Cytotoxic effects of some animal and vegetable extracts and some chemicals on liver and colon carcinoma and myosarcoma

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ABSTRACT

Objectives: To study, the cytotoxic effects of some biological and chemical agents on G_1, S, G_2, M and G_0 phases of liver and colon carcinomas and myosarcoma cells obtained with chemical carcinogens dimethylbenzanthracene (DMBA) and cadmium chloride.

Methods: Eight rabbit livers, colon carcinoma and myosarcoma cell lines were obtained by injection of DMBA in the Biology Laboratory, of the University of Dumlupinar, Kutahya, Turkey between January 2001 and June 2003. All lines were grown at 37°C and 5% carbon dioxide in sterile RPMI-1640 medium with 10% fetal bovine serum after addition of glutamate, penicillin (50 units/ml) and streptomycin (50 µg/ml) (complete medium). Cells were grown on standard tissue culture plastic flasks to 80% confluence and passed by trypsinization.

Results: Tortoise (*Testudo graeca*) shell, sponge (*Geodia cydonium*), medusa (*Aurelia aurita*), meat flies (*Calliphora erythrocephala*) larva, frog (*Rana ridibunda*) larva and juniper (*Juniperus communis*) berry extracts killed a large amount of the liver and colon carcinomas and the myosarcoma cells in G_2, M and G_0 phases ($p<0.01$). The mistletoe (Viscum album) extract had more effect in only the G_0 phase ($p<0.05$). Genistein, genistin, glycitein, glycitin, daitzein and daitzin have significantly decreased in the cancer cells tests, particularly, genistein and daitzein caused the apoptotic effect in G_2, M and G_0 phases ($p<0.01$). Cesium chloride, a mixture of cesium chloride with magnesium chloride had the most effect on tumor cells ($p<0.01$). AzhexSi, Azhex-AzhepSi, Et-Azhex-AzhepSi, AzhepSi, Hexamine and DL 54 have been inhibited in various levels of the cancer cells ($p<0.05$, $p<0.01$).

Conclusions: This data suggest that some biological extracts and chemicals tested may be useful chemotherapeutic agents to inhibit the growth of cancer cells. This study sheds some light for new anti cancerogenic experiments preventing various cancers on humans.

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One of the greatest challenges in the treatment of certain cancer remains its inherent lack of beneficial response to cytotoxic chemotherapy. Herbal extracts have been widely used for the treatment of various cancers, but objective information on their efficacy is lacking. Tumoral cancer continues to represent a significant oncological challenge. Although most patients present with advanced, unresectable disease, effective systemic treatment options remain sparse. Gemcitabine, vincristine, taxol and cisplatin currently represent the standard chemotherapeutic drugs for metastatic and advanced disease, but these only lead to a modest improvement in quality of life and survival.[1-38]

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A search did not yield literature regarding cytotoxic effects of tortoise (*Testudo graeca*) shell, medusa (*Aurelia aurita*), meat flies (*Calliphora erythrocephala*) larva, frog (*Rana ridibunda*) larva and juniper (*Juniperus communis*) berry extracts on liver and colon carcinomas and myosarcoma cells in vitro.

The sponge chemicals Agosterol A, Jasplakinolide, Naamidine A and Spongistatins, Agelastatins, Crellastatin, Bolinequinone, Haliclona, Cyclamines, Sclaren and Sesterstatins, Spongouridine, Spongosine, Spongothymidine have antitumoral and anticarcinogenic effects.[1-4] The Japanese sponge *Halichondria okadai* provided the halichondrins a new class of polyethers. Halichondrin B and homohalichondrin B, the most potent in the series, are highly cytotoxic in vitro and in vivo to P388 and B16 melanoma. They are active in vivo against P388 leukemia and B16 melanoma. They are also potently active in vivo against lung tumors. Both inhibit microtubule assembly dependent on microtubulic associated proteins.[2-4] The Spongistatins are inhibitors of tubulin polymerization and bind in a distinct region of the vinca domain. Jasplakinolide (*Jaspamide*) was isolated independently by 2 groups from an Indo-Pacific sponge *Jaspis species*, later identified as *Jaspis Johnstoni*.[5-16] Further investigations revealed that Jasplakinolide was active against 36 solid tumor cell cultures in the National Cancer Institute human tumor panel acting by disruption of the action cytoskeleton in mammalian cells. Mycaperoxide B obtained from a New Zealand sponge *Mycale species* exhibits antiviral activity and anticancer activity in vitro and in vivo ovarian and lung cancer.[13,16] Soybean isoflavonoids; genistin, genistein, glycitin, glycitein, daitzin, daitzein have a therapeutic effect on some cancer cells and these are important phytoestrogens. Research into the possible benefits of phytoestrogens has focused on cancer, menopause, osteoporosis and heart disease due to the antioxidant activity.[17,18]

Genistein not only prevents tumor cell growth; it acts as a powerful protective agent, namely, antioxidant, against the destructive effects of free radicals. Substances present in soybeans and soybean products are suggested to prevent the development of cancer in many different organ systems.[19-22] All dimethylsilane polyamines inhibited the cancer cells at micromolar concentrations. Cytotoxicity of these compounds has been tested on the various cells. In the various studies, AzhexSi, AzhexSi+DFMO, Azhex-AzhepSi, Et-Azhex-AzhepSi, AzhepSi, DL-54 and Hexamine decreased the putrescine, spermine and spermidine concentrations, therefore, dimethylsilane polyamines have a cytotoxic effect and inhibit growing of the cells.[23-32]

Radioactive cesium (Cs-137) is used in certain types of radiation therapy for cancer patients. However, there is no scientific evidence that non-radioactive cesium chloride (CsCl) supplements have any effect on tumors. Cesium chloride supplements increase the pH level of tumor cells back to a normal level, which may be detrimental to the cancer's growth. Since CsCl is claimed to work by raising the pH of tumor cells, its use in therapy has been called 'high pH therapy.'[33,34] We were interested to examine the direct effects of isoflavonoids, dimethylsilane polyamines, tortoise (*Testudo graeca*) shell, sponge (*Geodia cydonium*), medusa (*Aurelia aurita*), meat fly (*Calliphora erythrocephala*) larva, frog (*Rana ridibunda*) larva and juniper (*Juniperus communis*) berry extracts on liver and colon carcinomas and myosarcoma cells in vitro.

Methods. The present study was conducted at the Biology Laboratory, Faculty of Sciences and Arts, University of Dumlupinar, Kutahya, Turkey between January 2001 and June 2002. In this study, tortoise (*Testudo graeca*), frog (*Rana ridibunda*) larva, meat fly (*Calliphora erythrocephala*) larva, juniper (*Juniperus communis*) berries, mistletoe (*Viscum album*) were obtained from forestry and wet areas. Sponge (*Geodia cydonium*) and medusa (*Aurelia aurita*) were collected from Bodrum and Marmaris coasts of Turkey on the Mediterranean. Genistein was purchased from Genay, France, Daitzein, Daitzin, Genistin, Glycitein and Glycitin were purchased from Fujicco Co. Ltd., (Kobe, Japan). Cesium chloride and magnesium chloride ($MgCl_2$) were purchased from Merck, Dimethylsilane polyamines were purchased from Merck, (Darmstadt, Germany), and Sigma Chemical Co (St. Louis, Missouri). Also, cisplatin chloride and DMBA were purchased from Merck.

Two adult male tortoises were anesthetized with phenobarbital natrium and later decapitated with 5 ml of anesthetic matter. Anesthetic matter was injected as intra arteria jugularis. The plastron and carapax of the tortoise were sawn and its internal organs were taken out. The shells were cleaned from meats and fats and sterilized with 70% ethyl alcohol and dried. These shells were broken with the cracking machine and were ground with the grinder. The grinding particles were 43 micron in diameter. The meal of the shell was boiled in 10% of potassium hydroxide and the material was later extracted with methanol and hexane, and sterilized in the autoclave. Porifera specimens were washed with distilled water and cut into slices and these were washed over and over again, dried in etude and ground. The diameters of the slices were 43 micron and were extracted with methanol and hexane and the slices were sterilized.

The larva specimens were collected from watery habitats and they were washed with distilled water and were killed by ether inhalation. The larvae were homogenized with the cold bidistilled water as volume per volume (v/v) and were frozen in little bottles or vials. During assay, material was extracted with methanol and hexane, and later was sterilized. The meat flies were cultured in bovine meat medium and their eggs were cracked at 18^{0}C after 24 hours and the white pups came to light at approximately 5 days. These pups were killed by ether inhalation and were homogenized with the cold bidistilled water v/v, and were frozen in little bottles. During the experiment this material was extracted with methanol and hexane. The medusa specimens collected were put in glass flasks and immediately they were allowed by themselves melting and all medusa specimens were homogenized at the coast and were conserved in little bottles and later extracted with methanol and hexane. The fresh berries were washed with the bidistilled water and were dried between filter papers and later ground with the grinder. The meal of the berries was diluted with the bidistilled water and the samples were later extracted with methanol and hexane, and also sterilized in the autoclave. The mistletoe was collected from pines (*Pinus nigra*) and washed with the bidistilled water and dried between filtered papers. Later the fresh mistletoe was homogenized v/v and later extracted with methanol and hexane and sterilized in the autoclave.

The liver carcinoma, colon carcinoma and the myosarcoma tumors were obtained by injecting one and 3 ml of dimethylbenzanthracene (DMBA)/kg/day to female rabbits (*Lepus capensis*) at 36-42 days and as one dosage per day. After approximately 36-42 days, the rabbits were anesthetized with phenobarbital natrium injection, decapitated and their organs removed. In this operation, liver carcinoma, colon carcinoma, colon adenocarcinoma tumors, polyps and myosarcoma tumors were obtained in the ultraviolet sterilized chamber. All of the tumors were put in sterilized glass flasks. Immediately, the tumors were homogenized with 0.09% of sodium chloride (NaCl) v/v under sterilize conditions. Later, the homogenized cancer cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, Missouri) supplemented with 10% v/v fetal bovine serum (Gibco, Paisley, United Kingdom), 1.0 v/v L-glutamine, and 1% v/v penicillin, streptomycin and neomycin (PSN) antibiotic mix. Biorack does not provide a 5% carbon dioxide ($C0_2$) atmosphere; therefore, for experiments in the GCAK cassettes, the RPMI-1640 mix supplemented with 25 mm hepes, 12 mmol sodium carbonate (Na_2CO_3), and 1 mmol sodium pyruvate, all from Sigma. These supplements replace the requirement for atmospheric CO_2 by buffering the medium and providing a source of soluble bicarbonate. Additional serum was added to the cells at transfer to 37^{0}C as indicated in the experimental protocol to increase the serum concentration to 10%. Experiments were performed in both the GCAK-1 and 2 units. Each cassette chamber was loaded with approximately 0.5 ml of cell suspension using an Eppendorf multinjector. Viable and total cell counts were determined from trypan blue exclusion counts under the microscope using a hemocytometer. Two counts of at least 200 cells were performed for each sample in the cassettes. The initial viability of the cells was determined before loading of the cells into the cassettes. Moreover, flow cytometrical analysis was applied. As components, the caspase substrate reagent kits (cat. A304R1G-3) were used. This kit contains 3 vials (each vial contains 500 ml) plus one additional substrate vial containing at least 750 ml of 10 mmol substrate in RPMI-1640 medium. Additionally, one bottle (60 ml) of flow cytometry dilution buffer is included. Three caspase substrate reagent kits contain 6 vials (500 ml) plus 2 additional substrate vials (750 ml) of 10 mmol substrate RPMI-1640 medium and 2 bottles of the flow cytometry dilution buffer. All the medium was removed in order to minimize substrate dilution. Between 50-75 ml of 10 mmol substrate solution was added to each of centrifuged cell pellets. The cell number in these solutions was between 0.5 and 1 million per sample. The incubation was made in a 5% CO_2 incubator at 37^{0}C for 60 minutes before cytometric analysis. The present cancer cells were first diluted by adding 1 ml of ice cold buffer and then all the buffer was removed from the cells by centrifuging. The cell suspensions were kept on ice until analysis by flow cytometry. All samples were analyzed within 60-90 minutes after the end of the 37^{0}C incubation. After collecting propodium iodide (PI)+ cells if a drop of a 5-10 mg/ml PI solution was added and samples are rerun on the flow cytometer. The cytotoxic effects were tested on the cell analysis, that is, G_1, S, G_2, M and G_0 phases.[23-38]

Results. The findings related to the inhibition or apoptosis of the cancer cells are given in **Tables 1-3**. All of the vegetable and animal extracts showed more shrinkage in M and G_0 phases. Also, isoflavonoids and dimethylsilane polyamines tested have similar effects at the same phases. Tortoise shell and sponge extracts, genistein, CsCl + $MgCl_2$ had more effect in M and G_0 phases on tumor cells tested (**Tables 1-3**). Dimethylsilane polyamines have been more less cytotoxic effects on cancer cells, according to the Tables. On autopsy, it was seen that CsCl + $MgCl_2$, dimethylsilane polyamines tested, cisplatin chloride, juniper and frog extracts showed necrosis and bleeding in the kidneys and liver. Results of skin irritant and eye irritant tests were given in **Table 4**. All of extracts and

dimethylsilane polyamines, cisplatin chloride showed irritation of the skin and eye.

Discussion. All of the treatments effected the cancer cells and the mitotic phases of the cells. Tortoise shell has a cuticular structure and polypeptide and phenol compounds. Also, it has boron, $MgCl_2$, and pigments according to the qualitative analysis. Particularly, the cuticular structure has the protein-phenol polymer. Sulfur compounds in carapax and the plastron of the shell, for example methionine, cystine, cysteines are found. Therefore, shell extract had the apoptotic effect on the cancer cells. The protein polymer and the phenol and the other chemicals may have chelated with the DNA of the cancer cells or the chemistry of the shell may have broken the DNA ester bounds of the cancer cells, as in some assays in vitro apoptotic precipitations were found. These cancer cells do not have the cuticular chitinase enzyme, therefore, the shell extracts effected the different phases of the cancer cells. On the other hand, the sponge extract did effect the cancer cells. The sponge species has the anticarcinogenic agent spongistatin compound. However, some sponge species have agelastatin (alkaloid), balinaquinone (terpene), crellastatin (sterol), haliclona cyclamines (alkaloid), scalarane (terpene) and sestorstatins (terpene); these compounds are anticarcinogens. Further, a few sponges have agosterol (terpene), jasplakinolide, naamidine (imidazole), and these are also anticarcinogenic compounds. It is also known that sponges contain iodine, chlorure, natrium, calcium and potassium. In this investigation sponge extract contained the spongistatin, which acted as the anticarcinogenic agent according to the qualitative and quantitative analysis and the applications. The larval extract of the frog has defense chemicals, metamorphic particularities, and rich lymph systems. The larval extracts were not effected in the metamorphosis of the cancer cells, however these extracts inhibited the mitotic phases. The larval extract of the meat flies had an inhibitory effect on the cancer cells. In this study, the living larvae ate and digested some of the tumors. For this investigation, one tumor from each cancer case was placed in a sterilized glass flask, and the living larvae were placed on tumors, as a result of the experiment, the amount of the living larvae killed after eating the tumors was measured. As these larvae have proteolytic enzymes, larval therapy of the tumors may be considered.

Table 1 - The cytotoxic effects of the animal and the vegetable extracts and some chemicals on liver carcinoma. Dosages are one and 3 µmol/ml.

Treatment	**Inhibition (%) Cell phases**											
	G_1		**S**		**G_2**		**M**		**G_0**		**C**	
Dosages	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
Tortoise shell extract	19	27	35*	48*	43*	52*	56*	58*	73*	85*	18	23
Spongia extract	23	26	33*	45*	41*	53*	55*	70*	72*	84*	20	22
Frog larva extract	10	12	11	12	14	19	19*	19*	18*	20*	10	12
Meat fly larva extract	14	19	21*	23*	20*	21*	24*	28*	27*	33*	8	12
Medusa extract	20	28	30	36	40	52*	58*	71*	66*	87*	30	36
Juniper berries extract	15	18	17	21	23*	32*	31*	36*	37*	41*	11	14
Mistletoe extract	8	10	9	13	10	13	13	15	17*	19*	9	8
Genistein	18	19	18	23	24*	35*	36*	48*	56*	73*	12	17
Genistin	10	13	12	15	13	17	23*	28*	33*	42*	9	13
Glycitein	7	7	7*	8*	7*	8*	8*	9*	9*	9*	2	2
Glycitin	6*	6*	5	5	6*	8*	6*	8*	10*	11*	3	3
Daitzein	11*	12*	14*	16*	15*	17*	16*	19*	29*	38*	3	5
Daitzin	6*	6*	5*	5*	7*	7*	6*	7*	8*	10*	2	3
CsC1	26	38	45*	53*	53*	68*	62*	69*	68*	83*	20	26
CsC1+MgC1$_2$	33	39	48*	56*	50*	60*	53*	62*	68*	85*	28	33
AzhexSi	7	11	8	13	10	19	24	38	40*	42*	20	22
Azhex-AzhepSi	14	16	17	18	17	23	30	38	38*	43*	19	23
Et AzhexAzhepSi	19	23	19	26	28	35	36	38	40*	42*	20	23
AzhepSi	9	11	12	13	16	19	24	29	30*	36*	17	22
Et Azhepsi	10	12	13	15	14	18	23*	27	29*	35*	12	18
Hexamine	12	16	13	17	14	19	26	30	34*	39*	19	22
DL 54	11	12	11	16	14	19	27	37	30*	38*	20	21
Cisplatin chloride	20	21	22	22	49*	51*	56*	63*	58*	66*	20	24

CsCl - cesium chloride, $MgCl_2$ - magnesium chloride, *$p<0.01$ according to the control
± SEM values calculated between 2.28 and 3.56, C - control

The medusa extract has proteolytic enzymes, however it has also other enzymes. The medusa extract effected the cancer cells and the various cell phases. In this way, the ester bonds of DNA of the cancer cells may have been broken. The juniper berries have a diuretic and antiseptic effect in alternative medicine. The berry extract decreased the amount of cancer cells ($p<0.01$). The extract of the mistletoe decreased the longevity of the cancer cells ($p< 0.01$). This plant lives as an ectoparasite on other plants. Soybean isoflavonoids; genistein, genistin, glycitein, glycitin, daitzein and daitzin are very important phytoestrogens. These compounds decreased the cancer cells in various levels, particularly genistein and genistin, caused shrinkage of the cancer cells ($p<0.01$).

Cesium chloride and the mixture of CsCl + $MgCl_2$ enormously inhibited all the cancer cells ($p<0.01$). Dimethylsilane polyamines, AzhexSi, Azhex-AzhepSi, Et-Azhex-AzhepSi, AzhepSi, Et AzhepSi, DL-54 and Hexamine played a great role in inhibiting of the cancer cells. The cytotoxic effects of all the chemicals and extracts were seen particularly in G_2, M and G_0 phases. The cell reactions were evaluated and scored after 24 and 48 hours. Dimethylsilane polyamines and cesium chloride, juniper berries and medusa extracts were judged as irritants on intact organs, particularly the kidneys and brain, whereas soybean isoflavonoids and $MgCl_2$ were considered as essentially nonirritants in normal dosages. The skin and eye of rabbits were tested for compounds and the animal and the vegetable extracts are summarized in **Table 4**.

The present agents caused the apoptosis of the cancer cells. Apoptosis, which is a morphologically and biochemically defined form of cell death, occurs in response to a variety of stimuli under physiological and pathological circumstances. The apoptotic volume decrease (AVD) is coupled to potassium ion (K^+) release from the cells, presumably via K^+ channels. At same time these agents led to cancer cell shrinkage. In this situation, these agents may have broken down their cell

Table 2 - The cytotoxic effects of the animals and the vegetable extracts and some chemicals on colon carcinoma cells. Dosages are one and 3 µmol/ml.

Treatment	**Inhibition (%) Cell phases**											
	G_1		**S**		**G_2**		**M**		**G_0**		**C**	
Dosages	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
Tortoise shell extract	20	25	28*	34*	60*	68*	65*	73*	68*	72*	17	20
Spongia extract	23	28	26*	29*	45*	57*	59*	68*	63*	75*	16	21
Frog larva extract	8	9	9*	9	8	9	10*	12*	11*	13*	5	7
Meat fly larva extract	8	10	13*	13*	10*	11	19*	14*	18*	18*	5	8
Medusa extract	21	27	33*	34*	37*	43*	48*	59*	63*	78*	18	23
Juniper berries extract	19	23	27*	38*	48*	59*	58*	63*	60*	71*	17	21
Mistletoe extract	9	11	9	10	17*	28*	24*	34*	33*	39*	10	10
Genistein	19*	26*	33*	48*	40*	42*	44*	59*	63*	78*	5	6
Genistin	6*	6*	7*	7*	7*	8*	10*	11*	10*	11*	2	2
Glycitein	5*	6*	5*	5*	6*	7*	7	10*	9	12*	1	1
Glycitin	6*	6*	7*	8*	7*	8*	8	10*	12*	12*	3	3
Daitzein	13*	17*	14*	16*	18*	23*	36*	48*	50*	56*	4	5
Daitzin	6*	6*	5*	5*	8*	8*	9*	11*	10*	12*	1	1
CsC1	30	33	36*	44*	40*	53*	49*	68*	65*	81*	24	30
CsC1+MgC1$_2$	32	36	37	50*	49*	60*	63*	84*	81*	88*	35	37
AzhexSi	10	12	11	18	13	17	19	21	20	24	18	19
Azhex-AzhepSi	21	26	27	33	28	36	32	40	33	40	20	26
Et AzhexAzhepSi	23	27	30	36	32	35	36*	41	36*	40	20	31
AzhepSi	12	14	13	14	15	17	17*	18*	17*	20	9	31
Et Azhepsi	12	13	13	15	13	14	15	17	17*	21*	10	13
Hexamine	11	14	11	14	16*	19	17*	24*	26*	38*	9	13
DL 54	14	15	14	17	16	18	16	17	18*	23*	10	12
Cis platin chloride	24	25	28	33	56*	63*	61*	68*	68*	69*	25	27

CsCl - cesium chloride, $MgCl_2$ - magnesium chloride, C - control
*$p<0.01$ according to the control ± SEM values calculated between 4.20 and 4.98.

Table 3 - The cytotoxic effects of the animal and the vegetable extracts and some chemicals on myosarcoma cells. Dosages are one and 3 µmol/ml.

Treatment	Inhibition (%) Cell phases											
	G_1		S		G_2		M		G_0		C	
Dosages	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**	**1**	**3**
Tortoise shell extract	36*	38*	40*	44*	48*	65*	50*	68*	55*	73*	17	20
Spongia extract	36*	40*	42*	48*	56*	71*	56*	70*	58*	74*	18	21
Frog larva extract	3	3	1	2	4	5	8	9	9	9	6	7
Meat fly larva extract	4*	4*	2	3	3	3	3	6*	4*	5*	2	2
Medusa extract	30*	32*	32*	32*	31*	33*	36*	40*	35*	39*	12	18
Juniper berries extract	25	27	26	27	29	33	39*	46*	49*	57*	17	20
Mistletoe extract	5	6	6	6	7	8	7	7	9	11	10	10
Genistein	17*	23*	17*	26*	23*	32*	29*	38*	44*	49*	6	6
Genistin	6*	6*	5*	7*	5*	6*	8*	8*	8*	9*	2	3
Glycitein	1	1	2	2	2	2	2	3	2	2	1	1
Glycitin	1	1	2	2	2	2	2	3	2	2	2	3
Daitzein	9*	10*	9*	11*	10*	13*	10*	16*	19*	21*	4	5
Daitzin	2	2	1	1	2	2	1	1	3	3	2	2
CsC1	38	40	44*	56*	44*	52*	53*	69*	65*	78*	26	30
CsC1+MgC1$_2$	38	42	46	58*	56	63	59*	74*	67*	81*	36	37
AzhexSi	8	10	11	11	15	23	19	37	20	40*	14	16
Azhex-AzhepSi	19	21	19	23	26	38*	29	38	30	38	18	21
Et AzhexAzhepSi	23	26	30	36	40*	44*	40*	43	48*	56*	20	26
AzhepSi	10	12	12	18	13	19	14	22	15	18	14	16
Et Azhepsi	19	20	14	19	23	30	24	32*	26*	38*	15	18
Hexamine	14	17	14	18	16	19	20*	21*	23*	27*	10	13
DL 54	12	15	12	13	12	14	16	18	19	24	14	17
Cisplatin chloride	24	26	27	27	48*	56*	55*	57*	59*	68*	25	27

CsCl - cesium chloride, MgCl$_2$ - magnesium chloride, *p<0.01 according to the control
± SEM values calculated between 5.21 and 5.58, C - control

Table 4 - Results of skin and eye irritant tests in the rabbits.

Agent	Skin irritant test	Eye irritant test
Tortoise shell extract	Mild irritant	Irritant
Spongia extract	Mild irritant	Mild irritant
Frog larva extract	Irritant	Irritant
Meat fly larva extract	Irritant	Irritant
Medusa extract	Irritant	Irritant
Juniper berries extract	Mild irritant	Irritant
Mistletoe extract	Mild irritant	Mild irritant
Genistein	-	-
Genistin	-	-
Glycitein	-	-
Glycitin	-	-
Daitzein	-	-
Daitzin	Irritant	Strongly irritant
Cesium	Irritant	Irritant
AzhexSi	Irritant	Irritant
Azhex-AzhepSi	Irritant	Strongly irritant
Et AzhexAzhepSi	Mild irritant	Mild irritant
AzhepSi	Irritant	Irritant
Et Azhepsi	Irritant	Irritant
Hexamine	Irritant	Irritant
DL 54	Irritant	Strongly irritant
Cisplatin chloride		

message systems or communications, and the calmodulsin receptor released the natrium at a higher level, and C-kinase and C-AMP message systems may have failed. Thus isotonic balance break down and the reproduction capacity of the cancer cells was inhibited at various levels. When some cancer cells were recultured after the killed cells did not reproduce. Cl^- and K^+ channels of these cells are disordered, thereby inducing the AVD during the apoptotic processes.[35-38] The persistent physical shrinkage induced by hyperosmotic stress leads to apoptosis in cancer cells.

Treatment for 48 hours with the animal and the vegetable extracts and the present chemicals resulted in significant reduction of mean cell volume in the cancer cells. Application for 48 hours of CsCl, $CsCl^+$ $MgCl_2$, isoflavonoids and dimethylsilane polyamines also induced shrinkage in all cancer cells. Within 48 hours after apoptogenic stimulation, cell volume distribution exhibited no additional apoptotic body formation. The last phases (G_2, M and G_0) cell shrinkage associated with apoptosis, termed AVD, was the most level inhibited by a Cl^- channel blocker, these blockers are agents in this research. These biological and chemical agents may have affected to block volume-sensitive Cl^- channels. When these apoptotic cancer cells were compared with that in the control cells, the death ratio of controls was very much less, but CsCl and $CsCl^+$ $MgCl_2$ have been affected at higher levels in the control cells. All of apoptotic events examined in the present study were found to be prevented by the present agents. Three mmol/ml of all agents exhibited an inhibitory effect on the cancer cells. A major hallmark of the programmed cell death is normotonic shrinkage cells. The most normal cells can regulate cell volume even under anisotonic conditions. The regular volume increase (RVI) caused osmotic swelling and shrinkage in the cancer cells. The AVD is known to be associated not only with significant reduction of the intracellular K^+ concentration but also with reduced Cl^-.[35-38]

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