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Anti-inflammatory and anti-tumour effects of *Abies georgei* extracts

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Abstract

Chloroform (AGC), ethyl acetate (AGE) and *n*-butanol (AGB) extracts of *Abies georgei* were investigated for anti-tumour and anti-inflammatory activities in-vitro and in-vivo. AGC exhibited potent antiproliferative effects against A549, LOVO, QGY-7703 and 6T-CEM tumour cells, with EC50 values of 77.5, 7.8, 11.1 and 32.8 $\mu g\,mL^{-1}$, respectively. It also inhibited the growth of S180 sarcoma implanted into mice; tumour growth inhibition ratios were 46.7, 53.1 and 31.0% of controls at doses of 100, 200 and 400 $mg\,kg^{-1}$, respectively. AGE showed significant anti-inflammatory activities in the carrageenin-induced acute pedal oedema model in rats and dimethylbenzene-induced ear oedema in mice at doses of 140 $mg\,kg^{-1}$ and 200 $mg\,kg^{-1}$ p.o., respectively. Primary mechanism studies in-vitro showed that AGE inhibited platelet aggregation induced in rabbits by arachidonic acid (AA), with an IC50 of 14.4 $\mu g\,mL^{-1}$. Its effect on AA metabolism was also studied in mouse peritoneal macrophages stimulated by A23187. Formation of prostaglandin E_2, leukotriene B_4 and 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE) was significantly inhibited in a concentration-dependent manner. In addition, AGE inhibited lipopolysaccharide-induced nitric oxide production in RAW246.7 macrophages and nuclear factor κB activation induced in 293 cells by tumour necrosis factor α.

Introduction

Abies is an important genus of the Pinaceae family, with about 50 species occurring in the highlands of Asia, Europe, North and Middle America and North Africa (Zheng et al 1978). Several species have been used as folk medicines against cold, stomach ache, indigestion, and vascular, pulmonary and venereal diseases (Fujita et al 1995; Yesilada et al 1995). Some chemical constituents isolated from *Abies* species and their derivatives have exhibited various bioactivities, including anti-tumour, antimicrobial, anti-ulcerogenic, anti-inflammatory, antihypertensive and anti-tussive activities, and effects on the central nervous system (Yang et al 2008a).

A. georgei is indigenous to China. Phytochemical investigations indicated that norditerpenoids are its main chemical constituents, in addition to several flavanoids (Yang et al 2008b; 2008c) The current investigation was designed to evaluate anti-tumour and anti-inflammatory activities of *A. georgei* extracts in-vitro and in-vivo.

Materials and Methods

Plant material and chemicals

The aerial parts of *A. georgei*, collected in Zhongdian City, Yunnan Province of China in July 2006, was identified by Professor Li-Shang Xie of the Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, China). A voucher specimen (no. 2006-07-016) was deposited in the School of Pharmacy, Second Military Medical University (Shanghai, China).

Arachidonic acid (AA), 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE), prostaglandin E_2 (PGE_2), PGB_2 and leukotriene B_4 (LTB_4) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The calcium ionophore A23187 was purchased

from Sigma Chemical Co. (St Louis, MO, USA). Other reagents were purchased from Sinopharm Chemical Reagent Company Ltd (Shanghai, China).

Extraction procedure

The dried sample of *A. georgei* (22 kg) was pulverized and extracted using 80% ethanol under reflux for 3×3 h. The extract was concentrated to a small volume and then partitioned sequentially with chloroform, ethyl acetate and *n*-butanol. Each of these three fractions was evaporated in-vacuo to give 906 g of the chloroform extract (AGC), 282 g of the ethyl acetate extract (AGE) and 1220 g of the *n*-butanol extract (AGB). For the in-vitro study, extracts were dissolved in DMSO to yield 20 mg mL^{-1} stock solutions and stored at −20°C. For in-vivo studies, the extracts were suspended in normal saline containing 0.5% carboxymethyl cellulose (CMC)-Na.

HPLC

Qualitative HPLC fingerprint analysis was performed using an LC2010AHT HPLC system (Shimadzu Corporation, Kyoto, Japan) with a ZORBAX Extend C_{18} analytical column (25 cm×4.6 mm, 5 μm, Agilent Corporation, MA, USA). A 10 mL volume of extract sample dissolved in MeOH was eluted at a flow rate of 0.8 mL min^{-1} with a linear gradient of MeOH and water as follows (% MeOH): 0 min, 10%; 80–90 min 100%; 91–100 min 10%. Detection was at 236 nm. Data acquisition was performed using an LC-Solution workstation (Shimadzu).

Cell lines

RAW 264.7, 293, A549, LOVO, QGY-7703 and 6T-CEM cell lines were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and maintained in media recommended by the suppliers, supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), penicillin (100 UmL^{-1}) and streptomycin (100 μg mL^{-1}) in a humidified 5% CO_2 atmosphere at 37°C.

Antiproliferative activity

Antiproliferative experiments were carried out 24 h after cells were seeded according to the protocol reported by Alley et al (1988). Briefly, different concentrations of the extracts were added, and incubation continued for 72 h. Cell viability was evaluated by measuring the optical density of the colour produced by MTT dye reduction with a microplate reader at 570 nm.

Animals

ICR mice (male, 14–16 g), Sprague–Dawley rats (160–200 g) and New Zealand rabbits (2.5–3.0 kg) were purchased from the Shanghai SLAC Laboratory Animal Company Ltd (Shanghai, China). The animals were maintained in controlled conditions of 22±1°C with food and water ad libitum. All animal treatments were strictly in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. The experiments were carried out with the approval of the Committee of Experimental Animal Administration of the University.

Evaluation of anti-tumour activity in S180-inoculated mice

S180 tumour cells (2.0×10^6 per mouse) in 0.9% NaCl solution were inoculated intradermally into the right axilla of ICR mice. Tumour-bearing mice were randomly assigned to seven groups. After 24 h, mice were given the different extracts (dissolved in 0.5% CMC-Na, p.o.) for 12 days. For model and cyclophosphamide (CTX; positive control) groups, mice were given 0.5% CMC-Na p.o. or CTX dissolved in 0.9% NaCl, i.p., respectively. On the final day, tumours were excised and weighed to calculate the inhibition ratio.

Carrageenin-induced acute paw oedema in rats

AGE (70, 140 and 280 mg kg^{-1}, p. o.) was administered to rats for 5 days, or the cyclo-oxygenase (COX) inhibitor aspirin (210 mg kg^{-1}) for 3 days. Thirty min after the last administration, oedema was induced by intraplantar injection of 0.1 mL 1% (w/v) carrageenin suspension in normal saline into the right hindpaw. The volume of the paw was measured using a plethysmometer (UGO BASILE, comerio VA, Italy) at the initial time and 2 and 6 h after the phlogistic agent.

Dimethylbenzene-induced ear oedema in mice

AGE (100, 200 and 400 mg kg^{-1}, p. o.) was administered to mice for 5 days, or aspirin (300 mg kg^{-1}) for 3 days. Thirty min after the last administration, 40 μL dimethylbenzene was applied to the anterior and posterior surfaces of the right ear of each mouse. Mice were killed 1 h later, the ears were cut off and discs were taken from each ear using a cork borer (ID 8 mm). The oedematous response was measured as the difference in weight between the two ear samples. The anti-inflammatory activity was expressed as percentage inhibition of oedema in treated mice compared with control mice.

Platelet aggregation

Blood was withdrawn from the carotid arteries of New Zealand rabbits and collected with a one-tenth volume of 3.8% trisodium citrate solution, as described previously (Penz et al 2005). Platelet aggregation was measured by using an aggregometer (General Technological Research Institute, Shanghai, China). Platelet suspensions were pretreated with either AGE or aspirin at 37°C for 3 min in the aggregometer. Aggregation was induced by AA (20 μM) and the extent of platelet aggregation was recorded and compared with control.

Measurement of arachidonic acid metabolites in A23187-stimulated peritoneal macrophages

Rats were given 3 mL i.p. injections of thioglycolate broth 3 days before use. Elicited peritoneal macrophages were prepared as described previously (Ishida-Fujii et al 2007). The macrophages collected were resuspended in phosphate-buffered

saline (PBS, pH = 7.4) supplemented with 2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at a concentration of 2×10^7 cells mL^{-1}. After pretreatment with drugs for 20 min, the aliquots (1 mL) were incubated with AA (50 μM) or A23187 (5 μM) for 15 min. The reactions were stopped by adding 2 mL cold alcohol, then the internal standard (PGB_2) at 200 ng was added and the samples mixed. Eicosanoids formed were extracted with solid-phase extraction and assayed using an Agilent 6410A triple quadrupole LC-MS system (Agilent Corporation, MA, USA) equipped with a G1311A quaternary pump, a G1322A vacuum degasser, a G1329A autosampler and a G1316A therm. column compartment. The system was controlled by MassHunter software (Agilent). The separation was performed using a ZORBAX SB-C18 column (3.5 μm, 2.1×100 mm; Agilent) and a C18 guard column (5 μm, 4.0×2.0 mm, Phenomenex, CA, USA). The AA metabolites were identified by comparing their retention times and MS fragments with those of authentic standards and were quantified from the peak area. The result was expressed as the amount relative to that in the A23187 group (negative control).

Measurement of nitric oxide in lipopolysaccharide-activated macrophages

RAW 264.7 macrophages were seeded into 24-well cell culture plates (10^5 cells per well). The cells were co-incubated with drugs and lipopolysaccharide (LPS; 1 μgmL^{-1}) for 24 h. The amount of nitric oxide (NO) was assessed by determining the nitrite concentration in the culture supernatants with Griess reagent. Aliquots of supernatants (100 μL) were incubated in sequence with 50 μL 1% sulphanilamide and 50 μL 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. The absorbance at 570 nm was read using a microplate reader.

Nuclear factor-κB activation assay

Nuclear factor (NF)-κB activation was assayed using stable pNF-κB-luc-293 cells (Baran et al 2007). Cells seeded into 96-well plates at 1.0×10^5 cells per well were pretreated with test drugs for 15 min, and then incubated with 10 ngmL^{-1} recombinant human tumour necrosis factor (TNF)α for 6 h. The cells were lysed, and luciferase activity was measured with a luciferase assay system (Promega, WI, USA).

Statistical analysis

Results are given as mean ± s.d. The bioactivities of tested samples were compared using the Kruskal–Wallis test, followed by Dunn's test. If the data were normally distributed and the variances of the different treatments were equal, parametric analysis of variance (ANOVA) was performed followed by Dunnett's post-hoc test. Differences were considered statistically significant for $P < 0.05$. All calculations were carried out using SigmaStat 3.5 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Three extracts of *A. georgei* were tested for antiproliferative activities against four human tumour cell lines: A549, LOVO, QGY-7703 and 6T-CEM. However, only AGC showed potent activities, with EC50 values of 77.5, 7.8, 11.1 and 32.8 μgmL^{-1} in the four cell lines, respectively (Table 1).

Table 1 Antiproliferation activities of the three extracts from *Abies georgei* against four human tumour cell lines. Values are EC50 (μgmL^{-1})

	A549	LOVO	QGY-7703	6T-CEM
Doxorubicin[a]	0.0114	0.237	0.00106	0.0419
AGC	77.5	7.8	11.1	32.8
AGE	> 100	> 100	> 100	> 100
AGB	> 100	> 100	> 100	> 100

[a]positive control.
AGC/AGE/AGB, chloroform/ethyl acetate/n-butanol extracts of *A. georgei*.

Based on the positive activity in-vitro, the in-vivo anti-tumour effect of AGC was tested in S180 tumours grown in mice. Interestingly, AGC still exhibited potent activity. Doses of 100, 200 and 400 mgkg^{-1} gave tumour growth inhibition ratios of 46.7, 53.1 and 31.0%, respectively. Furthermore, no loss of body weight was observed in the concentration range tested (Table 2).

Triterpenoids are the main chemical constituents of many *Abies* plants, and some have showed significant antiproliferative activities in-vivo (Kim et al 2001, 2004; Wada et al 2002; Tanaka et al 2004). Thus, triterpenoids may be partially responsible for the anti-tumour activities of *A. georgei*.

The content of AGB was mostly similar to that of AGE on the basis of HPLC profiles of the three extracts from *A. georgei* (Figure 1). At concentration below 100 μgmL^{-1}, AGC displayed significant cytotoxic effect on RAW 264.7 and 293 cells, while AGE did not (data not shown). Thus, only AGE was used in the anti-inflammatory experiments.

The anti-inflammatory effect of AGE was evaluated in two popular in-vivo models: dimethylbenzene-induced ear oedema in mice and carrageenin-induced paw oedema in rats. Data showed that AGE (200 mgkg^{-1}) significantly inhibited dimethylbenzene-induced ear oedema in mice, with an inhibition ratio of 18.7% (Table 3). At the same dose, AGE showed significant inhibitory effects on rat paw oedema 2 h and 6 h after carrageenin stimulation (Table 4), with inhibition ratios of 28.2% and 35.6%, respectively.

Table 2 Effects of the three extracts from *Abies georgei* on the growth of S180 sarcoma implanted into mice

Groups	Dose (mgkg^{-1})	Animal weight[a] (g)	Tumour weight (g)	Inhibition ratio (%)
Model[b]		32.39 ± 3.31	5.33 ± 1.89	
CTX[c]	40	30.13 ± 3.41	0.42 ± 0.24**	92.2
AGC	400	30.53 ± 2.62	3.68 ± 1.53*	31.0
	200	31.77 ± 2.84	2.50 ± 0.83**	53.1
	100	30.38 ± 1.82	2.88 ± 1.12**	46.7
AGE	400	27.67 ± 2.59	5.18 ± 1.21	2.8
AGB	400	30.83 ± 1.38	5.07 ± 1.01	5.0

[a]After removal of tumour; [b]negative control; [c]cyclophosphamide, positive control.
AGC/AGE/AGB, chloroform/ethyl acetate/n-butanol extracts of *A. georgei*.
Data are mean ± s.d. (n = 10). *$P < 0.05$; **$P < 0.01$ vs model.

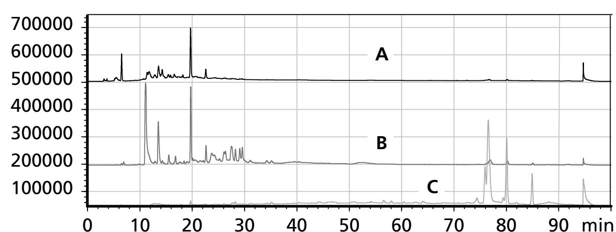

Figure 1 HPLC chromatogram for the *n*-butanol (A), ethyl acetate (B) and chloroform (C) extracts of *Abies georgei*

Table 3 Effect of the ethyl acetate extract of *Abies georgei* (AGE) against dimethylbenzene-induced ear oedema in mice

Group	Dose (mg kg^{-1})	Oedema weight (mg)	Inhibition
Model[a]		19.7 ± 4.6	
Aspirin[b]	300	13.0 ± 7.9*	34.0%
AGE	100	17.4 ± 5.8	11.6%
	200	16.0 ± 2.8*	18.7%
	400	18.2 ± 5.7	7.6%

[a]negative control. [b]positive control.
Data are mean ± s.d. (n = 10). *$P < 0.05$ vs model.

Table 4 Effect of the ethyl acetate extract of *Abies georgei* (AGE) against carrageenin-induced hindpaw oedema in rats

Group	Dose (mgkg^{-1})	Oedema (mL)	
		After 2 h	After 6 h
Model[a]		0.39 ± 0.06	0.45 ± 0.08
Aspirin[b]	210	0.06 ± 0.06**	0.20 ± 0.06**
AGE	70	0.40 ± 0.13	0.48 ± 0.11
	140	0.28 ± 0.05 *	0.29 ± 0.06**
	280	0.40 ± 0.09	0.38 ± 0.15

[a]negative control; [b]positive control.
Data are mean ± s.d. (n = 6). *$P < 0.05$; **$P < 0.01$ vs model.

Many anti-inflammatory compounds of plant origin interfere with three relevant targets involved in the inflammatory process: AA metabolite pathways, NO and NF-κB (Calixto et al 2003). The key enzymes involved in AA metabolism include COX-1 and COX-2 and several lipoxygenase (LOX) isozymes. PGs and LTs are the major metabolites of COX and LOX pathways, respectively (Simmons et al 2004). To better understand the anti-inflammatory action of bioactive extracts, it is important to monitor their effects on COX-1 and -2 and on the balance between COX and LOX pathways.

Platelets contain only COX-1, which can convert AA to the potent pro-aggregatory eicosanoid thromboxane A_2. In this work, inhibitory potency against COX-1 was measured in platelets. AGE inhibited AA-induced rabbit-platelet aggregation in a concentration-dependent manner (Table 5), with an IC50 of 14.4 $\mu g mL^{-1}$. The potency of AGE for inhibition of LOX and COX was also estimated in elicited peritoneal macrophages. As shown in Figure 2, AGE (20,

Table 5 Effect of the ethyl acetate extract of *Abies georgei* (AGE) against arachidonic-acid-induced platelet aggregation in rabbits

Group	Dose ($\mu g mL^{-1}$)	Aggregation (%)	Inhibition (%)
Model[a]		73 ± 4	
Aspirin[b]	0.18	68 ± 8	7 ± 12
	1.8	46 ± 12	37 ± 16
	9.0	13 ± 6*	82 ± 8
	18	12 ± 4**	84 ± 1
AGE	2.5	70 ± 4	4 ± 5
	10	46 ± 8	36 ± 10
	20	34 ± 12*	53 ± 17
	100	1 ± 1**	98 ± 1

[a]negative control; [b]positive control.
Data are mean ± s.d. (n = 4). *$P < 0.05$; **$P < 0.01$ vs model.

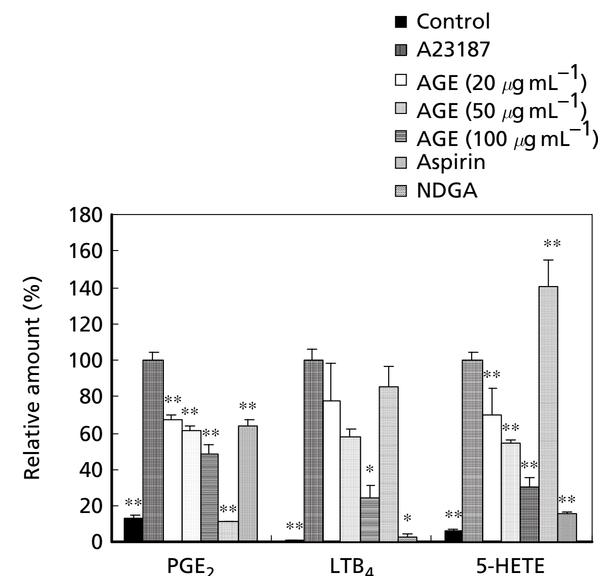

Figure 2 Activities of the ethyl acetate extract of *Abies georgei* (AGE) on concentrations of eicosanoids in elicited peritoneal macrophages. The cyclo-oxygenase inhibitor aspirin (100 μm) and lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 40 μm) were used as positive controls. Data are mean ± s.d. (n = 4). *$P < 0.05$; **$P < 0.01$ vs A23187 groups. 5-HETE, 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4.

50 and 100 $\mu g mL^{-1}$) significantly inhibited the production of PGE_2, 5-HETE and LTB_4 in macrophages stimulated with A23187. Maximal inhibition ratios at 100 $\mu g mL^{-1}$ were 51.6% for PGE_2, 75.4% for 5-HETE and 69.5% for LTB_4. The COX inhibitor, aspirin, inhibited only the production of PGE_2 (COX metabolite), while the LOX inhibitor, nordihydroguaiaretic acid (NDGA), showed potent activities against LTB_4 and 5-HETE (LOX metabolites). Our data also show that aspirin can divert arachidonate to LOX pathways, which resulted in the enhancement of LTB_4 and 5-HETE production (LOX pathway eicosanoids). Such effects are known in the clinic as aspirin-induced asthma. Therefore, compared with the conventional anti-inflammatory

Table 6 Effect of the ethyl acetate extract of *Abies georgei* (AGE) on nuclear factor (NF)-κB activity induced by TNFα

Group	Dose (μg mL^{-1})	Relative light units ($\times 10^{-5}$)
TNFα[a]		13.3 ± 2.1
LGT[b]	0.1	0.6 ± 0.2**
AGE	10	16.0 ± 1.5
	50	7.0 ± 2.9
	100	3.7 ± 2.0*

[a]Tumour necrosis factor α, negative control; [b]triptolide, positive control.
Data are mean ± s.d. (n = 4). *$P < 0.05$; **$P < 0.01$ vs TNFα group.

Table 7 Effect of the ethyl acetate extract of *Abies georgei* (AGE) on nitric oxide (NO) induction by lipopolysaccharide (LPS)

Group	Dose	NO (μM)	Inhibition (%)
LPS[a]	1 μg mL^{-1}	15.6 ± 1.0	
Aminoguanidine[b]	50 μM	3.9 ± 0.6**	75.0
AGE	25 μg mL^{-1}	12.6 ± 1.0*	19.2
	50 μg mL^{-1}	9.6 ± 0.7**	38.4
	100 μg mL^{-1}	6.5 ± 0.5**	58.3

[a]negative control; [b]positive control.
Data are mean ± s.d. (n = 4). *$P < 0.05$; **$P < 0.01$ vs LPS group.

medicines, dual COX/LOX inhibitors are supposed to be more efficacious and safe. Interestingly, AGE contains both COX and LOX inhibitors.

NF-κB is also an important therapeutic target for anti-inflammatory agents. Luciferase activities in stable pNF-κB-luc-293 cells were measured to assess NF-κB activity. Recombinant human TNFα (10 ng mL^{-1}) can significantly elevate luciferase activity. However, the effect could be inhibited by AGE at 50 and 100 μg mL^{-1} with inhibition ratios of 47.3 and 72.2%, respectively (Table 6).

Inhibition of NO release is beneficial in many inflammatory diseases. The ability of AGE to inhibit NO production was therefore measured in RAW264.7 macrophages stimulated by LPS. LPS significantly increased NO production ($P < 0.01$). AGE (50 and 100 μg mL^{-1}) markedly inhibited the increase of NO caused by LPS, with inhibition ratios of 38.4% and 58.3%, respectively (Table 7).

Since some norditerpenoids isolated from *A. georgei* have been shown to have strong activities against LPS-induced NO production in RAW264.7 macrophages (Yang et al 2008b), they could be partially responsible for the anti-inflammatory activities of *A. georgei*.

Conclusions

AGE showed anti-inflammatory effect in-vitro and in-vivo. It inhibited both COX and LOX, and NO production and NF-κB activity. An advanced concept of anti-inflammatory therapy proposes the use of dual inhibitors of COX-1/2 and LOX pathways (Coruzzi et al 2007). It is therefore worth analysing the anti-inflammatory components purified from *A. georgei*, and evaluating their potential in the treatment of inflammation.

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