Antioxidization and antiproliferation of extract from leaves of *Toona sinensis*

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ABSTRACT

Objective: To determine the antioxidization and antiproliferation of extract from leaves of *Toona sinensis* (LTS).

Methods: The total phenolic extract of LTS was obtained by solvent and polyamide resin to determine the content. The antioxidization of the LTS extract was measured by TOSC assay. Antiproliferation was studied in vitro with different human cancer cells.

Results: The total phenolic content in the LTS was (427.53±4.31) mg/g and antioxidization was 807.64 μmoL vitamin C equivalents/g in the sample. The extract significantly inhibited the colon cancer cell Caco-2, human liver cancer cell $HepG_2$ and breast cancer cell MCF-7 proliferation with EC_{50} (4.00±0.39), (153.16±13.49) and (193.46±14.68) μg/mL, respectively. The bioactivity index (BI) of the LTS extract was nearly 283. Caco-2 was more sensitive than MCF-7 and $HepG_2$.

Conclusion: Extract from LTS has anticancer properties useful for preventing chronic diseases.

KEY WORDS *Toona sinensis*; phenolics; antioxidant; antiproliferation; cancer

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香椿叶提取物抗氧化和抑制癌细胞增殖的研究

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[**摘要**] **目的：**研究香椿叶提取物抗氧化和抑制细胞增殖的活性。**方法：**借助溶剂浸提和聚酰胺树脂获得香椿叶的总酚提取物进而考察其含量。采用 TOSC 实验衡量香椿叶提取物的总抗氧化活性，同时利用体外实验观察其对人类不同类型癌细胞生长的抑制作用。**结果：**香椿叶提取物的总酚含量为 (427.53 ± 4.31) mg/g，每克样品的抗氧化活性达到 807.64 μmoL 维生素 C 当量。提取物可以显著抑制人肠癌细胞 Caco–2、肝癌细胞 $HepG_2$ 和乳腺癌细胞 MCF–7 的生长，其 EC_{50} 分别为 (4.00 ± 0.39)，(153.16 ± 13.49)，(193.46 ± 14.68)μg/mL。香椿叶提取物的生物活性指数 (BI) 约 283，肠癌细胞 Caco–2 对提取物的敏感性超过了乳腺癌细胞 MCF–7 和肝癌细胞 $HepG_2$。**结论：**香椿叶提取物具有一定抗癌功效，对慢性疾病的预防具有应用价值。

[**关键词**] 香椿；酚类物质；抗氧化；抑制增殖；癌症

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Biography: LIU Jinfu, master, professor, mainly engaged in the research of natural product chemistry and functional food.

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Toona sinensis (Meliaceae, *T. sinensis*), a perennial deciduous tree vegetable, is widely distributed in Asia. Its fresh, young leaves and shoots are edible and nutritious. Many compounds, such as phenolics and flavonoids compounds, were identified and isolated from *Toona sinensis* [1-2]. Nearly every part of *Toona sinensis* has medicinal effects on human health.The leaves of *Toona sinensis*(LTS) have been used to treat enteritis, dysentery, carbuncles, boils, dermatitis, scabies, tinea blanca, heliosis, and improve body health in traditional Chinese medicine[3]. In Korean, *Toona sinensis* are used to remove the odor of meat, tenderize the meat and improve the meat quality[4]. It has been speculated that LTS may possess other properties including inhibiting Leydig cell steroidogenesis[5], being a potent anti-LDL glycative agent[6] and improving the dynamic activity of human sperm[7]. The extracts of *Toona sinensis* have been used to lower blood pressure and enhance glucose uptake and lipolysis in 3T3-L1 adipocytes[8-9]. Furthermore, Methyl gallate extracted from *Toona sinensis* was showed to protect against hydrogen-peroxide-induced oxidative stress and DNA damage in Madin-Darby canine kidney (MDCK) cells[10]. In recent years, it has been reported that *Toona sinensis* has anticancer activity. The extracts induced apoptosis in human premyelocytic leukemia cells[11] and human ovarian cancer cells[3].

In this study, total phenolics from LTS was extracted by solvent and polyamide resin.Total antioxidant activity of the extracts was measured by the total oxyradical scavenging capacity (TOSC) assay and antiproliferative activitiy was also studied in vitro by human colon cancer Caco-2 cells, human liver cancer $HepG_2$ cells and breast cancer MCF-7 cells with MTS assay.

1 Materials and methods

1.1 Materials

Sodium hydroxide, methyl tert-butyl ether and ethanol were purchased from Fisher Scientific (Pittsburgh, PA, UAS). The polyamide resin was obtained from Naikai University Chemical Factory(Tianjin, China). Gallic acid was obtained from ICN Biomedical Inc.(Costa Mesa, CA, UAS). 2, 2′-azobis(2-amidinopropane)dihydrochoride(ABAP) was obtained from the Wako Chemicals(Richmond, VA, USA).Folin-Ciocalteu reagent, hyrdrochloric acid, α-keto-ç-methiolbutyric acid (KMBA), MTS and DMSO were purchased from Sigma Chemical Company(St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO Life Technologies Corporation(Grand Island, NY, USA).All the reagents used in the study were of analytical grade.

1.2 Cell culture

$HepG_2$ human liver cancer cells were maintained in William's medium E (WME), containing 10 mmol/L Hepes, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 2 μg/mL glucagon, and 5% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin in an incubator at 37 ℃ with 5% CO_2. Caco-2 human colon cancer cells were maintained in DMEM, containing 10 mmol/L Hepes, 5% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin. MCF-7 human breast cancer cells were maintained in α minimum essentail medium (MEM-α), containing 10 mmol/L Hepes, 10 μg/mL insulin, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL gentamicin and 10% FBS.

1.3 Preparation of extract from LTS

Toona sinensis were cultivated in the garden of Tianjin University of Science & Technology(Tianjin, China). A flowchart for the extracts of LTS was provided in Figure 1. The plants were collected and dried in the shade. Then the leaves of the plants were separated from the stems and grounded to pass through 40 meshes. The powders (200 g) were macerated with 60% ethanol (1:10, *w/v*) at 60 ℃ for 8 h. It was repeated for 3 times and the combined solution was put into a column filled with polyamide resin. Then, at

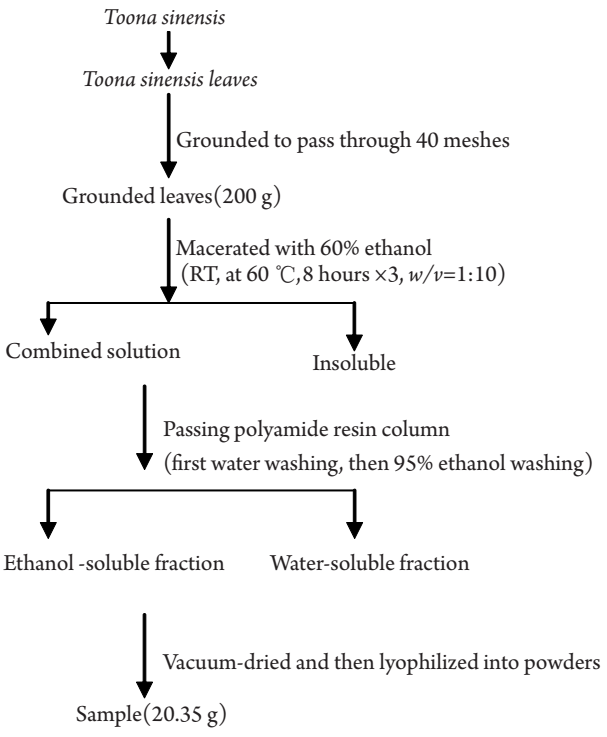

Figure 1 Flowchart of phytochemical extraction for Leaves of *Toona sinensis*.

least 6 volumes of water flowed through this column to get rid of the water-soluble impurities including protein and polysaccharides, etc. Then 95% ethanol was used to run through the column. The obtained solution was combined, vacuum-dried and then lyophilized into powders(20.35 g). The samples were stored at –20℃ until used.

1.4 Determination of total phenolic content

The content of total phenolics was analyzed spetrophotometrically using a Folin-Ciocalteu colorimetric method described previously[12] with modifications[13]. Briefly, the appropriate dilutions of samples were oxidized with Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue colour was measured at 760 nm after 90 min by a MRX II Dynex spectrophotometer (Dynex Technologies, Inc., Chanilly, VA). Gallic acid was used as standard.

1.5 Quantification of the total antioxidant activity

The total antioxidant activity of the extracts from LTS was determined by a modified TOSC assay[14] in Dr.Liu's laboratory[15]. Antioxidant activity was measured at four different time points (10, 20, 30, and 40 min) and with four different concentrations (50, 100, 250, and 500 μg/mL) to determine the TOSC value. The TOSC value for each concentration of the sample was calculated using the integration of the area under the kinetics curve. The TOSC value for each was quantified according to the following equation:

$$TOSC = 100 - (\int SA / \int CA \times 100)$$

where ∫SA and ∫CA are the integrated areas from the sample and control reaction, respectively. The TOSS value was calculated as μmoL of vitamin C equivalents per gram of sample for 3 replications. The median effective dose (EC_{50}) was determined from the dose-response curve of concentration of the sample.

1.6 Measurement of cytotoxicity of human cancer cells

Caco-2 cells, $HepG_2$ cells and MCF-7 cells were placed in 96-well plate (5.0×10^4 cells/well) and incubated for 24 h at 37 °C. The growth medium was discarded and added different concentrations extract from LTS. After 24 h treatment, the medium was removed and the plate was washed with PBS. A volume of 50 μL/well methylene blue staining solution [98% Hank's balanced salt solutions (HBSS), 1.25% glutaraldehyde, 0.6% methylene blue] was applied to each well and the plate was incubated at 37 °C for 1 h. Methylene blue was discarded and rinsed the plates several times by immersion in Milli-Q H_2O. The excess water should be tapped out of the wells and the plate allowed to air-dry briefly before addition of 100 μL elution solution (49% PBS, 50% ethanol, 1% acetic acid) to each well. The plate was then placed on a bench-top shaker for 30 min to allow uniform elution. The absorbance was read on plate reader at 570 nm and calculated each concentration.

1.7 Measurement of Caco-2, HepG_2, and MCF-7 cell proliferation

The antiproliferative activity of LTS extracts was assessed by measuring inhibition of Caco-2, $HepG_2$, and MCF-7 human cancer cell proliferation. Antiproliferative activities were determined by the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) reported previously[16-17]. Cancer cell concentrations of 2.5×10^4/well in the growth media were placed in each well of a 96-well flat-bottom plate. Cell proliferation was measured by the ability of viable cells to reduce MTS to formazan. After 4 h of incubation at 37 °C in 5% CO_2, the growth medium was removed and media containing various concentrations of extracts of LTS were added to the cells. Control cultures received the extraction solution minus the extracts, and blank wells contained 100 μL of growth medium without cells. After 96 h of incubation, cell proliferation was determined with a tetrazolium reagent. The MTS absorbance was read at 490 nm using MRX II DYNEX spectrophotometer. The EC_{50} was determined.

1.8 Statistial analysis

Statistial analyses were conducted using Mini Tab software. All values were expressed as mean± standard deviation ($\bar{x}\pm s$) for at least 3 replications. Differences amang each treatment were determined using *t* test. For relationship plots, significance of the relationship was determined by using analysis of variance. $P<0.05$ was considered statistically significant.

2 Results

2.1 Total antioxidant activity

The total phenolic content of extracts from LTS was (427.53±4.31) mg gallic acid equivalents/g. While, the results of the TOSC assay for total antioxidant activity were expressed as μmoL of vitamin C equivalents/g of LTS. The concentrations of ascorbic acid and the sample required to cause 50% inhibition of the reaction (EC_{50} or PSC unit=0.5) were (2.47±0.13) and (17.34±0.83) μg/mL,

respectively. The total antioxidant activity of sample was significantly (807.64 ±14.6) μmoL of vitamin C equivalents/g of sample (P<0.01).

2.2 Cytotoxicity and antiproliferative activity of extracts toward human cancer cells

As shown in the Figure 2, within the 500 μg/mL doses, the extracts from LTS showed no cytotoxicity toward Caco-2 cells, $HepG_2$ cells and MCF-7 cells by methylene blue cytotoxicity assay.

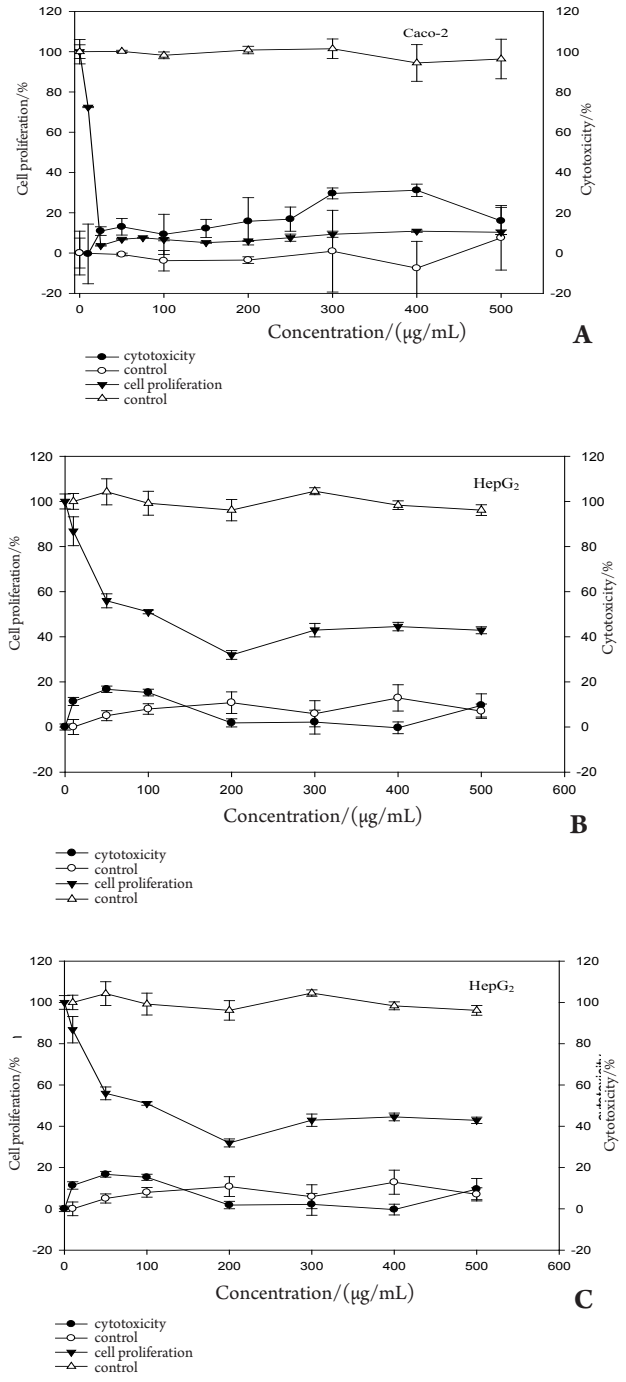

Figure 2 Dose-response curve of cytotoxicity and antiproliferative activity of extract from LTS. A: Caco-2 cells; B:$HepG_2$ cells; C:MCF-7 cells.

The antiproliferative activities of different concentrations from LTS were summarized in vitro to the Caco-2 cells, $HepG_2$ cells and MCF-7 cells in Figure 2. Caco-2 cell proliferation was significantly inhibited in a dose-dependant manner as its concentration was lower, under 5 μg/mL (P<0.05). At the dose of 10 μg/mL, approximately 27% inhibition was observed compared to the control (P<0.05). At the dose of 25 μg/mL, the inhibition rate was approximately 96 % (P<0.05). The EC_{50} of the extract from LTS for inhibition to Caco-2 cell was (4.00±0.39)μg/mL, with a lower EC_{50} value indicating a higher antiproliferative activity.At the dose of 200 μg/mL, the inhibition rate to $HepG_2$ cells was approximately 78.30% (P<0.05). The EC_{50} to $HepG_2$ was (153.16±13.49) μg/mL.As to the breast cancer cell MCF-7, there was not significantly different between solvent control cells and cells treated with 50 μg/mL the extract from LTS. At the dose of 400 μg/mL, the inhibition rate was approximately 85% (P<0.05). The EC_{50} to MCF-7 was (193.46±14.68) μg/mL.

3 Dissusion

Cells in human and other organisms are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. Overproduction of oxidants can cause oxidative stress, which is associated with chronic diseases. Therefore, increased consumption of fruits and vegetables containing high levels of antioxidants has been recommended to prevent or slow the oxidative stress.

In this study, we investigated the profiles of total phenolics from LTS by applying solvent extraction, separated by the polyamide resin column, vacuum-dried and lyophilized methods. The total phenolic of sample was (427.53±4.31)mg gallic acid equivalents/g, which was 4.35% of LTS weight and equal to 653 mg gallic acid equivalents/100 g of fresh weight of the leaves, higher than the common fruits and vegetables[16-18]. So there was abundance phenolics in LTS. The phytochemical extracts of fruits and vegetables, especially for phenolics, showed potent antioxidant activities and the combination of phytochemicals and synergistic mechanisms matrix may be responsible for the potent antioxidant activities[19]. Other experiments showed that there was a direct linear relationship between the phenolic contents and total antioxidant activities in the 11 fruits tested (R2=0.9788, P<0.01), indicating phenolics may be the major contributor

to the total antioxidant activities of fruits[16]. The phenolic content of extract separated from LTS by us was (427.53±4.31)mg gallic acid equi/g, indicating higher antioxidant activity. The total antioxidant activity of the sample was significantly 807.64 μmoL of vitamin C equivalents/g. The resent study showed that the old leaves of *Toona sinensis roem* had ferric reduing/antioxidant power (FRAP) value [(10 578.71 ±149.00) μmol/L FRAP/g] and strong DPPH radical scavenging capacity[20]. LTS were mainly used as vegetable and traditional Chinese medicine to cure treating diseases[3]. Nowadays, antioxidant activity and antiproliferation of *Toona sinensis* are deeply studied. Oxidative stress has been linked to many chronic diseases, such as cardiovascular diseases and cancer.

Some recent studies showed that extracts of *Toona sinensis* had anticancer activity. They induced apoptosis via reactive oxygen species in human premyelocytic leukemia cells and human ovarian cancer cells.They inhibited tumor growth in a murine xenograft model[3, 11].

We selected designedly human cancer cells from different apparatus to investigate the effect of *Toona sinensis* on cell proliferation.The data clearly showed that extracts from LTS could inhibit the growth of human colon cancer Caco-2 cell, human liver cancer $HepG_2$ cell and human breast cancer MCF-7 cell at a dose range from 5 to 50 μg/mL, 10 to 200 μg/mL, and 50 to 400 μg/mL. At these doses, extracts from LTS showed no cytotoxicity to cells by methylene blue cytotoxicity assay.The colon cancer Caco-2 cells were more sensitive than to the breast cancer MCF-7 cell and human liver cancer $HepG_2$ cells.

Above results showed that extracts of *Toona sinensis* inhibited not only proliferation of the respiratory and gastrointestinal tract (e.g., lung and colon), but also the metabolizable apparatus cancer cells($HepG_2$ cells), the hormonally related cancers cells (human ovarian cancer cells and MCF-7 cells) and human premyelocytic leukemia cells.These results suggested that extracts of *Toona sinensis* were comprehensive and potentially valuable for prevention of cancer and chronic diseases.

The bioactivity index (BI) for dietary cancer prevention was proposed to provide a simple reference for consumers to choose vegetables on the basis of their beneficial activities[16]. Red pepper and spinach had the highest antioxidant(46.95 mol of vitamin C equivalents/g) and antiproliferative activities (42.51 mg/mL, $HepG_2$) respectively, so the BI value was calculated by the followinge quations:BI=1/2(score of total antioxidant activity +score of antiproliferative activity); score of total antioxidant activity= LTS TOSC value/red pepper TOSC value; score of antiproliferative activity= spinach EC_{50} value/LTS EC_{50} value. So the BI of LTS extract was nearly 283. It indicated that extracts from LTS may be potent to cure gastrointestinal tract (e.g., esophagus, stomach, and colon) diseases. Obviously, the antioxidant and anticancer activity mechanism of *Toona sinensis* was still needed to be further studied, which may cause by single component or complex component.But it is sure that the extracts of LTS may have anticancer properties and functional food valuable for application in chronic diseases prevention.

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