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RESEARCH ARTICLE

SOY AND ITS ISOFLAVONES ANALYSED AS PREVENTIVE AND THERAPEUTIC EFFECT ON B16F10 TUMOR MODEL

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ABSTRACT

Study the therapeutic and preventive effects of soybean and its Isoflavones on B16F10 melanoma tumor in mice. Among various oral treatments, results indicated maximum regression in tumor formation in terms of the volume doubling time and growth delay for the therapeutic as well as preventive treatments. The difference for both the parameters were significantly high with ($P<0.001$) as compared to the control. The response of silent period for preventive effect was also highly significant ($P<0.001$). The long-term survival studies demonstrate that the mean survival time of animals kept on diet. Supplemented with full fat soy flour (FFSF) and Isoflavones was observed to be 39.25 days and 41.5 days respectively as against control (30.76days). The respective % increases in life span of mice were 33.33% and 41.37%. The result on the therapeutic effect of FFSF and isoflavones indicated the MST of 40 days and 44.9 days respectively as compared to control (28.2days while the respective % ILS was 41.38 days and 58.89 days. As against 29.2 days of MST for control group in case preventive effect, the MST of 38.33 days and 55.33 days. Both the group i.e. therapeutic and preventive, exhibited highly significant increase in the MST ($P<0.005$) as well as % ILS compared to control. It may be emphasized that more than 25% increase in life span of cancer patient is considered as effective antitumor response.

INTRODUCTION

Soybeans and soy foods contain a variety of bioactive components, including Saponins, protease inhibitors, phytic acid, and isoflavones. Isoflavones belong to a class of compounds generally known as phytoestrogens, plant compounds that have estrogen-like structures. The dominant isoflavones in soy is genistein, with daidzein and glycitein composing. [1] Within soy, isoflavones are almost entirely bound to sugars, producing the respective compounds genistein, daidzein, and glycitein.[2-3] Few study reported that when Genistein taken in conjunction with chemotherapy have shown to make chemo more efficient and work faster by helping the drugs kill the tumor cells or stop those tumor cells from dividing.[4-5]

There are several approaches to deal with established cancer like surgical excision, radiotherapy, chemotherapy, hyperthermia, photodynamic therapy and gene therapy, manipulation of immune system, inhibition of angiogenesis and stimulation of normal hematopoietic elements.[6] However the radiotherapy and chemotherapy are the most common methods used the cancer treatment, besides the surgery. Surgical resections of malignant tissues, radiotherapy and chemotherapy have develops as three solid columns for tumor therapy [7]. Despite many improvement and refinements of these therapy modalities, it had proved impossible to develop cures for a great number of individual malignancies [8]. Moreover many of the potent drugs are very expensive. Hence there is a need to find alternative drugs, which are effective at the non-toxic doses, inexpensive and acceptable to the common man [9]. Natural compounds may have in advantaged over these drugs because being constituents of living systems they may be less toxic and hence more acceptable for human application [10-11]. In this study soybean flour and pure soy-isoflavones were included in the diets in order to find the effect on soybean. USFEA has recently approved a health claim in favor of 25g intake of soy protein daily, due to nutritional and health benefits associated with soybeans consumption [12-17]. As there are to reports concerned so far on its antitumor property the present investigations plan for antitumor effect of his plant shows that this could be effective in the treatment of the cancer.

MATERIAL AND METHODS

First group was treated orally after tumor volume reaching upto100 mm after intradermal inoculation is called Tumor therapeutic effect, whether in case of the

Second group the oral treatment was started one month before the inoculation of tumor cell which is Tumor prevention effect.

Animal Model

CPCSEA registration number CPCSEA/a/500/2001and IAEC latter Reference number 186/Research/01, dated 21/07/05.The agouti strain (C57BL strain X Swiss albino) were selected from a random breed colony and maintained in the animal house Department of Research, Jawaharlal Nehru Cancer Hospital, and Research Centre, Bhopal, Madhya Pradesh, India. The mice were housed in polypropylene cages containing sterile paddy husk as bedding material and maintained under controlled conditions of temperature (23 ±2 0C), humidity (50 ± 5 %) and light (12h: 12 h of light dark respectively). The animals were fed standard mice feed and filtered acidified water ad labitum. Mice of either sex, 6 – 8 weeks old and weighing 23 ± 2 g were selected from the above colony for the experiments.

Preparations of reagents and solutions

A chemical has been purchased from Sigma U.S.A. Eagles, Minimum Essential Media, Methanol (Laboratory Grade), Trypan blue was dissolved in 100 ml of physiological saline (0.9%NaCl) and stored at the 40^{o}c

Tumor Transplantation: Melanoma B16F10 tumor model originally procured from Cancer Research Institute, Mumbai, India, was used in the study. These have since been propagated and maintained in adult agouti mice. Tumor-bearing mice were sacrificed by cervical dislocation and the whole animal was dipped in 70% alcohol. The tumor was dissected out and single cell suspension was prepared in phosphate buffered saline by mechanical dispersion. The cell suspension was filtered through a 45µ nylon mesh and centrifuged at 800 rpm for 5 min. The supernatant was discarded and the pellet suitably diluted. Prior to transplantation, a small portion of the tumor cell suspension was treated for microbial contamination (Department of Microbiology, JNCH&RC) and the only contamination free tumors were used for propagation and experiment.

Cell viability by Trypan blue exclusion test: Principle - Living cell membrane has the ability to prevent the entry of the dye in to the cell. Hence, the viable cells remain unstained and can be easily distinguished from the dead cells that take up the dye and appear blue under the light microscope.

Determination of the percent viable cells-

Equal volume of the cell suspension and 0.1% Trypan blue solution (dilution factor is 4 were mixed thoroughly. The diluted suspension was loaded into hemocytometer (Reichert, Buffalo, N.Y., U.S.A.). The viable and dead cells were counted separately in four WBC chamber under the light microscope and the mean number of cells was calculated.

Total number of cells was calculated as:

Total number of cells/ ml = Mean of (dead + viable cells) X Dilution factor x 10^4

Percent viable cells = (Total no. of cells - Dead cells)/100

Tumor volume Measurement:

Tumors were grown on the dorsal skin of healthy adult mice by intradermal inoculation of 5X 105 viable cells. Once a palpable tumor has developed (after 5-6 days), the diameter was measured in three perpendicular planes (D1, D2, D3) using a Vernier calipers. The tumor volume (V) was calculated using the formula V = π/6 D1 D2 D3.Tumors measuring 100 ±10 mm3 were used for the experiments [6]. Volume doubling time (VDT) is calculated as the time in days required for the tumor to attain double time treatment volume. VDT for each tumor was calculated from the growth curve. Growth delay (GD) is measured as the difference in time between the treated (T) and untreated (C) tumors to reach five times the treatment volume. GD=T-C. Body weight: The animal body weight was measured on alternate day [18-19].

Animal survival:

The animals were observed for 120 days or till death. The mean survival time for each group were calculated. Increase in life span and %T/C value was calculated by the Formula: Increase Life Span (%ILS) = (Mean Survival Time of treated group – Mean Survival Time of control group) × 100/ Mean Survival Time of control group.

Statistical analysis:

All the values were expressed as Mean ± SE. The data of the volume doubling time and mean survival time were statistically analyzed by Student's' test and data of growth delay were analyzed by one-way ANOVA using micrococal origin version 6.0, Graph Pad In-Stat (GPIS) statistical software, U.S.A, and Chi plot. $P < 0.05$ was considered to be significant.

Tumor growth measurements:

Once a palpable tumor developed, tumor diameters were measured using a Vernier caliper in three perpendicular plans ($D_1xD_2xD_3$), and the tumor volume (V) was calculated using the formula, V = π/6

$D_1xD_2xD_3$.Tumors measuring 100± 10 mm^3 were used for the experiments.

Experiment - I: **Tumor Therapeutic effect**

An agouti mouse has been treated after reaching tumor volume of 100 $mm^{3;}$ Control group has been treated with double distilled water for one month. Sham control: Induce tumor in animals, Second group: Soy diet Keep on soy supplements diet for a month and induce tumor and the Third group animals treated with Pure isoflavones, Keep on isoflavones fortified diet for a month and induce tumor continue with this diet 10 animals in each groups has been taken.

Experiment-II: Tumor preventive effect.

RESULTS

Tumor therapeutic effect of soy flour and isoflavones on melanoma

Volume Doubling Time

The volume doubling time for the control group was 1.31 days. When it was compared with the other treatment groups, an extremely significant increase in VDT was observed in Soy flour treated group (2.3 days $p<0.001$) and in isoflavones treated group (1.93) also it was significant ($p<0.05$) (Table 1).

Growth Delay

Both the treatments resulted in significant delay in tumor growth compared to control. The GD was 2.47 days in soy flour treated group, and 2.36 days in isoflavones treated group, which was very significant ($p<0.01$) compared to the control group. Comparison between soy flour and isoflavones group showed that the difference was significant ($p<0.05$) (Fig. 1)

Survival Analysis (Percentage Survival)

None of the treatment resulted in tumor free survival of animal up to 120 days. In tumors resulted with soy flour, the mice survival only up to 44 days. The mean survival and median survival times were calculated for the different treatment groups. The Percentage survival was plotted with Mean survival time.

Mean survival time

The MST was 28.6 days in control group and 40 days in soy flour treated group, which was 11.4 days higher than the control MST for isoflavones group was 44.9 days, which was 16.3 days more than in control. Inter group comparison revealed that response to isoflavones in terms MST was significantly higher ($p<0.05$).

Median survival time: (MdST)

MdST has been calculated for the soy flour (41 days) and isoflavones (45.5 days) treatments, which was significant ($p<0.05$) compared to the control. When soy flour

treated group was compared with isoflavones treated group, significant ($p<0.05$) increased in the survival has been observed.

Percent increase in life span: (I1.S %)

ILS was calculated from MST for different treatment groups. It was 41.37% for soy flour and 56.89% for isoflavones treatments.

EXPERIMENT II.

Tumor preventive effect of soy flour and isoflavones on melanoma

Silent period: The silent period (i.e. time taken for palpable growth) for the control group was found to be 7.6 days. In case of soy flour and isoflavones it was found to be 13.9 and 15.5 days, respectively and the delay in appearance of tumor was highly significant ($p<0.01$).The isoflavones produced not significant increase in silent period (Table 2)

Time taken to reach 100 mm

In control group the time taken to reach the 100 mm volume was found to be 3.06 days. The time taken in case of soy flour and isoflavones treated groups was found to be 3.98 days and 3.85 respectively. Comparison between control group and those treated with soy flour and isoflavones showed that the effect was significant ($p<0.05$).

Volume Doubling Time (VDT): The VDT for the control group was 1.31 days. When it was compared with the other treatment groups, an extremely significant increase in VDT was observed in soy flour treated group (11.94 days, $p<0.001$) and isoflavones treated group 1.8 5days very significant ($p<0.01$).Comparison between soy flour and isoflavones showed that the difference was very significant ($p<0.01$) (Table 2)

Growth Delay (GD): Both the treatments resulted in significant delay in tumor growth compared to control. The GD was 1.47 days in soy flour treated group, which was significant (($p<0.05$). Delay in growth was found to be 0.74 days in isoflavones treated group was very significant $p<0.01$). Comparison between soy flour and isoflavones showed that the difference was not significant (Fig. 2).

Survival Analysis (Percent Survival)

None of the treatments resulted in tumor free survival of animals up to 120days. Animals treated with soy flour survived only up to 40 days. The mean survival and median survival times were calculated for the different treatment groups. The Percentage survival was plotted with Mean survival time.

Mean survival Time: (MST)

The MST was 29.2 days in control group and 42 days in soy flour treated group, which was 12 days higher than in the control group MST for isoflavones group was 46.6 days, which was 19.4 days more than in control. Inter group comparison revealed that response to isoflavones in term MST was significantly higher ($p<0.05$) than that to Soy flour.

Median survival Time (MdST)

MdST has been calculated for the Soy flour and isoflavones treatments which was significant ($p<0.05$) compared to the control, soy flour 43days and isoflavones 48.6 days treated groups at the dose of 1g/kg and 0.88 mg/kg respectively. When isoflavones treated group was compared with isoflavones treated group, significant ($p<0.05$) increased in the survival has been observed.

Percent increase in life span (ILS%)

ILS percent was calculated from for different treatment groups. It was 43.33% for Soy flour and 55.33% for Isoflavones treatments.

Tumor growth studies revealed that both treatments were effective only in delaying the growth in tumor growth kinetics, but both treatments were not effective in prolonging the lifetime or enhancing the survival time. The Soy flour and isoflavones produced most effective increase in life span (41.37%, 56.89%). In long-term survival studies, none of the Soy flour treatments were effective in increasing MST and prolonging the % ILS and %T/C values.

Survival studies revealed both the treated groups were having effective results in prolonging the life span.

Similarly the %ILS was found most significantly increased when the dose of soy flour and isoflavones was increased from 1g/kg to 0.88mg/kg in *43.33%, 55.33% treated group.

A compound is most effective if it show T/C value >120. When T/C value was calculated for all the treatment groups it was found that only isoflavones treated groups were having effective % T/C value. Data indicated that the highest antitumor activity was obtained with the soy flour and isoflavones with the dose of 1g/kg, 0.88mg/kg. The antitumor activity was higher. It showed that the T/C value was markedly increased from 133%. The % T/C value was significant in soy flour and isoflavones treatment was as effective as the significant (139% 156% and 143%, 155% groups respectively.

DISCUSSION AND CONCLUSION

Melanoma therapeutic/preventive agents that belong to the phytochemical category are being investigated for their potential to stop the increasing trend of the disease. Isoflavones was shown, in association with inhibition of topoisomerase II and tyrosine kinases, to suppress growth and increase melanin content in several different human melanoma cell lines [19 20 21]. The up-regulation of the cyclin-dependent kinase inhibitor (cdki) p27 (KIP1) by genistein is likely responsible for the inhibition of cyclin-dependent kinase (cdk)-2, whereas p21 (CIP1) is dispensable for Genistein-induced G2 arrest [22 23]. Genistein-induced growth inhibition of melanoma cells might depend on cellular p53 content. Transfection studies showed that high levels of p53 make melanoma cells resistant to the growth inhibitory action of genistein. Induction of differentiation of melanoma cells by genistein is also regulated by cellular p53, as cells lacking p53 responded readily to genistein-induced dendrite-like structure formation [24]. It has been prove that genistein-induced cellular differentiation occurs through stabilization of protein-linked DNA strand breakage [26]. A correlation between the growth inhibitory activity of genistein and miR-27a has been reported. Functional assays revealed that the levels of miR-27a and its target gene ZBTB10 were significantly altered in genistein-treated melanoma cells [26]. Darbon et al. showed that genistein treatment resulted in arrest of melanoma cells in the G2 phase of the cell cycle, whereas daidzein, which lacks a hydroxyl group, induced accumulation of cells in the G1 phase. Genistein exerted this effect by impairing the Cdc25C-dependent tyrosine dephosphorylation of cdk-1, in addition to activating the checkpoint kinase (Chk)-2. Dietary supplementation with genistein and daidzein reduced tumor size and number as well as lung metastasis in mice injected with melanoma cells as per the study of Le 1999. In this study data also support the previous findings which are quite promising and open way for further investigation.

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EXPERIMENTAL RESULT

Experiment – I Tumor therapeutic effect

Table: 1. Effect of Soyflour and Isoflavones treatment (1gm/kg, 2mg/kg, oral Daily for 1 month) on tumor take in Hybrid mice B16F10 Melanoma cells.

S. No	Treatment groups	No. of animals	VDT (Days) (Mean ±SE)	GD (Days) (Mean ±SE)
1.	Control (DDW)	10	1.39±0.08	0
2.	Group I (Soya flour) 1gm/kg	10	2.3 ± 0.10^{a}	2.47 ± 0.34^{a}
	Group II (Isoflavones)0.88 mg/kg	10	1.93 ± 0.13^{b}	2.36 ± 0.24^{a}

a: p <0.05, b : p <0.01, c: p <0.001 compared to control; compared to Isoflavones

Exp. –II Tumor prevention effect

Table: 2. Effect of Soyflour and Isoflavones treatment (1gm/kg, 2mg/kg, orals Daily for 1 month) on tumor take in Hybrid mice B16F10 Melanoma cells.

S. No	Treatment groups	Silent Period (Mean ± SE)	Time taken to reach 100 mm^3 (Mean ±SE)	VDT (Days) (Mean ±SE)	GD (Days) (Mean ±SE)
1.	Control (DDW)	7.6 ± 0.163	3.71 ± 0.20	1.31± 0.12	0
2.	Group-I Soy flour 1g/kg	13.9 ± 0.31^{a}	3.98± 0.24	1.85 ± 0.23^{c}	1.452 ± 0.50^{a}
3.	Group-II Iso flavones 2mg/kg	15.5 ± 0.34^{a}	3.19 ± 0.10^{c}	1.64 ± 0.18^{c}	0.742 ± 0.10^{b}

a : p <0.05, b : p <0.01, c: p <0.001 compared to control; compared to Isoflavones

Fig1. Growth curve for B16F10 melanoma after treatment 1g/kg and 2mg/kg body Weight with soyflour and isoflavones of soybean.

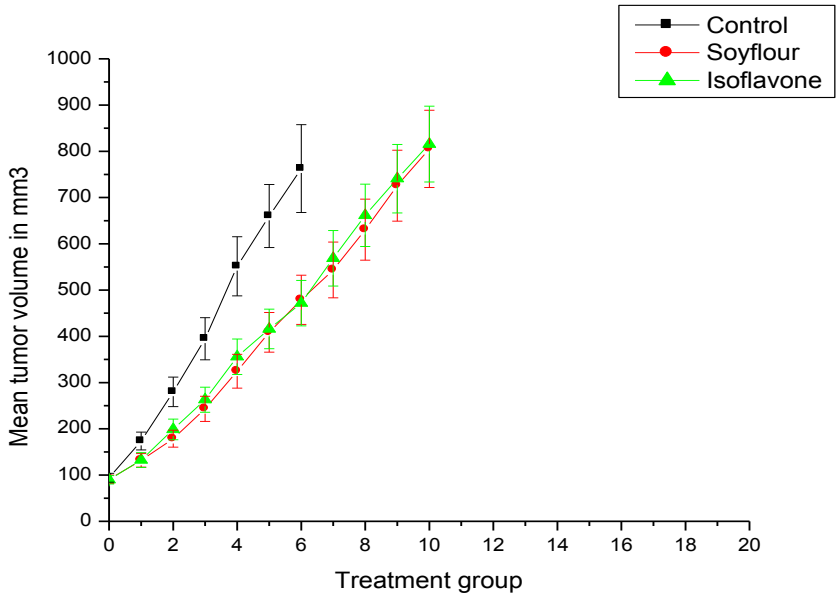

Fig2. Growth curve for B16F10 melanoma before treatment 1g/kg and 2mg/kg body Weight with soyflour and isoflavones of soybean.