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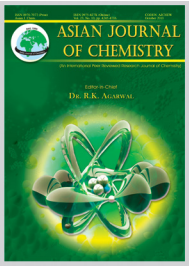

Inhibitition on H22 Allografts, HepG2 and SMMC-7721 Human Hepatoma Cells of Adlay Seed Extracts

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To examine the effects of effective chemical substances of adlay seed on the hepatoma *in vitro* and *in vivo*. H22 cancer-bearing mice were chosen to observe the effects of tumor inhibiting effective chemical substances of adlay seed, drug-containing serum were prepared by serum pharmacology method. MTT assay was used to observe the proliferation inhibition rate of HepG2 and SMMC-7721 cells after incubation with the drugs containing serum. The effective chemical substances of adlay seed could inhibit the growth of H22 cancer in mice, the tumor inhibition rate of the different concentration was 42.46, 38.05, 33.31 % and the drug-containing serum could obviously inhibit the growth of HepG2 and SMMC-7721 cells.The effective chemical substances of adlay seed has the effect on inhibiting the growth of hepatoma.

Key Words: Adlay seed, Hepatoma, Serum pharmacology, Effective chemical substances.

INTRODUCTION

Adlay (*Coix lachryma-jobi* L. var.ma-yuen Stapf.) has been used for more than thousand years as traditional Chinese medicine. It is well-known as medicinal and edible grass crop which grows throughout China[1]. It is distributed in the humid regions of China and has an ability to drought and water-logging. According to pharmacopoeia of China, adlay seed is derived from the dried kernel of *Coxi lachryma-jobi* and is an annual crop[2]. Chemical studies have shown that it consists of fatty acid and other components such as polysaccharides, sterol[3-6], *etc*. The adlay seed has been reported to exhibit immune antiinflammatory, stomachic, diuretic and antiseptic effects *in vivo*. It has been used in China for the treatment of warts and disorders of the female endocrine system rheumatism and neuralgia[7,8].

Recent studies demonstrated that adlay seed could inhibit the growth of Ehrlich ascites sarcomas and could increase the activity of cytotoxic T-lymphocytes and naturally kill cells in experimental animals[9,10]. However, the molecular mechanism by which adlay seed inhibits tumor development is largely unknown.

This study examine extracts and serum pharmacological method to evaluate the effective chemical substances of adlay seed on hepatoma cell lines to explore the mechanism in liver cancer cells, provide a theoretical basis for liver cancer in clinical application, and provides the information and evidences for further isolate components of anti-tumor active ingredient.

EXPERIMENTAL

Adlay (*Coxi lachryma-jobi*) was purchased from the BaoJian Pharmacy (Dalian, China) and was identified by Professor Yanjun Zhai (Liaoning University of Traditional Chinese Medicine). Acetonitrile,dichloromethane and methanol (chromatographic grade) were purchased from Tedia Co. (Fairfield, OH, U.S.A.). Water was purified using a Milli-Q purification system (Millipore Co., France). DMEM, RPMI 1640, trypsin, MTT, dimethyl sulfoxide and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Penicillin, streptomycin and cyclophosphamide were purchased from Harbin General Pharmaceutical Factory (Harbin, China). Olein (with the purity higher than 98 %) was from the Chinese National Institute of Control of Pharmaceutical and Biological Products (Beijing, China).

Cell lines: The H22 murine hepatocellular carcinoma cell line, HepG2 and SMMC-7721 human hepatocellular carcinoma cell lines were purchased from CAS Shanghai Institutes for Biological Sciences Cell Resource Center (Shanghai, China).

Animals: The healthy female and male KM mice (18 ± 2 g, 6 weeks old) were obtained from the Animal Center Laboratory of Dalian Medical University, the qualification certificate was SCXX(Liao)2008-0002.Animals were kept in an

environmentally controlled breeding room (temperature: 24 ± 2 °C, humidity: 60 ± 5 %, 12 h dark-light cycle) for 1 week before the experiment.

Instruments and chromatographic conditions: The chromatographic system (Shimadzu, Kyoto, Japan) consists of a pump (LC-10ATvp), an evaporative light-scattering detector (SEDEX 75), a column oven (AT-130) and a LC-Workstation. BCN-1360B Biological clean bench (Beijing East Union Hall Instrument Manufacturing Co., Ltd.), CO_2 Incubator (Nuaire Co., U.S.A.), Milli-Q system (Millipore, Bedford, MA, U.S.A.) AE31 inverted microscope (MOTIC CHINA GROUP Co., Ltd.), SUNRISE Microplate Reader (Tecan Trading Co., Shanghai)

Chromatographic system: C_{18} column chromatography was used (250 mm × 4.6 mm i.d., particle 5 μm, Dikma, Beijing, China). A mobile phase consist of acetonitrile-dichloromethane (60:40, v/v). The flow rate was 1.0 mL min^{-1}. The temperature of drift tube was 35 °C and the column temperature was kept at 25 °C.

Preparation of group of effective chemical substance: Adlay were powdered to a homogeneous size in a mill, sieved through No. 40 mesh. The powder was extracted 3 times, ethyl acetate was used as solvent, the extraction time was 0.5 h and was conducted for 3 times and then filtered. The resulting solution was evaporated to dryness in vacuum[11].

Preparation of standard solutions: Stock standard solutions of olein was prepared by dissolving in mobile phase at a concentration of 0.14 mg/mL.

Preparation of sample solutions: The effective chemical substances was dissolved in proper mobile phase. The sample was filtered through a 0.45 mm Millipore filter.

5 μL standard solution and 10 μL sample solution were injected for HPLC analysis. The results are shown in Fig. 1. Through experiment and literature review, the effective chemical substances[12] are shown in Table-1.

Drug administration

Treatment of H22 allografts and Preparation of drug-containing serum: Sixty rats were randomly divided into six groups including three groups treated with the effective chemical substances in three doses, one positive control group with cyclophosphamide (CTX), one negative control group and one blank control group. H22 cells (2×10^7 mL^{-1}) suspended in 0.2 mL of RPMI 1640 with 10 % FBS were inoculated subcutaneously in the right armpit of each mouse except the blank control group to establish a tumor model[13,14].

After 24 h of inoculation, gastric transfuse the effective chemical substances of adlay seed (3, 1 and 0.3 g/kg), gastric transfusion of CTX (20 mg/kg) as positive control and gastric transfusion of equivalent volume of distilled water as negative control, once per day, for 10 days. On the next day, after 1 h

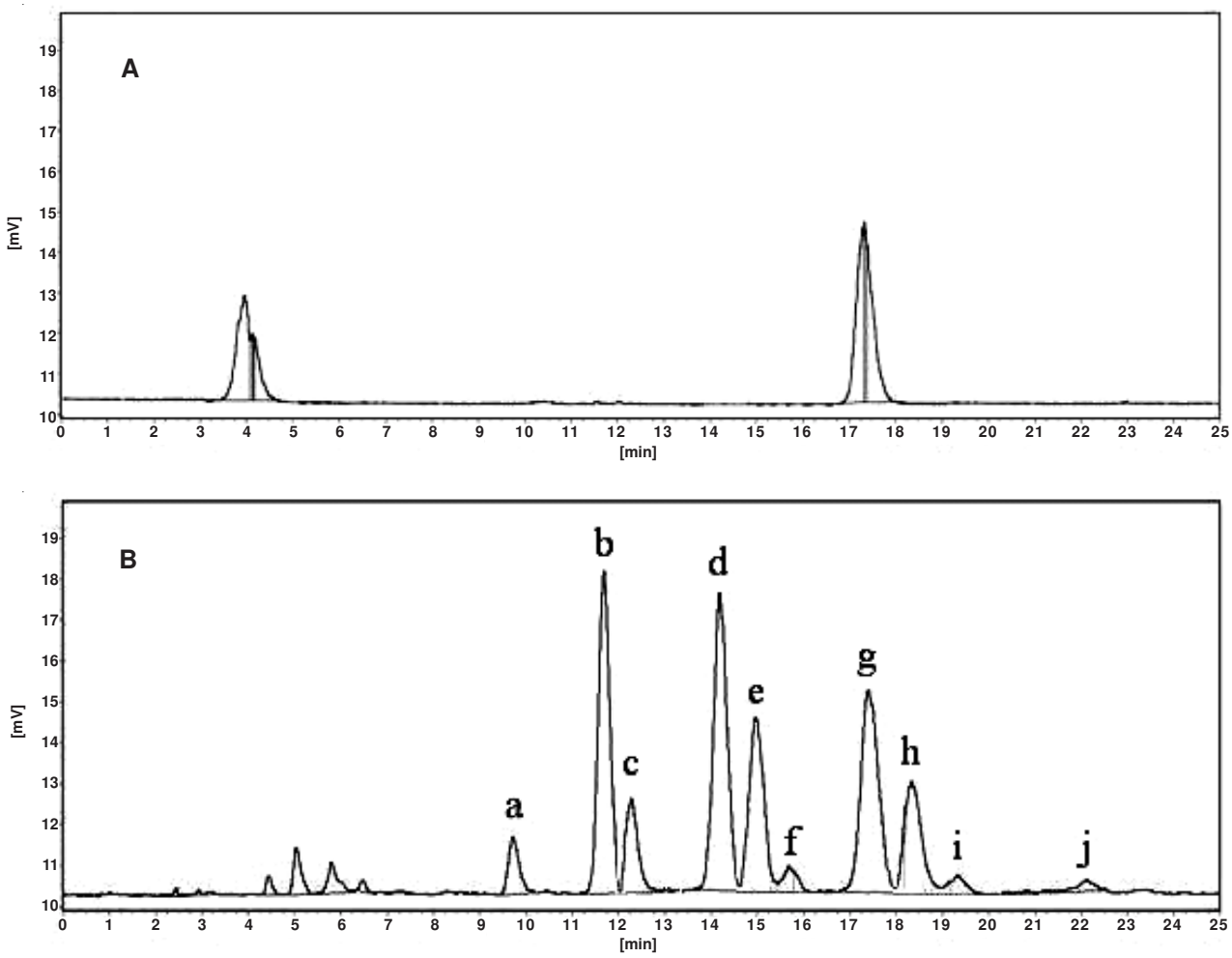

Fig.1 A Olein B Effective chemical substances of adlay seed

TABLE-1
EFFECTIVE CHEMICAL SUBSTANCES OF ADLAY SEED

n	Time (min)	Components	m.f.	m.w.
a	9.721	Linolein	$C_{57}H_{98}O_6$	878.7
b	11.679	Dilinoleic acid oleic acid glyceride	$C_{57}H_{100}O_6$	880.8
c	12.228	Palmitic acid dilinoleic acid glyceride	$C_{55}H_{98}O_6$	854.7
d	14.188	Linoleic acid dioleic acid glyceride	$C_{57}H_{102}O_6$	882.8
e	14.858	Palmitic acid linoleic acid oleic acid glyceride	$C_{55}H_{100}O_6$	856.8
f	15.584	Dipalmitic acid linoleic acid glyceride	$C_{53}H_{98}O_6$	830.7
g	17.370	Olein	$C_{57}H_{104}O_6$	884.8
h	18.327	Palmitic acid dioleic acid glyceride	$C_{55}H_{102}O_6$	858.8
i	19.317	Dipalmitic acid oleic acid glyceride	$C_{53}H_{100}O_6$	832.8
j	22.140	Dioleic acid stearic acid glyceride	$C_{57}H_{106}O_6$	886.8

the completion of drug administration, the mice were weighed and blood was drawn from eyeball, after 0.5 h centrifuged at 3500 rpm for 15 min, drain the upper serum. The sample was filtered through 0.45 mm and 0.22 mm Millipore filter and stored at -80 °C. The tumors were dissected and weighed to calculate the inhibition rate of tumor growth according to the following equation:

Tumor inhibition rate = (Average tumor weight of negative control group – Average tumor weight of test group)/ Average tumor weight of negative control group × 100 %

The differences between test groups and control group were compared by T-test.

Cell cultures: HepG2 and SMMC-7721 human liver cancer cells were cultured in DMEM medium supplemented with 10 % heat inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin in a 5 % CO_2 incubator at 37 ºC.

MTT Assays: To study cell viability, HepG2 and SMMC-7721 human liver cancer cells (5×10^3 cells/well)were respectively seed in 100 µL of medium in 96-well plates and grown overnight. The experiment contained eight groups: control (no cells, no drug), negative control (with media contained cells but no drug-containing serum), blank and four drug treatment groups (drug-containing serum of three doses groups, drugs containing serum of CTX, drug-containing serum of blank control group). Each group was further divided into four subgroups according to the time of incubated with drugs containing serum (24, 48,72 and 96 h).There were five parallel wells in each subgroup. After incubation, MTT (10 µL/well)was added and cultured under the same conditions for another 4 h. The supernatant was removed and the precipitate was dissolved with DMSO (150 µL) and shaked for 5 min. The absorbance at 492 nm (A492) of each well was measured with the microplate reader. The inhibition rate of cell proliferation was calculated according to the following equation:

Inhibition rate = (A492 of drug-containing serum of blank control group well-A492 of sample well)/A492 of drug-containing serum of blank control group well × 100 %

Statistical analysis: All observed data are displayed as mean ± SD. Statistical analysis was performed using the SPSS 16.0 software. The results were analyzed by one-way ANOVA. Differences were considered statistically significant when the *p* value was less than 0.05.

RESULTS AND DISCUSSION

Effect of the extracts of adlay seed on growth of H22 cells: Each dose inhibition rate are higher than 30 % and has the obvious inhibition. As shown in Table-2, each dose inhibition rate has significant differences ($p < 0.05$) compared with negative control group and doesn't have significant differences ($p < 0.05$) compared with positive control group.

Effect of drug-containing serum of adlay seed on HepG2 and SMMC-7721 human liver cancer cells: After data processing, different groups when added with 5, 10, 20 % drug containing serum shows different inhibition rate among which 10 % drug-containing serum shows highest inhibition rate, data are shown in Table-3. Compared with drug-containing serum of blank control group, three doses groups and CTX groups have significant differences($p < 0.05$) and has a good time - effect relation.

H22 cells were inoculated subcutaneously in the right armpit of each mouse, for the test of gastric transfusion ability of the effective chemical substances present in adlay seed. The inhibition rate of large, medium and small dose group are 42.46, 38.05, 33.31 %. Each dose group were more than 30 % that comply with Chinese medicine anti-cancer effect assessment standards and the inhibition rate of every dose group are close to or higher than positive control group which has obvious effect.

It is hypothetically known that it absorbed into the blood-stream and play pharmacological effect on the composition as

TABLE-2
EFFECTIVE CHEMICAL SUBSTANCES OF ADLAY SEED INJECTION ON GROWTH OF H22 TUMOR CELL XENOGRAFTS IN MICE (X ± S)

Treatment	Dose (g kg^{-1} d^{-1})	Weight		Tumor weight (g)	Inhibition rate (%)
		Before	After		
Negative control group	20	20.67 ± 0.34	26.89 ± 3.74	1.40 ± 0.47	–
Positive control group	0.02	19.39 ± 0.79	24.72 ± 2.33	0.86 ± 0.29*	38.33
Effective chemical substances of adlay seed	3.00	20.21 ± 0.96	28.26 ± 2.75	0.80 ± 0.36*	42.46
	1.00	20.65 ± 0.56	29.19 ± 0.37	0.86 ± 0.37*	38.05
	0.30	20.23 ± 0.58	26.98 ± 2.55	0.93 ± 0.52*	33.31

All values of tumor weight are presented as mean ± SD of 10 mice in relevant groups; *$p < 0.05$ *vs.* control group.

TABLE-3
EFFECT OF DRUG-CONTAINING SERUM ON HepG2 AND SMMC-7721 HUMAN LIVER CANCER CELLS

Treatment	Dose ($g\ kg^{-1}\ d^{-1}$)	Inhibition rate (%)							
		HepG2				SMMC-7721			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Blank control	-	-	-	-	-	-	-	-	-
CTX	0.02	33.41	10.47	39.72	67.13	3.09	4.28	6.22	10.72
Drug-containing serum	3.00	16.46	7.80	28.25	69.25	2.64	4.25	9.16	15.90
	1.00	17.95	7.52	31.82	73.52	1.25	1.69	3.70	9.81
	0.30	13.42	12.60	42.46	65.79	-1.86	1.38	3.60	9.27

the real active ingredient. This experiment reviews the influence on its antitumor function by observing medicated serum for tumor cell. The result shows, compared with blank serum, drugs containing serum of three doses groups have significant difference ($p < 0.05$) and inhibition rate was more than positive control group (CTX) serum. The result proves that it inhibits liver cancer cell, proliferates and also indirectly proves antitumor function of the drug in the body.

Animal medicated serum is divided into two kinds, normal physiological condition of medicated serum and pathologic condition of medicated serum. As a result of two kinds of condition for the animals to absorb medication, digestive function is different, the biotransformation is different, especially in drug, the action of the body respond varies and produce the endogenous effective component which is also differ. Therefore, this paper adopts preparation of pathologic state animal serum for the patients at actual situation.

In vitro experiments, the medicated serum inhibits human hepatocellular carcinoma cells, in the group of 5, 10, 20 % medicated serum inhibition rate compared to the role of the most obvious 10 % content, no dose dependent. Although 20 % group of serum contained more dosage of drug ingredients, but relative to the serum of cell growth promoting function, its inhibition rate is weak, so 10 % dosage of inhibitory effect is obvious.

Anticancer effect of adlay seed has been reported, but the effect on the evaluation reports *in vitro* and *in vivo* were rare. In this paper, extracts of adlay seed through the inhibitory H22 liver cancer in mice effects on solid tumor to determine efficacy *in vivo*, by medicated serum on inhibition of human tumor cell lines to determine the efficacy *in vitro*, also identified anticancer effect of adlay seed *in vivo* and *in vitro* simultaneously. Effective group and the composition of the effective chemical substances of adlay seed has been basically clear, its clinical application provides reasonable theoretical basis. Drugs into the blood components and mechanisms are further to be research.

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