 0		

Brine shrimp larvae were treated at the above doses continuously for 24 h under illumination, and the live shrimps were counted. The ED_{50} values were calculated with 95% confidence intervals by a Finney computer program.

Table 2. Anticancer activity of crude extract and pure licamichauxiioic-B compound of LMP plant on cancer and normal cell lines.

Cell lines	Crude extract LD_{50} (μg/ml)	Pure compound LD_{50} (μg/ml)	Selectivity index (SI)[a]
CL-187	88.1 ± 3.6	31.8 ± 4.2	>3.14
CRL-2547	58.8 ± 2.8	22.9 ± 0.2	>4.36
MCF-7	63.8 ± 1.6	21.6 ± 1.2	>4.62
MDA-MB-231	33.8 ± 0.9	22.4 ± 0.4	>4.46
Normal mouse adipose areolar (CCL-1 NCTC clone 929)	Not determined	>100	—

The cultures were treated with different concentrations continuously for 48 h at 37°C, 5% CO_2 in the incubator. Cytotoxicity was evaluated by the crystal-violet method as described before. Each value represents the mean ± SD ($n = 6$ to 8, $p < 0.001$). [a]SI = LD_{50} of pure compound on normal cell line/LD_{50} of pure compound on cancer cell line.

et al., 2003, 2004a,c; Couladis et al., 2002). The interaction of crude extract and pure licamichauxiioic-B compound of LMP with brine shrimps was not investigated earlier. It was reported that the fruits of this plant were consumed by Gopher tortoises and, thus, LMP plant is also called Gopher-apple. In the current study, it was observed that addition of crude extract and licamichauxiioic-B compound at 10, 30, 60, 100, and 1000 μg/ml resulted in immediate precipitation in the sea-salt solution. In the case of pure compound, the precipitation disappeared upon vortexing, while with the crude extract, it settled at the bottom of the vial slowly. As no precipitation occurred upon addition of DMSO (0.4% final, v/v) in control vials, it is obvious that precipitation resulted from the test materials. In this *in vivo* shrimp assay, both LMP crude extract and pure licamichauxiioic-B compound exhibited cytotoxic activity against brine shrimp larvae. However, a marked difference was observed in their activities. The pure compound was found to be more cytotoxic than the crude extract by about 4 times. The ED_{50} values of pure compound and the crude extract were 32.1 and 122.5 μg/ml, respectively (Table 1). Based on the observation that ED_{50} values of crude extract and pure compound are less than 1000 μg/ml, both are considered to be active in this assay as per the earlier standard (Meyer et al., 1982). In both control vials, shrimp larvae were alive and active even after 48 h of incubation, indicating lack of DMSO effect on shrimps. In fact, it was reported earlier that shrimps could tolerate DMSO up to 2.3% (Badisa et al., 2003).

Based on the results of the brine shrimp assay, the *in vitro* influence of crude extract and pure licamichauxiioic-B compound of LMP plant were investigated for possible selective anticancer activities in cultured cell lines. The lethal doses, where 50% cell population were killed, are shown in Table 2. As speculated, the pure compound was more active than the crude extract by 1.5- to 3-times. Of all cultures, hormone-dependent human breast carcinoma cell line (MCF-7) responded well, showing an LD_{50} of 21.6 ± 1.2 μg/ml, while human colon adenocarcinoma (CL-187) cell line responded least with an LD_{50} of 31.8 ± 4.2 μg/ml, which was consistent with earlier observation from our group (Chaudhuri et al., 2002). The close LD_{50} values in three of the cultures may reflect that the pure compound has the same target sites. This, however, needs to be confirmed. The LD_{50} value for the pure compound in mouse normal cell line was found to be greater than 100 μg/ml. The insolubility of the pure licamichauxiioic-B compound in DMSO at higher than 25 mg/ml caused the limitation to test at higher concentrations, where DMSO was found to be affecting the cell growths of the cultures (data not shown).

In the current study, the degree of selectivity of the compound is expressed as per the earlier report (Koch et al., 2005) with a minor modification:

$$\text{Selectivity index (SI)} = LD_{50} \text{ of pure compound in normal cell line}/LD_{50} \text{ of pure compound in cancer cell line}$$

As the value of SI demonstrates the differential activity of a compound, the greater the index value is, the better it is. The SI value of pure licamichauxiioic-B compound for each cell line was determined and is presented in Table 2. An SI value less than 2.0 indicates the general toxicity of the compound (Koch et al., 2005). Based on this, the SI data presented in Table 2 clearly reflect the high degree of selectivity of pure licamichauxiioic-B compound in all cell cultures studied. Interestingly, the human pancreatic carcinoma, hormone-dependent (MCF-7) and hormone-independent

(MDA-MB-231) human breast cancer cell lines responded very well with the pure compound by exhibiting a higher selectivity index than the human colon adenocarcinoma cell line.

Comparison of Tables 1 and 2 reveals a high degree of correlation of cytotoxic results between shrimp larvae and cell culture tested. This observation is consistent with previous reports (Anderson et al., 1991; Badisa et al., 2003).

The only approach in disseminated cancers is by chemotherapy. Although it is desired that the malignant cells are killed at lower doses by chemotherapeutic drugs, most often the chemically synthesized or modified compounds show side effects on normal body cells also. Because it is difficult to obtain many active natural products in nature, and because some pure and modified compounds show undesired side effects, the next obvious choice for consideration is to exploit those pure compounds that, in spite of low activity, show selective killing of cancer cells, and thus have a high therapeutic index. Such pure compounds may be utilized to determine the efficacy of anticancer activity in animal models. Because pure licamichauxiioic-B compound in this study showed selective toxicity, this observation warrants further investigations to determine the anticancer potential in animal models. Studies are under way in this regard.

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