in vivo *19*: 191-194 (2005)

Antitumour Activity of *Angelica archangelica* Leaf Extract

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Abstract. *Background: The purpose of this study was to examine the effect of a leaf extract from A. archangelica on the growth of Crl mouse breast cancer cells in vitro and in vivo. Materials and Methods: The antiproliferative activity of the extract was measured by ^{3}H-thymidine uptake in the Crl cells in vitro. Twenty mice were injected with the Crl cells, and 11 of them were fed A. archangelica leaf extract, and the progress of the tumours was followed. Results: The leaf extract was mildly antiproliferative on the Crl cells with an EC_{50} of 87.6 μg/ml. The antitumour activity of the extract was expressed in the mice by marked reduction in tumour growth. In the experimental animals, 9 out of 11 mice developed no or very small tumours, whereas control animals, not receiving the extract, developed significantly larger tumours ($p<0.01$), as estimated by Mann-Whitney U-test. The antitumour activity of the leaf extract could not be explained by the antiproliferative activity of furanocoumarins present in the extract. Conclusion: The results demonstrate the antiproliferative activity in vitro and antitumour activity in vivo of a leaf extract from A. archangelica*

Angelica archangelica or A. officinalis has been widely used in folk medicine and is one of the most respected medicinal herbs in northern countries, where it was cultivated during the Middle Ages (1,2). It grows wild in most parts of Iceland, where it and *A. sylvestris* are the only representatives of the genus *Angelica* (2). It was exported to central Europe during the Middle Ages (3).

The most characteristic secondary metabolites of *A. archangelica* are essential oils and furanocoumarins, both of which are more abundant in the roots and seeds than in the leaves. The whole plant has been used as a vegetable. In folk medicine *A. archangelica* has been used for respiratory catarrh, asthma, flatulent dyspepsia, anorexia nervosa, rheumatic diseases and peripheral vascular diseases (4).

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Key Words: Angelica archangelica, antitumour activity, Crl.

The bioactivity of coumarins, furanocoumarins and other constituents present in *A. archangelica* and in other species of the genus *Angelica* has been described previously. Inhibition of nitric oxide production has been demonstrated in a methanolic extract from *A. megaphylla* (5) and in polyacetylenes isolated from *A. gigas* (6). Polysaccharides from *A. sinensis* have been shown to protect against gastric damage (7), to promote healing of gastric ulcers (8,9) and prevent hepatic injury (10). Polysaccharides from *A. sinensis,* with gastric ulcer healing activity, have been shown to have an anti-angiogenic effect (9).

Coumarins isolated from the fruits of *A. edulis* have shown antitumour-promoting activity by inhibiting 12-O-tetradecanoylphorbol 13-acetate-stimulated ^{32}P incorporation into phospholipids of cultured cells (11). The same activity has been observed in furanocoumarins and chalcones isolated from *A. keiskei* (12). Furthermore, in recent studies inhibition of growth and metastasis of Lewis lung carcinoma (LLC) implanted in mice has been demonstrated for extracts from *A. keiskei* (13), as well as for chalcone derivates contained in it, specifically xanthoangelol (13) and 4-hydroxyderricin (14). The antiproliferative activity of extracts from *A. japonica* has been reported, as well as that of furanocoumarins isolated from it (15). Immunostimulating activity has been demonstrated for *A. gigas* (16) and antitumour activity has been described in immunostimulating polysaccharides from *A. acutiloba* (17) and *A. sinensis* (18). These studies were chiefly concerned with the roots of the plant.

The aim of this study was to evaluate the antiproliferative and antitumour activity of *A. archangelica* leaves. This is, to our knowledge, the first study of the antiproliferative and antitumour activity of the leaves of *A. archangelica*.

Materials and Methods

Preparation of tumour cells. Crl mouse breast cancer cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI-1640 medium with 10% foetal calf serum, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 0.01 M HEPES-buffer and 0.2 M L-glutamine (all from Gibco, Paisley, UK). The cultures were incubated at 37˚C in 95% humidity and 5% CO_2. The cells were trypsinized using a 1:30 dilution of standard Gibco trypsin-EDTA solution (Gibco), and washed

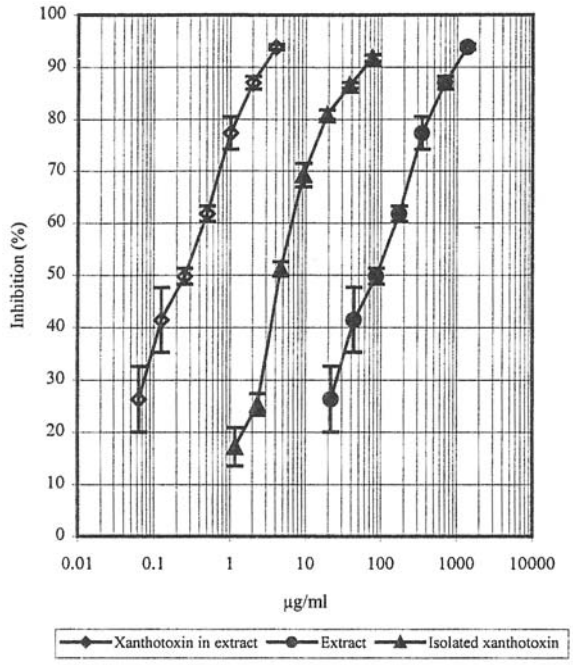

Figure 1. *Antiproliferative activity of Angelica archangelica leaf extract on Crl mouse breast cancer cells in vitro. On the right side is the dose response curve of the leaf extract with an EC_{50}=87.6 µg/ml. Each point represents the mean±SEM of 4 experiments, each carried out in triplicate. In the middle is the response curve for pure xanthotoxin, EC_{50}=4.54 µg/ml. On the left side is the dose response curve calculated for xanthotoxin present in the extract, EC_{50}=0.25 µg/ml.*

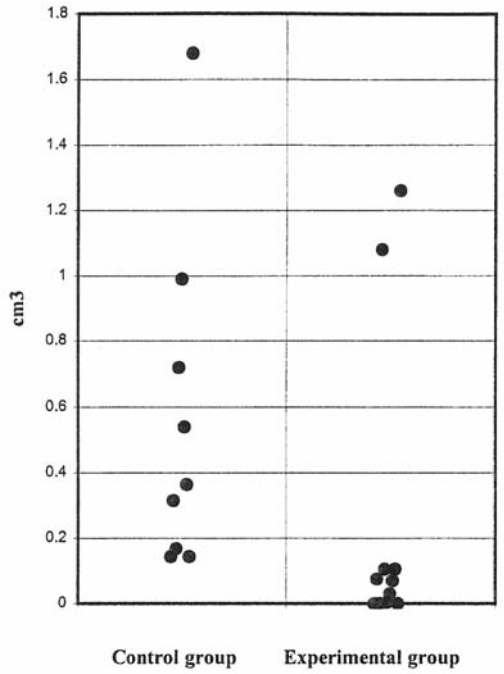

Figure 2. *Antitumour activity of the leaf extract expressed as reduced growth of tumours in an experimental group of mice fed a diet containing the extract. Tumours were measured and the volumes were calculated after decapitation on the 31st day after injection with mouse Crl breast cancer cells. The volumes of tumours in the control group fed a regular diet and the experimental group fed a diet supplemented with a leaf extract are shown. The tumours in experimental animals were significantly smaller than in control mice.*

in RPMI 1640 medium. The tumour cells were then ready to be used in the experiments.

In vitro assay. The cells were trypsinized, counted and placed in 96-well plates at 10^4 cells per well. The sample was added at the start, dissolved and serially diluted in water (extract) or 60% aqueous ethanol (xanthotoxin). In the case of xanthotoxin, the final ethanol concentration in all samples, including controls, was 3%. After 24-h culture, [^{3}H]-thymidine was added at 1 µCi per well, and 4 h later the cells were washed, trypsinized and harvested in a Skatron Cell Harvester (Skatron Instruments, Lier, Norway) on to a Titertek Filter Paper for Cell Harvester (Titertek Instruments, Huntsville, AL, USA). These were dried and the radioactivity was counted in a liquid scintillation counter (Tri-Carb, Packard, Boston, MA, USA) using Opti-Fluor (Packard) scintillation fluid.

Food. The control group was fed standard mouse diet, RM1 (Special Diet Services, Essex, England). Leaf extract was made by boiling 100 g leaf in 2 L water for 30 min, when the final volume was about 1600 ml. The dry weight of the extract was typically about 30 mg/ml. To each gram of the standard diet RM1, about 0.93 ml of extract was added. The pellets were allowed to dry before being stored in a refrigerator. Thus, each gram of food contained approximately 28 mg dry weight from the extract. The mice in the experimental group were fed the diet containing the leaf extract for 14 days preceding injection with tumour cells. Standard diet was fed to 10 mice, but the experimental group contained 11 mice.

Tumours. The female BALB/c mice were injected with 130,000 cells in 0.1 ml of the culture, subcutaneously in the flank. On the 31st day after the injection of tumour cells, the mice were sacrificed, and the tumours were excised with surrounding tissue and fixed immediately in formalin. After fixation, their diameters were measured. The overall gross form of the tumours was ellipsoid, and their volume was calculated accordingly using their 3 measured dimensions. Appropriate sections were taken for histological examinations, using Hematoxylin and Eosin stain.

Results

Food consumption. The food consumption was relatively constant in each group of mice during the experiment. There was no significant difference between the average food consumption of the control group (2.48 g/mouse/day) and the experimental group (2.76 g/mouse/day) receiving the leaf extract. There was no significant difference in weight gain either. These values allow for estimation of the consumption of the extract, the mice in the experimental group consuming on average 77 mg leaf extract daily.

Antiproliferative activity in vitro. The dose response curve is shown in Figure 1. The extract was diluted four-fold in each step, the concentration range of the extract being from 21 to

1382 μg/ml. An antiproliferative effect resulting from the extract was observed, and the EC_{50} was estimated as 87.6 μg/ml. The antiproliferative activity of pure xanthotoxin, the most abundant furanocoumarin in the extract, was also examined. Xanthotoxin had an EC_{50}=4.54 μg/ml.

Antitumour activity in vivo. One mouse in the control group died prior to sacrifice, and its tumour was not measured. The tumours in the experimental group were 0.25±0.14 cm^3 compared with 0.56±0.16 cm^3 in the control group.

Figure 2 shows the distribution of the tumour size. In the experimental group (n=11), 3 mice had no tumours that could be detected in the injected area, which was serially sectioned. Another 6 mice in that group developed very small tumours. In the control group, only 3 tumours were in the range of the majority of the experimental group, but the others were evenly distributed in size through the range. Two mice in the experimental group, however, developed larger tumours. The Mann-Whitney *U*-test was applied to measure the difference of the medians, which proved to be highly significant ($p<0.01$).

Pathology of tumours. All thoracic and abdominal organs were removed and observed grossly. Microscopic sections were taken from lungs, heart, thymus, stomach, liver, spleen and kidneys. No metastatic tumours were detected.

Grossly, all the tumours, limited to the injection site, were well demarcated from the surrounding subcutaneous tissue. Most were single and ellipsoid in shape but a few were composed of two adjacent masses. In 5 mice in the experimental group no tumours were grossly visible, but in 2 of these cases, when the injection area was serially sectioned, microscopic tumours around 1 mm in diameter were present.

Microscopically, all tumours were identical in structure, being composed of a mixture of round, oval and spindly cells, with frequent mitoses and multinucleated giant cells. The round and oval cells formed small sheets and the spindly cells formed frequent streams or bundles intersecting the tumours. These three cell types seemed to be closely related, either the round cells transforming into oval cells which transformed into spindly cells, or the other way around. The tumour growth was mostly by a pushing border, but focally an invasive pattern was present either into fat or muscle tissue. A slight sprinkle of small round and polymorphonuclear inflammatory cells was present in the periphery of the tumours and in the surrounding area there was a more dense inflammatory infiltrate of the same cells, but intermixed with reactive fibroblastic cells. No obvious difference was seen in this inflammatory pattern between either large and small tumours or treated and untreated tumours. Evidence of tumour regression was not found, either in treated or untreated mice.

Tumour necrosis was present in all but three of the smallest tumours. The necrotic areas involved an estimated from <5% to 65% of the transsected sections. All necrotic areas were centrally placed, leaving the tumour surface viable. Polymorphonuclear inflammatory cells were abundant in the necrotic areas.

In the 3 mice from the experimental group without tumours, the injection site showed a reactive pattern, with scattered inflammatory cells and reactive fibroblasts in the subcutanous fat.

Discussion

The antitumour activity of *Angelica archangelica* leaf extract in mice was observed in this study. The consumption of the extract reduced tumour growth and could even prevent tumour development in some of the mice. In only 2 of the 11 mice receiving leaf extract did large tumours develop. The histological examination suggested a combination of failure of tumour cell growth and host inflammatory response.

There are several possible explanations for this antitumour effect that must be considered; direct cytotstatic and cytotoxic effects of furanocoumarins (19) and possible effects of other secondary metabolites such as flavonoids, on the one hand, and immunological or indirect effects on the cancer cells, on the other hand (16,17). *A. archangelica* contains cytotoxic furanocoumarins (19) in the seeds, roots and leaves. The furanocoumarin levels in the leaves were much lower than in the seeds and roots. The dietary intake of furanocoumarins by mice in the experimental group, getting the food containing the leaf extract, was 65 μg furanocoumarins/g feed or 177 μg/day, thereof 140 μg xanthotoxin. The xanthotoxin intake was thus 6.90 mg/kg body weight. Furanocoumarins have been shown to have antiproliferative activity against cancer cells *in vitro*, particularly imperatorin and xanthotoxin (19-21). The primary furanocoumarin in the leaf extract is xanthotoxin or about 80% of total furanocoumarins.

The antiproliferative activity of the leaf extract with an EC_{50} of 87.6 μg/ml was examined in relation to the furanocoumarin content of the extract or the xanthotoxin content, Figure 1. If we assume that the antiproliferative activity is due to the furanocoumarins, the dose response relationship suggests an EC_{50}=0.25 μg/ml for xanthotoxin. The antiproliferative activity of pure xanthotoxin was also examined, EC_{50}=4.54 μg/ml. The antiproliferative activity of xanthotoxin in the leaf extract appears, thus, to be about 18 times greater than the activity of pure xanthotoxin, indicating that the antitumour activity is not due to xanthotoxin or the furanocoumarins alone. Xanthotoxin could, however, play an important role if there are synergistic effects of furanocoumarins with other components in the leaf extract.

Other secondary metabolites in plants with potential antitumour activity are flavonoids (22). Although flavonoids have been shown to inhibit cancer cell growth *in vitro*, the ability of flavonoids to inhibit cancer progression is limited in animal studies (22-24). A potential application of flavonoids is the

possible synergism of flavonoids with chemotherapy agents (22) or other antiproliferative compounds such as furanocoumarins. Chalcone derivatives from the roots of *A. keiskei* have been shown to inhibit the growth of tumour implants in mice (13,14). At present, nothing is known of the presence or composition of chalcone derivates in *A. archangelica* leaf.

Another possible explanation for the antitumour activity observed in this study is due to the polysaccharides present in the leaves. A polysaccharide fraction from *A. acutiloba* showed a potent antitumour activity against several forms of cancer (17,18). The active polysaccharide fraction showed anti-complement activity. The immunopharmacological characteristics of angelan, a polysaccharide from *A. gigas* Nakai, were investigated in relation to the specificity to immune cells (18). A polysaccharide from *A. archangelica* leaves showed anti-complement activity but this polysaccharide has not been characterized (data not included). It is possible that there is synergy between the antiproliferative activities of the furanocoumarins and flavonoids or polysaccharides that is responsible for the observed *in vivo* antitumour activity of the leaf extract.

Additionally, it is possible that the extract contains a compound that could prevent growth of new blood vessels (angiogenesis) in the tumour and thereby limit its size. Crude extracts from *A. sinensis*, which mainly consisted of polysaccharides, significantly promoted gastric ulcer healing in animal models. Angiogenesis was inhibited by treatment with the crude extract (9). The authors suggested that, since the crude extract could reduce angiogenesis, the extract might have an anti-angiogenic effect on tumour growth induced by chronic ulcers (9). Such polysaccharides in the leaves of *A. archangelica* might explain the antitumour activity oserved in this study.

In conclusion, leaf extract from *A. archangelica* showed moderate antiproliferative activity against Crl mouse mammary carcinoma cells *in vitro*. This activity can partially be attributed to the xanthotoxin content of the extract. The formation of tumours in mice injected with Crl cells was significantly reduced in those fed pellets containing the extract.

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Received September 21, 2004
Accepted December 20, 2004