Journal of Biochemistry and Molecular Biology, Vol. 36, No. 4, July 2003, pp. 387-389

JOURNAL OF Biochemistry and Molecular Biology

The Effect of Willow Leaf Extracts on Human Leukemic Cells *in Vitro*

Hany A. El-Shemy[†,§,*], Ahmed M. Aboul-Enein[†], Mostafa I. Aboul-Enein[‡], Sohair I. Issa[‡] and Kounosuke Fujita[§]

[†]Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza, Egypt
[‡]Department of Clinical Pathology, National Cancer Institute, Cairo University, Cairo, Egypt
[§]Faculty of Applied Biological Science, Hiroshima University, Hiroshima 739-8528, Japan

Received 5 January 2003, Accepted 25 February 2003

The young developing leaves of willow (*Salix safsaf*, *Salicaceae*) trees have antileukemic activity. After a 24-h incubation *in vitro*, the crude water extracts of the leaves killed a majority of the blasts of acute myeloid leukemia (AML, 73.8%).

Keywords: Acute myeloid leukemia, Human leukemic cell, Willow leaf

Introduction

The genus Salix contains about 300 species in the world. A screening program was initiated by Leven *et al.* (1979), that identified the antibacterial and antifungal activities as well as the antiviral, antiparasitic, and other pharmacologically active substance activities in higher plants. The extracts of certain plants are known to yield active antimicrobial substances which have been documented as phytochemicals of the genus of the family, and reported as the toxicity of the plants. Many components that are derived from medical and dietary plants possess potential chemopreventive properties (Han *et al.*, 2002). To our knowledge, however, there has been no research concerning its effects on tumor cells. Therefore, this work investigated the effect of willow leaf extracts on the viability of leukemic cells.

Materials and Methods

The willow leaves (*S. safsaf*) were collected from the Salix farm of the Faculty of Agriculture, Cairo University, Giza, Egypt. The young leaves were directly extracted with hot water (7% concentration, 7 g of fresh leaves boiled (100°C) in 100 ml distilled. water for 20 min, then filtered through a sterilized Miracloth and centrifuged at 15,000 rpm for 15 min). The solvent extraction was carried out with the plant leaves as follows: 80 g leaves were extracted consecutively at room temperature with petroleum ether (40-60°C), that was followed by diethyl ether, chloroform, acetone, and finally with 70% ethanol. The solvent of each extract was removed by distillation at a low temperature, and each class of crude plant extract was separated for the next study.

The study was performed on adult leukemic patients, aged 18-65 years, that were admitted to the National Cancer Institute, Cairo University. The healthy volunteers (6 samples) and patients that included 15 AML (acute myeloid leukemia, immature monocytes) were diagnosed by peripheral blood and bone marrow examination, cytochemistry, and immunological markers, when needed. The healthy volunteers and patients were subjected to separation of mononuclear cells done by Ficoll hypaque density gradient (Pharmacia, Uppsala, Sweden). The cells were then washed three times with PBS and the counts were adjusted to 10^5 cells/0.1 ml (both mature and immature cells). The culture medium was prepared using modified Earles-salt with 1.2 g/l sodium carbonate and L-glutamine (Gibco, Grand island, USA), 10% inactivated fetal bovine serum (Gibco), and penicillin/streptomycin was added. The medium was then filtered through 0.22 μm Millipore filters, one ml of which was transferred into a 1.8-ml screw-capped sterile plastic tube. Next, 0.1 ml of the cell suspension containing 10^5 cells was added to 5 tubes. To three of these tubes, 0.1 ml of the willow extract was added, while the other two tubes served as negative and positive controls. Culture medium was used instead of the willow extract for the negative control and the willow extract was added in the cells from healthy volunteers as a positive control. The tubes were incubated at 37°C in the presence of 5% CO_2 for 24 h. The cells were tested for their viability using the trypan blue exclusion test (Bennett *et al.*, 1976). Two hundred cells were counted, then the percentage of viable cells was estimated.

*To whom correspondence should be addressed.
Tel: 20-2-7742-600; Fax: 20-2-5717-355
E-mail: helshemy@hotmail.com

Table 1. The effect of willow leaf extracts (7%) on the viability of human AML cells

Sample No.	Blasts (control)		Blasts+Extract	
	Dead %	Viable %	Dead %	Viable %
1	2	98	85	15
2	2	98	75	25
3	5	95	70	30
4	5	95	70	30
5	2	98	82	18
6	3	97	75	25
7	2	98	80	20
8	2	98	80	20
9	5	95	75	25
10	5	95	80	20
11	5	95	75	25
12	2	98	75	25
13	3	97	80	20
14	2	98	55	45
15	2	98	50	50
Mean	3.1	96.9	73.8	26.2
S	1.5	1.5	4.8	4.8
P			<0.01	

Results and Discussion

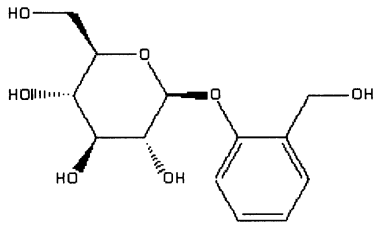
In the case of acute myeloid leukemia (AML), the mean viability was 26.2% when compared to the control (96.9%) (Table 1). The willow extract (in a 7% concentration) was incubated with normal cells as a positive control from healthy volunteers (6 samples). The results showed that there was no significant difference on killing the cells (mean 7.8% and 6.7%) when compared to 3.1% in the control (negative control). From these data, it is clear that younger cells are vulnerable to the extract.

The willow leaves were not studied in terms of antitumor effect; however, they have long been used in folk medicine as an antirheumatic, analgesic, and antipyretic herbal medicine. In connection with antileukemia, the allamandin derivatives which are extracted with water and/or ethanol from *Allamanda catharica* (Apocynaceae) showed significant activity *in vivo* against the p-388 leukemia in the mouse (Kupchan *et al.*, 1976).

Different leaf extracts (*Salix safsaf*) by successive solvent extractions were tested for their antileukemic activity on acute myeloid leukemia (AML) cells. The fractions of each crude extract were dissolved in a saline solution after removing the solvent. They were then incubated with the AML cells. The results showed that a fraction of the willow leaves extracted with nonpolar organic solvents (petroleum ether, ether, and chloroform; viability by the solvents was 90, 83, and 80, respectively) had a very low destructive effect on AML cells. Destruction ranged between 1.5-2.9% of each extract. However, a major destructive effect on AML cells was obtained by a fraction of the polar organic solvents (water and 70% ethanol). From this observation, it is clear that the antileukemic activity of the willow leaves was mostly due to compounds that were soluble in water and/or ethanol. These active ingredients were easily dissolved in hot water, and in turn could be used as a natural antitumor medicine.

The phenolic compounds, glycosides, and tannins usually dissolved in water or ethanol solutions (Bravo, 1998). Therefore, this group of compounds represents major active components for the destruction of leukemia. Salicin is the primary compound in salix leaves that can be dissolved in water and ethanol (GCMS and HPLC methods, data not shown). Salicin was identified by comparing their retention time and spectral characteristics to those of the reference compounds.

In our study, the salicin standard (Sigma, St. Louis, USA) was tested for its antileukemic effect. Salicin (0.1 ml of a 0.75 mg/ml) was added to the suspension of 10^5 AML cells. The results showed that the destruction of myeloblasts (70-75%) was significantly higher than those in the control specimens. From these observations, salicin is probably the major component that shows the antileukemic effect.



The Structure of Salicin

Transport of salicin and saligenin into erythrocytes was rapid for saligenin (1 min to saturation) and delayed for salicin (4 h to saturation). The process was reversible, exhibiting a rapid release for saligenin and slower release for salicin (Matsumoto *et al.*, 1993). Both saligenin and salicin bind to human serum albumin, but saligenin has the significantly higher affinity (Matsumoto *et al.*, 1993). Salicin is partially metabolized to saligenin and salicylic acid (effective compound) after incubation with homogenized kidneys from rats (Metzner *et al.*, 1989). Saligenin was transformed to salicylic acid by homogenized liver, lung, and kidney. Gentisic acid was qualitatively detectable in the homogenized liver after incubation with saligenin (Metzner *et al.*, 1989). The transport of salicin and saligenin through the isolated intestinal wall was confirmed using the closed-off posterior section of the male rat intestine. When salicin and saligenin were injected into the closed intestine, both passed unchanged through the ileal wall. Saligenin appeared to penetrate the intestinal wall faster than salicin (Adamkiewicz and Fortier, 1961). Steinegger and Hel (1972) showed that the metabolites that were equivalent to more than 86% of the administered salicin were recovered in 24-h urine: salicyluric acid (51%),

salicyl glucuronide (14%), salicylic acid (12%), gentisic acid (5%), and saligenin (49%) together with a small amount of unchanged salicin.

Based on these findings, it is speculated that the leukemic cell may emit some signaling substances for salicin and saligenin receptors. Therefore, the compounds bind with the receptors on the surface of leukemic cells and penetrate into the cells. The cells could be killed through denaturation of some enzymes and proteins that are induced by salicin and saligenin. Therefore, we propose that future clinical studies should be pursued.

References

Adamkiewicz, V. W. and Fortier, A. A. (1961) Passage of salicin and saligenin across the wall of the rat ilcum. *Can. J. Biochem. Physiol.* **39**, 1097-1099.

Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A. G., Gralnick, H. R. and Sultan C. (1976) Proposals for the classification of the acute myeloid leukemia. *Br. J. Haematol.* **33**, 451-458.

Bravo, L. (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **56**, 317-333.

Han S. -S., Keum Y. -S., Seo, H. -J. and Surh, Y. -J. (2002) Curcumin suppresses activation of NF-κB and AP-1 induced by phorbol ester in cultured human promyelocytic leukemia cells. *J. Biochem. Mol. Biol.* **35**, 337-342.

Kupchan, M. S., Uchida, I., Branfman, A. R., Caily, R. G. and Yufei, B. (1976) Antileukemic principles isolated from Euphorbiaceae plants. *Science* **191**, 571-572.

Leven, M., Berghe, V. D., Martins, F., Vlietinck, A. J. and Lammas, E. (1979) Screening of higher plants for biological activities, 1. Antimicrobial activity. *J. Med. Plant Res.* **36**, 311-321.

Matsumoto, Y., Ohsako, M., Takadate, A. and Goto, S. (1993) Reduction of erythrocyte membrane permeability and protein binding of low molecular weight drugs following glycoside gerivatization. *J. Pharm. Sci.* **82**, 399-403.

Metzner, J., Hirschelmann, R., Hiller, K., Fotsch, G., Pfeifer, S., Bartoszek, M., Franke, P. and Hiller, K. (1989) Biotransformation of phenolglycosides leiocarposide and salicin. *Pharmazie* **44**, 555-558.

Steinegger, E. and Hel, H. (1972) Analytische und biologische Untersuchungen an Salicaceen Wirkstoffen, insbesondere an Salicin. II. Biologische Untersuchungen. *Pharm. Acta Helv.* **47**, 222-234.