CYTOTOXIC AND IMMUNOMODULATION EFFECT OF ROASTED FLAXSEED AND SOYBEAN EXTRACTS

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ABSTRACT

Research is focusing on the search for new types of natural chemotherapeutic agents derived from plant sources which are providing to be excellent sources of new compounds. Thus, Soybean \textit{(Glycine max (L))} and flaxseed \textit{(Linuim usitatissimum L.)} are known to have a number of health benefits ranging from the well documented many chronic diseases. The present research article was aimed to study the cell viability and cytotoxic activity of roasted form of soybean and flaxseed extracts. The \textit{in-vitro} methods used for the determination of cell viability by trypan blue dye exclusion method and cytotoxicity by MTT and SRB assay against MCF-7. The cell viability also showed significant result. The IC\textsubscript{50} value of roasted form of soybean and flaxseed are 125µg & 80µg, 135 µg & 140µg respectively in MTT and SRB assay whereas this extracts were found to be non-toxic to normal PBMC. It is proved that the extracts of roasted soybean and flaxseed exhibits significant anticancer potential non-toxicity on MCF-7 cell line.

KEY WORDS: soybean, flaxseed, MTT, SRB, PBMC and MCF-7 cell line.

INTRODUCTION

Immune system is a remarkably sophisticated defence system within vertebrates, to protect them from invading agents. It is able to generate varieties of cells and molecules capable of recognizing and eliminating limitless varieties of foreign and undesirable agents. Modulation
of the immune system denotes to any change in the immune response that can involve induction, expression, amplification or inhibition of any part or phase of the immune response. Thus, immunomodulator is a substance used for its effect on the immune system. There are generally of two types immunomodulators based on their effects immunosuppressants and immunostimulators. They have the ability to mount an immune response or defend against pathogens or tumors. (19) Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases. [3] The suppression of the immune system is characterised by reduction in the number and phagocytic function of the neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells. This immunosuppression allows opportunistic pathogens to overwhelm the host to cause secondary infections. [4] Activation of immune system by "non-self" antigen (alloantigen) or "self" antigen (auto antigen) is generally believed to require processing of the antigen by phagocytic cells such as macrophages, monocytes, or related cells [2]. This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs. [5]

Indian traditional systems of medicines like siddha and Ayurveda have suggested increasing the body’s natural resistance to disease (21). Many plants used in traditional medicines are reported to have immunomodulating activities. Some of these stimulate both humoral and cell mediated immunity while other activates only the cellular components of the immune system. i.e. phagocytic function without affecting the humoral or cell immunity (20). Recent screenings with plants have revealed many compounds (e.g. alkaloids, flavonoids, quinines, and terpenoids) with pronounced antioxidant, antineoplastic, antiulcer, anti-inflammatory and immunostimulating potential (18). There is a strong consensus that nutrition plays a role in modulating immune function and that the immune system needs adequate supply of nutrients to function properly. The complexity of the immune system supports this idea because its optimal functioning involves a variety of biological activities including cell division and proliferation, energy metabolism, and production of proteins. The micronutrients most often cited as being important to immune function include vitamins A, C, E, and B6, folate, iron, zinc, and selenium. Other nutrients mentioned as playing a role in immune function include beta-carotene (a precursor to vitamin A), vitamin B12, and vitamin D. On the other hand, over-activation of the immune system can lead to detrimental effects such as chronic inflammation or autoimmune diseases. Chemotherapeutic agents available today have mainly immunosuppressive activity. Most of them are cytotoxic and have various side effects. This
has led to the search for investigating natural resources showing immunomodulatory activity. Medicinal plants, which are used for their immunomodulatory effect, provide alternative potential to conventional chemotherapy for a variety of diseases, especially in relation to host defence mechanism. [15]

Immune functions are indispensable for defending the body against attack by pathogens or cancer cells, and thus play a pivotal role in the maintenance of health. However, the immune functions are disturbed by malnutrition, aging, physical and mental stress or undesirable lifestyle. Therefore, the ingestion of foods with immune-modulating activities is considered an efficient way to prevent immune functions from declining and reduce the risk of infection or cancer.

Several reports have shown that the improvement of depressed immune functions by ingesting foods reduced infection rates and mitigated the severity of infectious disease(17). Therefore, foods capable of enhancing the immune responses of cancer patients with disturbed immune functions are valuable(16). Moreover, the proliferation and metastasis of cancer cells accelerate when immune functions are disturbed. It has been found that cancer patients have lower NK cell activity than healthy controls and persons with lower NK cell activity are subject to higher rates of cancer incidence, metastasis and aggravation of cancer (18). Identification of medicinal plants with significant cytotoxic potential useful for the development of cancer therapeutics has gained increasing importance in the last decade, and research in this field is expanding(5). By virtue of the presence of physiologically active food components that may provide health benefits beyond basic nutrition, flaxseed and soybean are often grouped into one of several categories: “functional food”, “bioactive food” and an “endocrine active food” (Hasler et al., 2000).

Soybean (Glycine max(L)) and flaxseed (Linuim usitatissimum L.) are known to have a number of health benefits ranging from the well documented many chronic diseases(6). Soybeans are an important source of edible vegetable oil and protein throughout the world. Soybeans are used in a multitude of food and industrial applications. Several important nutrient, including vitamin-E, isoflavones, saponins and phytosterols are found in soybeans. Some of these compounds are known for health benefits (8). Flax is an important oilseed and its seeds are available source of many bioactive compounds (9). It is one of the richest sources of α-linolenic acid, an important source of high quality protein and soluble fibre (10). moreover, seeds contains phenolic compounds (13) such as lignans, phenolic acids.
Among the phenolic compounds, flax lignans are in focus because of their estrogenic and antioxidant activity (14). Therefore, flaxseed and soybean are used as a component of functional food (13). Recently flaxseed consumption may accord as effective radio protectors. The present study was therefore, undertaken to explore the cytotoxic and immunomodulatory effects of roasted flaxseed and soybean.

METHODS AND MATERIALS

Lymphocyte transformation test

The healthy peripheral blood was collected in a sterile syringe containing sufficient heparin to give a final concentration of 100 units/ml. Mononuclear cells were obtained by centrifuging (25 C, 400 g, 30 minutes) the mixture of blood and normal saline of v/v:1/1 on Ficoll-Hyapque gradients as described in the protocol (Sigma). Mononuclear cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G and 100 µg/ml streptomycin.

The experiments were conducted in test tube containing 0.05 ml of unfractioned peripheral blood mononuclear cell, 0.15ml of fetal calf serum and 0.75ml of RPMI 1640 and 0.05 ml (100 µg/ml) of soybean extract and flaxseed extracts were added in two separate experiments set. PHA was used as a positive control. The medium only with DMSO was used as a negative control. The plate was gently mixed, 200 µl per well of the mixture were added to a 96 well microtitre plate and incubated for 3 days at 37°C in a 5% CO₂ incubator. At the end of the incubation, 20ul per well of BrdU labelling solution were added and the culture plate was re-incubated for 24 hrs at 37°C. The plates were centrifuged at 300g for 10 minutes, the labelling medium was then removed while the cells were dried at 60°C for 1hr. 200ul per well of Fix Denat solution were added to the cells, which were then incubated for 90 minutes at room temperature. The wells were rinsed 3 times with 200ul per well of washing solution. After washing 100ul of substrate solution was added to each well followed by incubating the plate for 30 minutes. At the end of the incubation, 25ul per well of H₂SO₄ (1M) was added, the culture plate was further incubated in the shaker at 200 rpm for 1 minute. Absorbance of sample was determined at 450nm (test) and 690nm (reference) using an ELISA reader. the stimulation index was determined by the ratio of optical density our test samples to the optical density of negative control. Experiments were done in triplicates and results are expressed as mean±standard errors.
Trypan blue dye exclusion method (Boyum, 1968)

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

An aliquot of cell suspension being tested for viability was centrifuged for 5 minutes at 100 × g and discard supernatant. The size of the aliquot depends on the approximate number of cells present. The aliquot has contain a convenient number of cells to count in a hemocytometer when suspended in 1 ml phosphate buffer saline (PBS) and then diluted again by mixing with 0.4% trypan blue (5 ×10³ cells/ml). The cell pellet was suspended in 1 ml PBS or serum-free complete medium. One part of 0.4% trphan blue and one part cell suspension was allowed to incubate for three minutes at room temperature.

The cells were counted within 3-5 minutes of mixing with trypan blue. Mixing was performed in a microtitre plate contains 20µl of cell suspension and trypan blue and a drop of mixture was placed in a hemocytometer for counting using a binocular microscope. Unstained and stained cells were counted separately. The percentage of unstained cells was calculated that represent the percentage of viable cells.

Sulforhodamine B assay (Papazisis et al., 1997)

The assay relies on the ability of SRB (Sulforhodamine B) to bind protein components of cells that have been fixed to tissue culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass. 10 µl of test samples diluted in 10% (v/v) DMSO were added to each well of a 96-well tissue culture plate in triplicate. Negative control wells were provided only with DMSO and positive wells contain only 10µl doxorubicin diluted in 10% (v/v) DMSO. 190 µl of homogenous cell suspension (MCF-7 with appropriate cell seeding density) prepared prior were added to the assay wells filled with diluted samples in DMSO. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 72 hours.
Without removing the cell culture supernatant, 100µl cold TCA 10% (w/t) was added to each well and incubated at 4°C for one hour. 100 µl of 0.06 % (wt/vol) SRB solution was added to each well. Left at room temperature for 30 minutes and quickly rinsed four times with 1% (v/v) acetic acid to remove unbound dye. The plates were dried with a blow dryer at room temperature. 200 ml of 10mM Tris base solution (pH 10.5) was added and the plates were placed in a gyratory shaker for 5 minutes to solubilize the protein-bound dye. OD was measured at 510nm in a microtitre plate reader. The percentage of cell growth inhibition was calculated using the formulae

\[
\text{Mean OD}_{\text{sample}} - \text{Mean OD}_{\text{day 0}} \\
\frac{\text{Mean OD}_{\text{neg. control}} - \text{Mean OD}_{\text{day0}}}{\times 100}
\]

% of control cell growth = 

% growth inhibition = 100 - % of control cell growth

**MTT assay**

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma) assay as described by Mosmann (1983) with little modifications. The human breast cancer cell line (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). For maintenance, cultures were passaged weekly, and the culture medium was changed twice a week. The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x10^5 cells/ml. 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO2, 95 % air and 100 % relative humidity. After 24 hrs the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following drug addition, the plates were incubated for
an additional 48hrs at 37 °C, 5 % CO2, 95 % air and 100% relative humidity. The medium without samples were served as control and triplicates were maintained for all concentrations. After 48 hrs of incubation, 15 μl of MTT (5mg/ml) in phosphate buffered saline PBS) was added to each well and incubated at 37 °C for 4hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 μl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

\[
\% \text{ of cell inhibition} = \frac{1 - \text{Abs sample}}{\text{Abs control}} \times 100
\]

Nonlinear regression graph was plotted between % cell inhibition and Log10 concentration and IC<sub>50</sub> was determined using GraphPad Prism software.

**RESULTS AND DISCUSSION**

**Lymphocyte transformation test**
The results showed that, concentrations at 100µg/ml, both roasted soybean extract and flaxseed extracts showed significant stimulation through proliferation of human PBMC.

**Trypan blue dye exclusion method**
The cytotoxic effect of soybean and flaxseed extracts were determined using a cell viability assay. In this assay both the processed (roasted) seed extracts showed significant results than raw seed extracts of both soybean and flaxseed used in this study. The results are depicted in the following graph.
**Sulforhodamine B assay**

The toxicity screening of compounds selected for this study was performed using SRB assay. In this assay the roasted soy bean and flaxseed showed significant effect on cell lines by inhibiting cell growth. Whereas the raw soybean and flaxseed extracts has no significant cytotoxic effects on cell lines. The IC$_{50}$ values of roasted soybean and roasted flaxseed are 125µg and 80µg respectively. The results are depicted in the following graph.

![SRB assay graph](image)

**SRB assay**

A- Raw Soybean  
B- Raw Flaxseed  
SRO – Soybean roasted  
FROF– Flaxseed roasted

**MTT assay**

MTT assay is a rapid and high accuracy colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan. The cytotoxicity effect of flaxseed and soybean were investigated using MTT assay on human breast cancer cell lines (MCF-7). A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. Both the roasted soy bean and flaxseed showed significant cytotoxic effect on MCF-7 cell lines by inhibiting cell growth. Whereas the raw soybean and flaxseed extracts has no significant cytotoxic effects on cell lines. The IC$_{50}$...
values of roasted soybean and roasted flaxseed are 135µg and 140µg respectively. The results are depicted in the following graph.

**Effect of extracts on MCF-7**

![Graph showing effects of extracts on MCF-7](image)

A- Raw Soybean  
B- Raw Flaxseed  
SRO – Soybean roasted  
FROF– Flaxseed roasted

**CONCLUSION**

Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when host defence mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. There is great potential for the discovery of more specific immunomodulatory which mimic or antagonize the biological effects of cytokines and interleukins, and the refinement of assays for these mediators will create specific and sensitive screens. Natural remedies should be revisited as important sources of novel ligands capable of targeting specific cellular receptors.

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