Research Journal of Agriculture and Biological Sciences 1(3): 284-287, 2005

Molecular Changes on Cancer Cells as Affected by Willow Extracts

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Abstract: The aim of the work was to study the mode of action of willow (*salix safsaf*) extracts on two types of tumors, Ehrlich ascites carcinoma cells (EACC) and acute myeloid leukemia (AML). The fresh water extract of willow (5.4 mg/ml of cell media) gave 86% dead cells in EACC and 63% in AML. The cell membrane damage of tumor cells was evaluated by LDH increased activity. GST activity was also increased as antitumor marker and/or affected the redox system. Apoptosis was induced by the natural tested materials and showed an increase in DNA fragmentation and expression of P^{53} protein in tumor cells by willow extract treatment. The extract increased the percentage of diploid cells and S-phase as well as metaphase of tumor cells. Suggested mechanisms have been given for the action of willow extract as anticancer.

Key words: Tumor cells, apoptosis, antitumors, leukemic cells, DNA fragmentation, glutathione, metaphase

INTRODUCTION

The effect of plant extracts as antitumors was widely studied due to their low toxicity and side effect. The inhibition of ascites tumor cells by garlic extracts was investigated[1]. Soybean seed extracts showed antitumor activity which is due to the presence of trypsin inhibitor[2]. The tumor inhibitors of plant origin depend upon the type of cancer cells and plant specie as well as the extract used. Extract of *Allamanda cathartica* gave significant activity against P-388 leukemia in mouse[3]. Different plant species (from eight families) growing in Egypt showed anticancer activity[4]. The principles separated from plants were also studied such as alkaloids[5,6], terpenes[7], flavonoids[8,9], or chlorophyll[10].

The metaphase of tumor cells was highly arrested by vincristine and vinblastine as alkaloids which have antimitotic effect[5].The abnormalities in chromosomes of tumor cells such as sister chromatid change and abberations[11,9] and DNA fragmentation[12] by natural extracts were illustrated. The induction of apoptosis in the cancer cells by natural plant extracts was studied by[13-17].

We previously used willow extracts as anticancer agents in an in vitro study[18]. Willow has antileukemic activity and the water extract of the leaves killed the majority of the blasts of acute myeloid leukemia (AML) and acute lymphatic leukemia (ALL). The data showed that Salicin and Saligenin represent the more active gradients against the cancer cells. The powerful effect was found to be due to Salicin[19] and the formula of the compound was confirmed[20].

The mode action of the willow extracts and its active principles on the cancer cells was not clear. Therefore, we searched for mechanism of action of willow (*Salix safsaf*) extracts as anticancer by different biochemical and molecular assays. Two types of cancer cells (EACC and AML) and different biochemical and molecular parameters were performed in the study.

MATERIALS AND METHODS

Materials:

Animals: Female Swiss albino mice 2-2.5 months old, weighing 17-22g were used in the present study. The animals were kept under normal nutritional and environmental conditions. After 2 weeks adaptation period, each animal was i.p injected with Ehrlich ascites carcinoma cells (EACC), 200µl containing $2x10^6$ cells. The tumor cells were taken from National Cancer Institute (NCI), Cairo, Egypt for cell line preparation.

Willow extract: Extracts of *salix safsaf* were prepared from green leaves by slicing 2-3g and homogenizing with 10 ml of distilled water and boiling for 10 minutes. The clear extract was obtained after filtering and centrifugation at 3500 rpm.

Human cancer cells, Acute myeloid leukemia (AML) were taken from patients after clinical diagnosis in National Cancer Institute, Cairo, Egypt. Blood samples of patients were collected then the mononuclear cells were

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separated and fractionated through Ficoll-histopaque gradient density sedimentation (1077)[21].

Methods:

1- Viability of EACC was measured by the modified cyotoxic Trypan blue-exclusion method[22].
2- Glutathione was determined according the method of redox reaction[23].
3- Glutathione-S-Transferase, (GST) activity was measured using the method of[24].
4- Lactate Dehydrogenase, (LDH) activity was determined by the method of[25].
5- Apoptosis assay was done according to[26].
6- DNA fragmentation was evaluated by two techniques: a) Ladder detection[27] and b) Diphenylamine (DPA) colorimetric assay[28].
7- Image cytometeric analysis was done using Image Analyzer Becton Dickinson Model[29].
8- Antimitotic analysis was measured according to[5].
9- Cytogenetic analysis test was used as[30].
10- P^{53} expression was detected in EACC (*in vitro*) using Flow Cytometer technique[31].

RESULTS AND DISCUSSIONS

Salix species had been commonly used in folk medicine, antimalarial, laxative, hypoglycemic, contraceptive in treatment of sores, burn and slow continuous fever[32]. The leaf extracts found to inhibit the growth of leukemic cells hence it contain active ingredients against tumor cells[18]. In our previous studies salicin and saligenin showed the most of the antitumor effects in the willow extracts[19,20].

To understand the mechanism of action of willow extracts as anticancers we did a lot of biochemical and molecular analysis. Results in Table (1) showed that the addition of fresh willow extract at 4.5% of tumor cell media containing two million of EACC or 10^5 of AML killed about 85% of these cells.

It was found that , the willow extracts increased the contents of reduced glutathione in the tumor cells to more than 5-folds (Table 2). Also the activity of GST and LDH was enhanced by incubation of tumor cells with willow extracts (Table 2). The active ingredients in willow leaf extracts may disturb the metabolic behavior of tumor cells in special GSH/GSSG Redox System. This phenomenon was previously illustrated[33] and studied the relationship between cancer growth, glutathione redox cycle and antioxidant system in blood and Ehrlich ascites. The increase in the GST activity in general, used as indication for the antitumor activity of the tested materials

Table 1: Effect of willow extracts on the viability of tumor cells*

Sample No.	Extract Conc.	Dead cells %	
	(mg/ml)	EACC	AML
1	0	3	2
2	1.2	30	24
3	1.8	55	53
4	3.0	61	58
5	4.2	68	65
6	5.4	86	85
7	6.6	85	85

*One ml of EACC containing $2x10^6$ cells or AML containing 10^5 cells
Data presented as mean of three determinations triplicates.

Table 2: Effect of willow extracts on the activity of GST and LDH

Extract conc (mg/ml)	GST activity (µM/min)	LDH activity (U/L)
0	2	41.4
1.2	5	36
1.8	9	54
3.0	10	73
4.2	9	75
5.4	9	71

One ml of EACC containing $2x10^6$ cells.
Data presented as mean of three determinations triplicates.

in both normal and tumor transplanted animals. Therefore, this enzyme has been used as antitumor (as tumor factor[34] and high amounts of GSTP-1 in tumor cells). In the tumor cells, the increase of cellular enzymes that regulate the cell oxidative stress such as SOD and GST and antioxidants such as GSH induced cancer regression and stimulated large number of tumor necrosis factor-alpha (TNF-"). This factor is related to GSH level in cancer cells and the sensitivity of these cells to TNF- " (*in vivo*) depends on GSH content and their rate of proliferation[35].

In the present study, The willow extracts affected the tumor cell chromosomes by degrading the DNA which appeared as smear and ladder when subjected to agarose gel electrophoresis (data not shown) and by DNA fragmentation (Table 3). Also the cytometric analysis of EACC after incubation with extracts showed great changes in the nuclear grade of the tumor cells (Table 3). The effect included the appearance of high diploid cells, increase of S-phase and accumulation of tumor cells in G2/M phases.

The death of tumor cells by willow extract may be induced by different mechanisms such as cell membrane damage as observed by the increase in LDH activity. The enzyme is rapidly released into the cell culture supernatant when the plasma membrane is damaged[9].The cell damage will lead to the leakage of cell constituents (of cancer cells) such as enzymes (GST and LDH, Table 2) and GSH (Table 2). In another way the anticancer effect of willow extract may be due to stopping the cell life cycle at the metaphase (or other phases) and then inhibits cell division.

In some cases the plant extract may give more DNA fragmentation (Table 3) and in turn enhances tumor cells

Table 3: Effect of willow extracts on cytogenetic, nuclear grade and cell life cycle of EACC

Treatment	Extract conc. (mg/ml)	% DNA Fragmentation	Diploidy	S-phase	Tetra ploidy	>Tetra ploidy
Saline+Tumor*	0	1.6	1.73	31.12	17.75	49.40
Willow+Tumor*	1.2	1.4	15.7	54.81	16.72	25.73
		Metaphase change (%)				
		2h	**6h**		**24h**	
Saline+Tumor*	0	0.44	1.42		1.42	
Willow+Tumor	16.6	5.55	5.79		3.86	

One ml of EACC containing $2x10^6$ cells.
*Mean of six slides from 3 animals
Saline+Tumor represents control

Table 4: Effect of willow extract on expression of P^{53} in EACC media.

Treatment	Willow Conc. (mg/ml)	% Positive cells
Saline+Tumor cells	0	51.0 ± 0'6
Extract+Tumor cells	1.2	53.5 ± 0.8
Extract+Tumor cells	3.0	65.6 ± 1.1
Extract+Tumor cells	4.2	87.6 ± 1.8

One ml containing 10^6 of EACC

to inter apoptosis. To confirm the apoptosis state we did an experiment to detect the expression of P^{53} by determinating the percentage of positive EACC which have P^{53} protein after incubation with willow extract for 2h at 37°C. A specific flourescent antibody of P^{53} was used to measure the amount of the corresponding protein (of P^{53}) after interaction with laser beam hence the photons of light that are scattered and emitted by cells (that contain P^{53}) can be detected by specific detectors of the flow cytometer. The obtained results are given in Table (4) and showed the increase in number of tumor cells expressing contain P^{53} protein and the increase was concentration dependent.

The increase of tumor cells containing P^{53} protein confirms the suggestion of apoptosis induction by extract treatment more than repairing of cells. The induction of apoptosis was detected by: inhibition of tumor proliferation, nucleosomal DNA fragmentation, release of soluble nuclear mitotic apparatus protein, and presence of hypodiploid peaks in flow cytometric DNA analysis. The obtained results are in a good agreement with[17] observations and also with[5] by using *atharanthus roseus* plant extract and[36] by using annatto, ccurcumin and *salix* extracts. The latter study showed that these materials greatly changed the polyploidy (90-94%) of tumor cells to diploidy (normal morphology). The reduction of abnormalities in tumor cells by the extracts may stimulate the cells to divide normally or go to die (through apoptosis) if they cannot remove chromosomal abnormalities.

More work will be done using specific genes for apoptosis such P^{53} and BCl-2 and Caspases to understand more about these mechanisms.

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