Antitumor activity of *Ailanthus excelsa* (Roxb) stem bark fractions and Canthin-6-one

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Abstract

The alkaloidal compound was isolated from chloroform fraction of methanol 70% extract of *Ailanthus excelsa* stem bark of Egyptian origin. Successive extracts of *A. excelsa* stem bark and the isolated compound canthin-6-one were screened for chemopreventive activity. The chloroform extract showed strong inhibitory effect on short term *in vivo* assay for antitumor promotors, Epstein-Barr virus early antigen induction assay, compared with other fractions petroleum ether, diethyl ether and methanol 70% extracts. The isolated compound canthin-6-one also showed strong activity in the course of this assay. Further, these useful materials were investigated for the inhibitory effects in two-stage mouse skin carcinogensis test. Chloroform extract and its active canthin-6-one decrease actually the average number of papillomas per mouse and percentage papillomas in the promoting stage. These materials were found to exhibit the excellent anti-tumor promoting activity in the in vivo carcinogensis test.

Keywords: *Ailanthus excelsa*; Stem bark; Alkaloids; Canthin-6-one; Antitumor activity; Mouse skin carcinogensis.
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(Received:19.07.2009 Accepted:14.03.2011)

Introduction

Biologically active compounds from natural sources are of interest as possible new drugs for many infectious diseases. *Ailanthus excelsa* is a decidous tree belonging to the family Simaroubaceae and widely distributed in Asia and north Australia. *A. excelsa* is a tree up to 10 m tall. The root bark of this tree has quassinoids which have anticancer activity Ogura et al. 1977, the heartwood has triterpenes Srinivas et al. 2006, its bark is used as anthelminthic, expectorant, antispasmodic, Kirtikar and Basu 1933 and antipyretic, Suresh and Dhansekran 1990 stem bark has antifertility Dhanasekaran et al. 1993 antibacterial Shrimali et al. 2001 and antifungal, Joshi et al. 2003 activities. This study was to investigate the antitumor activity of *A. excelsa* fractions and of the alkaloidal compound canthi-6-one, and this provides relevant information regarding further biological effects of alkaloids.

Materials and methods

Plant material

Stem bark of *A. excelsa* was collected from Zoo garden, Giza, Egypt in April 2006. The plant was identified by Dr. Kamal El Batanony, Professor of Taxonomy and Botany, Faculty of Science, Cairo University. Specimens were deposited in the herbarium of National Research Centre (NRC), Giza, Egypt with No. 34254.

Extraction

One kilogram of air dried powder of *A. excelsa* stem bark was successively extracted

with 3 liters of petroleum ether, 3 liters of diethyl ether, 3 liters of chloroform and 3 liters of methanol 70% in a continous extraction apparatus (Soxhlet apparatus) until exhaustion and each extract was concentrated under reduced pressure to give 9 g of petroleum ether extract, 7 g of diethyl ether extract, 11.5 g of chloroform extract and 67.5 g of methanol 70% extract, respectively. Methanol extract (67.5) g was dissolved in 500 ml of distilled water and then acidified with 10%HCl and basified with NH_4OH to pH 9 and then extracted with chloroform several times till exhaustion. The chloroform fraction from methanol (70%) was dried over anhydrous sodium sulphates and then was concentrated to give 1.7 g of crude alkaloids.

Isolation of active constituents of chloroform fraction of methanol (70%) extract of A. excelsa stem bark

Chloroform fracrion was subjected to silica gel column chromatography (E. Merck, type 60-230 mesh, 100 gm of silica gel) and the column eluted with chloroform to afford compound 1 with R_f value 0.75 in $CHCl_3$/ MeOH (95:5). Elution with chloroform: methanol mixture (98:2) afforded another alkaloidal compound which was detected as one spot with violet colour under UV lamp by using TLC silica gel F_{254} and chloroform:methanol (96:4) as solvent system and the amount of this compound was very trace for identification. Also, elution with chloroform:methanol mixture (95:5) afforded another alkaloid compound which was detected as one spot with violet colour under UV lamp by using TLC silica gel F_{254} and chloroform : methanol mixture (90:10) as solvent system and the amount of this compound was also very trace for identification. Elution with further chloroform: methanol mixtures, detection with TLC silica gel F_{254} with different solvent systems and using Dragendroff reagent (specific reagent for alkaloids) revealed that no other alkaloids were detected. The structure of compound **1** was identified by ^{1}H-NMR, ^{13}C-NMR and MS.

Antitumor promoting effect in vitro (Epstein-Barr virus activation test)

The successive extracts of *A. excelsa* stem bark and canthin-6-one were tested for Epstein-Barr virus (EBV) genome carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Sigma, MS, USA). Spontaneous activation of EBV-EA (early antigen) in our sub-line Raji cells less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus non producer type) as described by Ito et al. 1981. The indicator cells (Raji cell, 1 x 10^6/ml) were incubated at 37°C for 48 h in 1 ml of medium containing n-butyric acid (4mmol), 12-*O*-Tetradecanoylphorbol-13-acetate [TPA] (32 pmol, 20 ng in DMSO, 2 µl) as inducer and various amounts of the test extracts in 5 µl DMSO. Smears were made from the cell suspension, and the activated cells that were stained by EBV-EA positive serum from nasopharyngeal carcinoma (NPC) patients were detected by an indirect immunofluoresence technique Henle and Henle 1966. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each extract. The average of EBV-EA induction of the test extracts was expressed as a relative ratio to the control experiment (100%) which was carried out only with n-butyric acid plus TPA. The viability of treated Raji cells was assayed by the trypan Blue staining method.

I. Antitumor activity of the chloroform extract of A. excelsa stem bark and of canthin-6-one using two-stage carcinogensis test on mouse skin tumor in vivo

Six-week old female SENCAR mice maintained under the following standard conditions: 24±2°C, 50±10% relative humidity, and 12/12h light/dark cycle. All animals were given drinking water and food.

II. In vivo two-stage mouse skin carcinogensis test

All the experimental procedures were performed with the approval of the Committee for Animal Research, Kyoto Prefectural University of Medicine. All animals were shaved with surgical clippers, then divided into 5 groups. In group 1, the non-initiating group, mice were treated twice a week with 1.7 nmol TPA in 100µl acetone to back of mouse skin for 20 weeks. In group 2, the positive control group, mice were topically treated with 390 nmol 7,12 – dimethylbenz [a] anthracence (DMBA) in 100µl acetone as an initiating treatment. In group 3, mice were initiated with 100 µl of chloroform extract of *A. excelsa* (topically applied 100µl acetone and at the same time *A. excelsa* chloroform extract was applied). In group 4, mice were initiated with 100 µl of *A. excelsa* chloroform extract. Mice in groups 3, 4 were then promoted twice a week with 1.7 nmol (TPA) in 100µl acetone for 20 weeks starting 1 week after initiating. In group 5, mice were initiated with 390 nmol (DMBA) in 100µl acetone. One week after initiation, they were promoted twice a week with 33 µl of *A. excelsa* chloroform extract for 20 weeks. The incidence of papillomas was observed weekly for 20 weeks, and mice were sacrificed by cervical dislocation at 20 weeks after starting promoting. The average number of papillomas per mouse was analysed with Student's *t*-test.

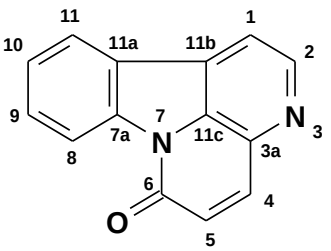
Results

Identification of compound 1

Compound 1 (Canthin-6-one) [100 mg]: R_f: 0.75 in $CHCl_3$/MeOH (95:5). Crystallized from acetone as a pale yellow needles, it gave one spot on TLC in a solvent system chloroform:methanol mixture (99:1) with an orange-red colour sprayed with Dragendroff reagent, MS m/e: 220 (M^+, base peak).

^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 6.98 (d, J = 9.9 Hz, H-5), 7.50 (t, J = 7.6 Hz, H-10), 7.68 (t, J = 7.6 Hz, H-9), 7.95 (d, J = 4.8 Hz, H-1), 8.01 (d, J = 9.9 Hz, H-4), 8.09 (d, J = 7.6 Hz, H-11), 8.64 (d, J = 8.1 Hz, H-8), 8.79 (d, J = 4.8 Hz, H-2).

^{13}C-NMR (100 MHz, $CDCl_3$): δ_c 116.49 (C-1), 117.30 (C-8), 122.74 (C-11), 124.37 (C-11a), 125.72 (C-10), 128.95 (C-5), 130.47 (C-11b), 130.95 (C-9), 132.07 (C-11c), 136.09 (C-3a), 139.49 (C-4), 139.49 (C-7a), 145.76 (C-2) and 159.59 (C-6).



Canthin-6-one

Antitumor promoting effect of A. excelsa stem bark extracts

The results in Table 1 shows that at the concentration of 100 µg/ml of the extracts of *A. excelsa* stem bark, the petroleum ether extract gave decrease of the tumoral Raji cells from 30% to 16.5%, for the diethyl ether from 40% to 12.9%, for chloroform extract from 50% to 8.9% and for methanol (70%) from 60% to 10.5%. For the concentration of 10 µg/ml of the extracts of *A. excelsa* stem bark, the petroleum ether extract gave decrease of the tumoral Raji cells from 60% to 54.7%, for the diethyl ether from 60% to 43.6%, for chloroform extract from 60% to 42.8% and for methanol (70%) from 60% to 49.3%. At the lowest concentration 1 µg/ml of the extracts of *A. excelsa* stem bark, the petroleum ether extract gave no response toward the the tumoral cells while the extract of diethyl ether gave decrease of the tumoral Raji cells from 100% to 95.8%, chloroform extract from 100% to 91.2% and for methanol (70%) from 100% to 98%. The results show that canthin-6-one is active than curcumin but less than beta-carotene. Table 3 shows that the chloroform extract of *A. excelsa* stem bark has inhibitory effect in two-stage mouse skin carcinogensis test where it decreases the average number of papillomas per mouse and

percent papillomas these results with comarsion of curcumin in the Table 4. Table 5 shows the inhibitory effect of canthin-6-one on Peroxynitrile-TPA mouse skin carcinogensis.

Table 1. Inhibitory effects of the extracts of *A. excelsa* on TPA-induced EBV-EA activation.

Test Materials	Relative Ratio of The Extracts With Respect To Positive Control		
	TPA(32 pmol, 20 ng) = 100% % to control-(% viability)		
	Concentration (µg/ml)		
	100	10 (60)	1
Petroleum ether extract of (*A. excelsa*) stem bark	16.5 (30)	54.7 (60)	100 (100)
Diethyl ether extract of (*A. excelsa)* stem bark	12.9 (40)	43.6(60)	95.8 (100)
Chloroform extract of (*A. excelsa*) stem bark	8.9 (50)	42.8 (60)	91.2 (100)
Methanol (70%) extract of (*A. excelsa*) stem bark	10.5 (60)	49.3(60)	98.0 (100)

Values in parentheses are viability percentages of Raji cells

Table 2. Inhibitory effects of canthin-6-one on TPA-induced EBV-EA activation.

Test compound	Relative Ratio of Compounds Activation With Respect to Positive Control			
	(mol ratio/TPA)			
	1000	500	100	10
Canthin-6-one	6.5 (60)	42.9 (60)	75.0 (100)	95.9 (100)
Curcumin	5.7 (60)	41.2 (60)	73.1 (100)	93.7 (100)
Beta-carotene	9.5 (60)	50.8 (60)	794 (100)	100 (100)

Values in parentheses are viability percentages of Raji cells

Table 3. Inhibitory effect of chloroform extract of *A. excelsa* stem bark on DMBA-TPA tumor promotion.

Week	Positive control		Extract of *A. excelsa* stem bark (50 µg)	
	DMBA(100 µg) + TPA (1.7µg)			
	Papillomas (%)	Papillomas /Mouse	Papillomas (%)	Papillomas /Mouse
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	6.6	0.5	0	0
7	20.0	1.2	6.6	0.5
8	40.0	2.1	13.3	0.8
9	80.0	3.2	20.0	1.2
10	100	4.8	26.6	1.5
11	100	5.7	26.6	1.9
12	100	6.5	33.3	2.1
13	100	7.3	40.0	2.4
14	100	7.9	46.6	2.6
15	100	8.1	46.6	3.0
16	100	8.2	53.3	3.2
17	100	8.4	60.0	3.5
18	100	8.6	66.6	3.9
19	100	8.8	80.0	4.3
20	100	9.1	86.6	4.7

Table 4. Inhibitory effect of curcumin on DMBA-TPA tumor promotion.

Week	Positive control DMBA(100 µg) + TPA (1.7µg)		Curcumin (85 nmol)	
	Papillomas (%)	Papillomas /Mouse	Papillomas (%)	Papillomas /Mouse
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	6.6	0.5	0	0
7	20.0	1.2	0	0
8	40.0	2.1	13.3	0.5
9	80.0	3.2	20.0	0.9
10	100	4.8	26.6	1.5
11	100	5.7	33.3	1.9
12	100	6.5	33.3	2.3
13	100	7.3	40.0	2.5
14	100	7.9	46.6	2.7
15	100	8.1	46.6	3.0
16	100	8.2	53.3	3.3
17	100	8.4	53.3	3.5
18	100	8.6	66.6	3.8
19	100	8.8	73.3	4.1
20	100	9.1	73.3	4.4

Table 5. Inhibitory effect of canthin-6-one on Peroxynitrile-TPA mouse skin carcinogensis.

Week	Positive control Peroxynitrile (35µg) + TPA (1 µg)		0.0025% Canthin-6-one 2 weeks oral feeding (before and after initiating)	
	Papillomas %	Papillomas /Mouse	Papillomas %	Papillomas /Mouse
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	33.3	0.9	0	0
8	60.0	2.0	0	0

Discusssion

The extracts of *A. excelsa* stem bark have an inhibitory effect on activation of the early antigen (% EBV-EA positive cells) in a short-term *in vitro* assay of the EBV-EA activation induced by TPA in Raji cells. The chloroform extract of *A. excelsa* stem bark has the most inhibitory effect against TPA induced activation than the other extracts of *A. excelsa* stem bark where chloroform extract showed the inhibitory effect to 91.2% at the lowest concentration 1 µg/ml in respect of the positive control while petroleum ether extract gave no response toward the the tumoral cells at the same concentration and while diethyl ether and methanol 70% decreased the ratio of tumoral Raji cells from 100% to 95.8% and 100% to 98%, respectively. The significant inhibitory effect (antitumor promoting effect) was obtainted by the isolated compound canthin-6-

one from this chloroform extract where this compound causes inhibitory effect against TPA induced activation to 95.9%. In the comparison of these results with both beta-carotene and curcumin, we found that canthin-6-one is more active than beta-carotene (100%) and has less inhibitory effect against TPA induced activation than curcumin (93.7%) at the lowest concentration of the compounds at 10 µg/ml and in comparison of these values with the control value [TPA (32 pmol) =100%]. Further investigation of both chloroform extract of *A. excelsa* and its active compound canthin-6-one as anti-tumor agents on two-stage mouse skin carcinogensis confirmed the anti-tumor activity of these significant test materials. The chloroform extract showed a significant inhibitory effect for mouse skin carcinogensis by decreasing the average number of papillomas per mouse and the percentage of papillomas in the promoting stage. In week 7, chloroform extract of *A. excelsa* stem bark decreases the percentage of papillomas in the promoting stage from 20.0 to 6.6 and in week 8 from 40.0 to 13.3 ,… etc. and finally in week 20 from 100 to 86.6. These significant values were compared with the results of the popular anti-tumor agent curcumin. Also canthin-6-one showed a significant inhibitory effect on TPA mouse skin carcinogensis where at the stage point (weeks 7 and 8 after initation), canthin-6-one showed no papillomas. The significant results of chloroform extract of *A. excelsa* stem bark and its active compound canthin-6-one which was previously isolated from the root bark of this plant Cordell et al. (1978) as inhibitors for Epstein barr-virus early antigen activation (EBV-EA) and for mouse skin carcinogensis, confirm the possible use of these materials as significant antitumor agents.

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