In vitro tumor cytotoxic activities of extracts from three Liriodendron plants

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Abstract: The extracts prepared from *Liriodendron tulipifera* Linn., *L. chinense* (Hemsl.) Sarg., and their hybrid *L. chinense* x *L. tulipifera*, were investigated for their cytotoxic abilities in vitro against five human cancer cell lines: MDA-MB-231 and MCF-7 breast cancer cells, SGC-7901 gastric cancer cells, HuH-7 hepatocarcinoma cells, and HCT-15 colon carcinoma cells, and then measured their phenols and alkaloids contents. Of these plant extracts, some of them, especially the lower polar extracts from barks, exhibited potent cytotoxic effects on five tested tumor cell lines.

Keywords: Liriodendron; polyphenol; alkaloid; tumor cells; cytotoxic activity.

INTRODUCTION

Liriodendron is a genus in the Magnoliaceae family consisting of two species of large deciduous trees, one named *L. tulipifera* native to eastern of North America, and the other named *L. chinense* to South China. Both of these two species showed diversity in medications in America, China, and other countries. *L. tulipifera*, known as the tulip tree or yellow poplar, is a majestic tree often reaching one hundred feet height, which is specific species in the eastern United States (Keeler, 1902). The bark of *L. tulipifera* had been used by the native Americans as a tonic, stimulant and febrifuge, and likely to treat the intermittent fevers related with malaria (Rafinesque *et al.*, 1828). The historical use of *L. tulipifera* as an antimalarial remedy in the United States was supported deeply recently (Graziosea *et al.*, 2011). It had also been used by the later native Americans as a suitable replacement of the imported and often scarce cinchona bark (Thacher, 1967). In India, *L. tulipifera* was also used to treat restorative, rheumatism, and dyspepsia (Kuanar, 2006). In China, the barks, leaves, and roots of the sibling species *L. chinense* were used to treat rheumatism and cough due to wind-cold evil (named 'Feng Han' in China) as traditional Chinese medicines (TCM) (Jiangsu New College of Medicine, 2001). In 1963, a new hybrid strain, *L. chinense* x *L. tulipifera*, was successfully cultivated by Prof. Peizhong Ye. The great advances have been made no matter in the theory of forest tree genetics and breeding or the application in plantation and landscape (Wang, 2003). However, so far, the medicinal values of the hybrid tree have not been investigated yet.

Cancer has caused millions of deaths each year worldwide. Pharmaceutical intervention with plant-derived products alone or in combination to reverse, suppress, or even prevent cancer plays a key role in the fight against this terrible disease (Siveen and Kuttan, 2010). There has been increasing interest in alternative medicine and nontoxic therapeutic approaches to anti-tumoral treatment. It has been reported that some natural components from the ethnobotany or diet are effective against cancer and are safe to the normal cells (Hakimuddin *et al.*, 2006). To further investigate potential antitumor bioactive components in TCM (Wu *et al.*, 2010) or potential replaceable medicinal ethno-health-promoting plants, a screening of the crude extracts of two *Liriodendron* species and the hybrid was assessed against five tumor cell lines using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazodium bromide) assay *in vitro*.

MATERIALS AND METHODS

Plant materials, chemicals and cell lines

Fresh plant materials (leaves and barks ca 500 g respective) of each species were harvested in Nanjing Forestry University (NFU) in August, 2010. All collected specimens were identified systematically by Prof. Jisen Shi, and voucher specimens of each plant species are deposited in herbaria at Nanjing University of Science and Technology (NUST) (voucher reference numbers are LT-001-B, LT-001-L, LC-001-B, LC-001-L, LX-001-B, and LX-001-L).

Two chemicals to measure the total phenol contents as standards, gallic acid (GA), folin-phenol (2M), and three cytotoxic assay reagents, MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyltetrazodium bromide), fluorouracil (5-Fu), and adriamycin, were purchased from Sigma, USA. Norisoboldine (NIB) as standard to measure alkaloid contents was purchased from Zelang Medical Technology

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Co. Ltd., Nanjing, China; five tumor cell lines, MDA-MB-231, MCF-7, SGC-7901, HuH7, and HCT15 were presented from the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. All other chemicals were analytical reagents.

Preparation of plant extracts

Freshly collected plant materials were separated to leaves and barks (as required) and immediately air dried in an oven at 35°C. Dried materials (ca 350 g) were ground in a laboratory mill to a moderately fine powder (ca 40 meshes).

To investigate anti-tumor effects of three species systematically, two extract methods were used: The first was, to grind the air-dried barks of *L. tulipifera* to ca 40 meshes, then extract with 95% EtOH (500 mL × 3). After removal of the solvent by evaporation *in vacuo*, the residues (ca 35.0 g) were obtained and re-dissolved in 500 mL MeOH. The ethanol solution was filtrated, and the clear solution was subjected to evaporate under vacuum again. The final residue (30.4 g, LTB0 in table 1) was divided into two parts. 5.0 g was left for bioassay. The other 25.4 g was re-dissolved in 200 mL MeOH, and was mixed with 100-200 mesh silica gel, dried it *in vacuo*, eluted silica gel with MeOH, and then evaporated under reduced pressure. next, the extract eluted from silica gel was re-extracted with chloroform (250 mL × 3) and MeOH (250 mL × 3) successively to yield LTB1 (8.4 g) and LTB2 (13.3 g) finally; The second extract method was according to (Yan *et al.*, 2009) with some modifications, the powdered bark of *L. tulipifera* was infiltrated with 10% ammonia over night, filtrated, then refluxed with chloroform (2 L× 3). The solution was filtrated again. The organic part was concentrated under reduced pressure to about 500 mL. The chloroform solution acidified with 1 M HCl solution until no alkaloid was detectable. The solution was adjusted to pH ≥ 9 with ammonia solution. Finally, the basic solution was extracted with chloroform (100 mL × 3) again, and evaporated chloroform *in vacuo* to obtain the dry residue 1.34 g.

Other *Liriodendron* plant materials such as the barks of *L. chinense* and *L. chinense* x *L. tulipifera* and the leaves of these three species were extracted the same as above. The weights of the different fractions were shown in table 1.

All the extracts were stored sterile screw-capped bottles at -20°C until bioassay. For the bioassays, plant extracts were dissolved in dimethylsulphoxide (DMSO) at a concentration of 10 mg/mL and deposited in icebox at -20°C.

Determination of cytotoxicities against tumor cells

Cell culture

The cultural methods of all five human tumor cells, MDA-MB-231 and MCF-7 breast cancer cells, SGC-7901 gastric cancer cells, HuH-7 hepatocarcinoma cells, and HCT-15 colon carcinoma cells, were the same as described in literature (Wu *et al.*, 2010), and the cultural conditions of other three human tumor cells were the same as above two breast tumor cells (MCF-7 and MDA-MB-231) except for gastric cancer cells SGC7901 and colon cancer cells HCT15 in 1640, hepatic cellular cancer cells HuH7 in DMEM.

MTT assay on tumor cells

The MTT assay used to measure the cytotoxic activities of the extracts on five tumor cells was the same as described in literature (Wu *et al.*, 2010).

Determination of phenol and alkaloid contents in the extracts

Total phenols were estimated using a colormetric assay based on procedures described by Lizcano (Lizcano *et al.*, 2010) with some modifications. Briefly, 300 µL of the extracts were mixed with 2250 µL of 1:10 diluted (in Milli-Q water) Folin-Ciocalteau phenol reagent. 5 min later in the dark at 27°C, 2250 µL of $NaHCO_3$ was added to the mixture. The tubes were kept in the dark for another 90 min at 30°C, then the absorbance was measured at 725 nm. Gallic acid (5.14-51.40 µg/mL) was used for plotting the standard curve. Each result was expressed as weight percent of gallic acid (GA) equivalent per extract.

Table 1: The yields of different extracts from different plant tissues

L. tulipifera (500 g)*			*L. chinense* (500 g)			*L. chinense* x *L. tulipifera* (500 g)		
LTB0	LTB1	LTB2	LCB0	LCB1	LCB2	LXB0	LXB1	LXB2
30.4 g	8.4 g	13.3 g	27.2 g	7.2 g	12.1 g	25.3 g	6.5 g	10.4 g
LTB0	LTB1	LTB2	LCB0	LCB1	LCB2	LXB0	LXB1	LXB2
84.2 g	8.7 g	60.2 g	71.7 g	2.8 g	51.7 g	65.6 g	3.9 g	52.5 g
LTBA		LTLA	LCBA		LCLA	LXBA		LXLA
1.34 g		0.60 g	0.48 g		0.35 g	0.35 g		0.21 g

LT: *L. tulipifera*; LC: *L. chinense*; LX: *L. chinense* x *L. tulipifera*; *: weights of fresh plant materials; B: barks; L: leaves; 0: total ethanol extracts from plant firstly; 1: chloroform extract from the ethanol extract secondly; 2: the rest of 1 which dissolve in methanol finallv. The svmbols in table 2 and table 3 are the same as table 1.

The total alkaloid contents of the samples were assessed using a method previously described by Ribeiro (Ribeiro *et al.*, 2008) with some modifications. Total alkaloids were determined by a spectrophotometric method, in which they were precipitated by Dragendorff's reagent followed by the formation of a yellow bismuth complex in nitric acid with thiourea. Each Result was also expressed as weight percent of norisoboldine equivalent per extract.

RESULTS

Cytotoxic activities

The intoxication of the plant extracts on cancer cell lines were determined by MTT assays with Fluorouracil (5-Fu) and adriamycin as positive controls. All the data were illustrated in table 2.

Phenolics and alkaloid contents measurements

The weights of different fractions were shown in table 1. The total phenol and alkaloid contents of the different extracts from *Liriodendron* plants were shown in table 3. Total phenols and alkaloids contents were expressed as weight percent of gallic acid (GA) and norisoboldine (NIB) equivalents respectively. The percent values of phenols ranged from 5.6% to 24.6% in the extracts from the barks of *Liriodendron* plants, from 13.9% to 36.4% in the extract from leaves, and from 17.3% to 37.0% in the extract obtained with second method. As for alkaloids contents, the percents from 5.0% to 8.6% in the extracts from barks, from 7.6% to 15.9% in the extracts from leaves, and from 22.2% to 34.1% in the extracts obtained by second method were observed.

DISCUSSION

As shown in table 2, all the extracts tested had different IC_{50} values. Firstly, with the extracts obtained by the first method, the IC_{50} values ranged from 1.3 μg / mL (LTB1) to 65 μg / mL (LTL0) on MDA-MB-231 cells, from 0.4 μg / mL (LTL1) to 52 μg / mL (LXL2) on MCF-7 cells, from 0.42 μg / mL (LTB1) to 17 μg / mL (LXL2) on HuH-7 cells, from 0.5 μg / mL (LTB1) to 37 μg / mL (LCB0) on SGC-7901 cells, and from 0.61 μg / mL (LXL1) to 15 μg / mL (LCB2) on HCT-15 cells; With the second extract method, The IC_{50} values ranged from 0.3 μg / mL to 31 μg / mL on all five tumor cells. Regarding the cytotoxicity against cancer cells, effects of the extracts obtained by first method were better than those by the second method; Secondly, the extracts treated cells showed total 27 IC_{50} values distributed in 14 extracts lower than the positive controls; Thirdly, The cytotoxic activities of extracts from the barks of *Liriodendron* plants were generally higher than those from the leaves. Furthermore, the activities of lower polar solvent extracts from barks were higher than those in higher polar solvents; Interestingly, LTL1 showed higher effects on four kinds of tumor cells, MCF-7, HuH-7, SGC-7901, and HCT-15.

As shown in table 3, the highest phenol concentrations were found in the extracts LTL2 from the leaves of *L. tulipifera*, LCB2 from the barks of *L. chinense*, and LTLA from the leaves of *L. tulipifera* (36.4%, 24.6% and 37.0%, respectively). The highest total alkaloid contents were found in the extracts LXL1 from the leaves of *L. chinense* x *L. tulipifera*, LXB2 from the barks of *L. chinense* x *L. tulipifera*, and LTBA from the barks of *L. tulipifera* (15.9%, 8.6% and 34.1%) respectively. The phenol contents in the extracts from higher-polar solvent are higher than that in the lower-polar solvent with both leaves and bark samples. In general, the phenol contents of extracts from leaves are higher than that from barks. LXB1 (5.6%), LCL1 (13.9%), and LTBA (17.3%) showed the lowest phenol contents in the extracts from barks, leaves, and extracts obtained by second method

Table 2: Cytotoxicity (IC_{50}, μg/mL) of extracts from plants leaves and barks on five tumor cell lines

Fraction	LTB0	LTB1	LTB2	LCB0	LCB1	LCB2	LXB0	LXB1	LXB2
MDA-MB-231	8.0	1.3	5.2	20	6.0	17	14	12	23
MCF-7	18	1.8	19	9.0	2.5	21	20	13	17
HuH-7	1.8	0.42	16.9	6.5	2.4	14	2.1	7.0	15
SGC-7901	5.5	0.5	22	37	1.8	13	11	0.62	22
HCT-15	6.6	2.18	8.1	1.1	4.4	15	9.0	8.0	6.8
Fraction	LTL0	LTL1	LTL2	LCL0	LCL1	LCL2	LXL0	LXL1	LXL2
MDA-MB-231	65	15	-	36	17	52	7	11	44
MCF-7	21	0.4	-	20	16	23	32	21	52
HuH-7	1.7	0.5	6.41	6.9	13	6.4	1.0	4.1	17
SGC-7901	1.5	0.8	7.9	5.6	7.0	12	0.63	1.0	5.4
HCT-15	4.9	1.9	8.7	3.0	3.0	2.9	1.1	0.61	3.4
Fraction	LTBA	LCBA	LXBA	LTLA	LCLA	LXLA	5-Fu	A	
MDA-MB-231	14	22	30	29	19	28	1.2	0.1	
MCF-7	24	23	28	25	6.6	30	1.4	0.11	
HuH-7	19	21	17	11	1.5	11	0.51	2.40	
SGC-7901	31	23	19	5.0	0.3	9.2	0.19	0.69	
HCT-15	15	17	16	4.0	1.0	3.8	1.51	3.05	

Table 3: Phenol and alkaloid contents in the extracts from plants leaves and barks

Fraction	LT B0	LT B1	LT B2	LC B0	LC B1	LC B2	LX B0	LX B1	LX B2
Phenol	14.6	6.5	20.9	18.1	7.7	24.6	11.1	5.6	15.5
Alkaloid	6.1	5.7	6.9	7.5	7.5	7.4	5.0	6.4	8.6
Fraction	LT L0	LT L1	LT L2	LC L0	LC L1	LC L2	LX L0	LX L1	LX L2
Phenol	25.5	14.9	36.4	25.8	13.9	31.3	22.7	15.2	25.6
Alkaloid	11.4	11.7	7.6	9.3	13.2	9.0	9.6	15.9	8.9
Fraction	LT BA	LC BA	LX BA	LT LA	LC LA	LX LA			
Phenol	17.3	19.0	17.5	37.0	33.8	31.9			
Alkaloid	34.1	28.7	27.5	32.9	22.2	23.5			

respectively. These results are in good agreement with previous works reporting high leaf/bark phenol contents in other plants (Lizcano *et al.*, 2010; Grubesic *et al.*, 2005). Regarding alkaloids, differences of alkaloid contents in the extracts were also observed, the content in the extracts from leaves is higher than those from the barks. The reasons may be that the alkaloids contents are investigated here with NIB possessing two phenolic hydroxyl groups as inner standard sample.

Comparing the relationship between phytochemical contents and anti-tumoral activities, the phenol and alkaloid contents in the extracts from barks were lower than from leaves in general, but the cytotoxic activities in the extracts from barks were higher than those from leaves. More interestingly, the activities of the bark extracts in lower polar solvents were higher than in higher polar solvents. All these information suggests that the cytotoxic components might not be phenols and alkaloids. All researches published so far support our observation (Doskotch and El-Feraly, 1969; Doskotch and El-Feraly, 1970; Doskotch *et al.*, 1976; Dong *et al.*, 2009; Moon *et al.*, 2007). That is to say, the cytotoxic active components would be sesquiterpenes or other lower polar components in *Liriodendron* plants barks.

CONCLUSION

In this work, the phenol and alkaloid contents of different extracts from *Liriodendron* plants and their potential antitumor activities of the extracts were systematically studied for the first time. We observed antitumor activities of different *Liriodendron* plant extracts *in vitro*. The lower polar extracts from barks showed more elevated cytotoxic active than higher polar extracts no matter from barks or from leaves. A detailed analysis of their cytotoxic chemical composition, with regards not only to sesquiterpenes, but also other phytochemicals, as well as their *in vitro* cytotoxicity potential in cell systems merits further investigation.

It's well known that *L. tulipifera* and *L. chinense* are ethno-plants that can treat some diseases. However, their hybrid, *L. chinense* x *L. tulipifera*, has not been explored before although are expected to show potential medicinal values as an ethno-health-promoting plant.

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