Antioxidant, cytotoxicity, and anti-human lung cancer properties of Linum Usitatissimum seed aqueous extract in the in vitro condition: A pre-clinical trial study

Type
Research paper

Keywords
Cytotoxicity, Anticancer, Human lung cancer, Linum Usitatissimum seed, Chemotherapeutic supplement

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Introduction
Linum usitatissimum seed or flax seed is known as a potential candidate as a remedy to treat various diseases in many traditional medicines around the world. In the current study, the antioxidant, cytotoxicity, and anti-human lung cancer properties of Linum Usitatissimum seed were investigated in the in vitro condition.

Material and methods
Antioxidant activity of the plant was analyzed using radical scavenging activity and ferrous ion chelating assay. MTT assay was used to evaluation of anti lung cancer of the plant.

Results
The plant extract scavenged DPPH as a free radical with an IC50 of 34.2±0.9 µg/mL. The plant also was rich in phenolic compounds with an amount of 294.8±2.3 mg GAE/g for total phenolic content. Cell viability of Linum Usitatissimum seed was very low against lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines without any cytotoxicity on the normal cell line. The best anti-human lung cancer properties of Linum Usitatissimum seed against the above cell lines was in the case of the PC-14 cell line. According to the above findings, the Linum Usitatissimum seed may be administrated for the treatment of several types of human lung cancer in humans.

Conclusions
According to the results, the IC50 values of plant extract against lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines were found 392, 483, and 564 µg/mL, respectively.
Antioxidant, cytotoxicity, and anti-human lung cancer properties of *Linum usitatissimum* seed aqueous extract in the *in vitro* condition: A pre-clinical trial study

Abstract

*Linum usitatissimum* seed or flax seed is known as a potential candidate as a remedy to treat various diseases in many traditional medicines around the world. In the current study, the antioxidant, cytotoxicity, and anti-human lung cancer properties of *Linum usitatissimum* seed were investigated in the *in vitro* condition. Antioxidant activity of the plant was analyzed using radical scavenging activity and ferrous ion chelating assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to evaluation of anti-lung cancer of the plant. The plant extract scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical with an IC$_{50}$ of 34.2±0.9 µg/mL. The plant also was rich in phenolic compounds with an amount of 294.8±2.3 mg GAE/g for total phenolic content. Cell viability of *Linum usitatissimum* seed was very low against lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines without any cytotoxicity on the normal cell line. The best anti-human lung cancer properties of *Linum usitatissimum* seed against the above cell lines was in the case of the PC-14 cell line. According to the above findings, the *Linum usitatissimum* seed may be administrated for the treatment of several types of human lung cancer in humans. According to the results, the IC$_{50}$ values of plant extract against lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines were found 392, 483, and 564 µg/mL, respectively.

**Keywords:** *Linum usitatissimum* seed; Anticancer; Cytotoxicity; Human lung cancer; Chemotherapeutic supplement.
1. Introduction

There are some organs in the body with clear functions. The lung is the most important organ in respiratory gas transformation such as CO₂ and O₂ [1]. Cancer, emphysema, tuberculosis, pulmonary hypertension, COPD, cystic fibrosis, bronchitis, allergies, tuberous sclerosis, flu, pneumonia, and asthma are the main diseases of the lung. The common sign of lung adenocarcinoma are dysphagia, breath shortness, wheezing, coughing up blood, weight loss, chest pain, fatigue, weakness, shoulder pain, cough, and hoarseness. The lung adenocarcinoma symptoms are blurred vision, headaches, seizures, and weakness [1-3]. Of course the familial predisposition, air pollution, exposure to radon gas, diesel exhaust, asbestos fibers, smoking, and radiation therapy increase the occurrence of lung adenocarcinoma [3]. Radiation therapy, targeted therapy, immunotherapy, chemotherapy, and surgery are the therapeutical options of the lung adenocarcinoma [4]. Due to the high side effects of chemotherapy, the researchers are following new formulations such as herbal medicine to treat lung adenocarcinoma [5].

Science history is an enticing field of humanity's interdisciplinary knowledge and study. Medical science is one of the interesting fields of science history all of the world, as the ancient civilized nation, has a long medical history with world-famous citizens [6]. From ancient times and when man entered the world, he always tried to strive for a better livelihood and meet his needs. In this regard, gaining valuable experiences created only by chance has led to the use of nature around them to improve life for consecutive years [6,7]. The most valuable experience that is now a relic of the ancients and the wealth gained from them by modern man is plants as the most natural substances around him for the treatment and even prevention of diseases, which of course is easier than cure [8,9]. The science of using medicinal plants is one of the most important medical sciences in the world and its importance was such that some countries tried to plant and harvest some of the most important ones [6,7]. Today, despite the high volume of chemical products with chemical sources, as well as the occurrence of various and incurable diseases, as well as the rapid treatment of diseases with synthetic drugs that reduce the pain and suffering of the disease, replacing medicinal plants for their treatment, which has an almost longer treatment process than chemical drugs, seems difficult and even unlikely [8-10]. Ethnomedicinal herbs as a source of necessary chemical compositions gained much attention to treat, control, and prevent many ills and promoting body health [11, 12]. Many plants are used for their antibacterial property [13, 14]. Due to the current progression in the herbs extraction methodology, ethnomedicinal plants are extracted in various sorts [15, 16]. One of the medicinal plant compound extraction methods is an aqueous extract [7, 8]. In the current years, interest in the aqueous extract has been incremented for the pharmacological experiments and it appears that the aqueous extract has been useful to treat, control, and prevent animal and human bacterial infections [9-11].

In recent years, it has been shown that traditional medicine herbs play an important role in the prevention and treatment of various cancers. Some of these plants are used directly to treat cancer, and some reduce the toxic effects of chemotherapy drugs [4-6]. One of these plants is Linum usitatissimum. It is an annual, diploid, herbaceous plant with erect stems and lanceolate leaves with blue petals. Linum usitatissimum seeds are available
in two colors, brown and golden yellow. This plant is cultivated every year in a large number of countries in the United States, Argentina, Uruguay, India, Austria, Hungary, China, etc. It is also considered native to the Middle East and has grown as a plant in hot and dry climates of Iran [12-14]. *Linum usitatissimum* seed oil varies between 42-59%. *Linum usitatissimum* seed dry matter decomposition shown; it contains 41% fat, 28% dietary fiber, 21% protein, 4% ash and 6% other carbohydrates such as sugar, phenolic acid, lignan and hemicellulose. *Linum usitatissimum* seed is a valuable source of phenolic and antioxidant compounds and one of the richest sources of the healing chemical is α-linolenic acid. *Linum usitatissimum* seed has the most appropriate ratio of omega-3 and omega-6 fatty acids [15-17]. This seed contains a type of soluble fiber called mucilage. Of course, most plants contain the chemical Lignan, but *Linum usitatissimum* seed contains about 75 times more of this healing chemical than any other plant. The experiments measuring phenolic compounds, flavonoids and the percentage of free radical scavenging confirmed the existence of high amounts and inhibitory power of these compounds in oily *Linum usitatissimum* seed extract [16,17]. The presence of phenols, flavonoids, saponins, tannins, terpenoids, proteins, cardiac glycosides, and functional groups in *Linum usitatissimum* seed were confirmed [15-17]. *Linum usitatissimum* seed reduces the growth of existing tumors, and other chemical compounds in *Linum usitatissimum* seed called Lignans appear to prevent the formation of new tumors [12]. Lignans are plant compounds that inhibit the activity of estrogen in cells and reduce the risk of some cancers. The highest content of *Linum usitatissimum* seed lignan is SDG, which is converted to the biologically active lignans of introdiol and interlactone by bacteria in the large intestine of humans and other animals [18,19]. The structure of interodiol and interlactone resembles an endogenous estrogen. This similar structure gives them the ability to bind to estrogen receptors and exhibit anti-cancer and antioxidant activity. The α-linolenic acid in *Linum usitatissimum* seed protects against the formation of cancer clones due to its high antioxidant activity [12,18,19].

In the recent study, we determined anti-lung adenocarcinoma effects of *Linum usitatissimum* seed aqueous extract against PC-14, LC-2/ad, and HLC-1 cell lines.

2. Experimental

2.1. Preparation of the plant extract

The seeds of *Linum usitatissimum* were ground and macerated in ethanol:water (70:30) for 48 h. Next, the solvent was evaporated using a Heidolph evaporator (50 °C). Then the obtained extract was dried under a hood.
2.2. Determination of total phenolic content (TPC)

Mohsen Abadi et al. methods were run to evaluate the total phenolic content (TPC), total flavonoid content (TFC), radical scavenging activity (RSA) and ferrous ion chelating (FIC) of the plant extract [20].

A 1 mL of Folin-Ciocalteu’s reagent (10% in distilled water) was added to 1 mL of the plant extract (100 µg/mL in methanol) and 3 mL of distilled water. After 10 min, 4 mL of Na₂CO₃ (5%) was added and shaken vigorously. The reaction mixture was put in a dark place for 2 h at room temperature. The absorbance was read at 760 nm using a Cary 50 UV-Vis. instrument. The analyses were repeated for three times. The extract TPC was measured according mg GAE/g extract (GAE), that is, mg of gallic acid equivalent per gram of dried extract.

2.3. Determination of total flavonoid content (TFC)

A 1 mL of AlCl₃ in methanol (2%) was poured to 2 mL of the plant extract solution (100 µg/mL). The mixture was kept at room temperature for 30 min. Next, the absorbance was read at 415 nm. The analyses were carried out for triplicates. A standard curve rutin was used to calculate the extract TFC in terms of mg RuE/g extract.

2.4. Determination of radical scavenging activity (RSA)

A 3 mL of the extract in methanol (20-100 µg/mL) was added to 2 mL of DPPH (0.1 mM). Then, the reaction mixture was stirred and kept in a dark place for 1.5 h. Next, the optical density was read at 517 nm. The result was compared to the positive controls of butylated hydroxytoluene (BHT) and α-tocopherol (Toc). The assay was run in triplicates. The following equation was used to calculate of the RSA:

\[
RSA\% = \left(\frac{A_c - A_s}{A_c}\right) \times 100
\]

A_c for the control (DPPH solution without extract) absorbance; A_s for the extract absorbance (extract with DPPH solution).

2.5. Ferrous ion chelating ability assay

A 200 µL ferrozine (5 mM) was added to 100 µL of FeSO₄ (2 mM), 1 mL of the plant extract solution in methanol (80-320 µg/mL), and 2 mL distilled water. The reaction mixture was vibrated and incubated at room temperature for 10 min. The mixture absorbance was analyzed at 562 nm. All measurements were carried out
for three times. EDTA and AscA (ascorbic acid) were used as the positive controls. The following equation was used to express the plant extract FIC:

$$\text{% Inhibition} = \left[ \frac{(A_c - A_s)}{A_c} \right] \times 100$$

$A_c$ for the control (contains FeSO$_4$, ferrozine, and water) absorbance, and $A_s$ for the sample absorbance.

2.6 Determination of anti-human lung adenocarcinoma effects of *Linum usitatissimum* seed

In this assay, different human lung cancer cell lines i.e., lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines and also the normal cell line (HUVEC) were used to study the cytotoxicity and anticancer potential of human lung over the *Linum usitatissimum* seed aqueous extract using the common cytotoxicity test i.e., MTT assay. 15 mL of RPMI 1640 medium containing 10% FSC (10 mg/mL penicillin and 100 mg/mL streptomycin) in a culture flask, placed in a CO$_2$ incubator for 2 hours to equilibrate the medium. Under safe conditions (using insulated gloves and goggles) the frozen cell vial was removed from the nitrogen storage tank. To avoid the possibility of explosion of the vial (due to the possible entry of liquid nitrogen into the vial), loosen the lid, after disinfecting the outer surface of the vial with 70% alcohol, under the hood to remove nitrogen gas. Close the vial lid again and immediately melt it in a pan at 37 °C. The melting process should be completed in about 1 minute and the cells should be avoided from overheating. The medium was added dropwise to the vial and then its contents were taken out and centrifuged with the medium in 15 cc sterile test tubes. After centrifugation, the supernatant was removed and the cells were suspended again in the medium and transferred to a pre-prepared flask containing the medium and FBS and incubated [5].

Cell line used in RPMI 1640 medium containing penicillin (100 IU / ML), streptomycin (100 IU / ML), glutamine (2 mmol) and 10% fetal bovine serum (FBS). They were incubated at 37 °C and in an atmosphere containing 0.5 CO$_2$. Cells began to grow in 75 cm$^2$ T-flasks in 15 mL medium with an initial number of $1-2 \times 10^6$ cells. After three days and covering the flask bed with the cell, the adhesive layer to the bottom of the flask was separated enzymatically using trypsin-verson and transferred to a sterile test
tube for 10 minutes at 1200 rpm. The cells were then suspended in a fresh culture medium with the help of a Pasteur pipette and the suspension was poured into 100-well plate flat wells (for cell culture) using an 8-channel sampler of 100 µl. One column of wells was kept cell-free and as a plank containing only culture medium. In another column, it was considered to contain culture medium and healthy cells and in other columns, it was considered to contain culture medium and cell line cells. One of these columns, which contained culture medium and cells and did not contain *Linum usitatissimum* seed aqueous extract, was considered as a control [5,6].

The plates were incubated in the incubator for 24 hours to return the cells to normal from the stress of trypsinization. After this time, suitable dilutions of the prepared *Linum usitatissimum* seed aqueous extract (0-1000 µl / ml) and 100 µl of each dilution were added columnar to the plate wells (Thus, the final concentration of the studied compound in the wells was halved. Therefore, the concentrations were prepared twice as much to reach the final concentration after being added to the well). The cells were incubated for 37 hours at 37 °C and 5% CO₂ in the atmosphere. After 72 hours, 20 µl of MTT solution (5 mg/ml) was added to each well. The plates were incubated for 3 to 4 hours and then the residue was removed and 100 µl of DMSO was added to each well to dissolve the resulting formazan. After 10 minutes, using shaking the plates, the optical absorption of Formazan at 570 nm was read using a plate reader. Wells containing cells without *Linum usitatissimum* seed aqueous extract were considered as control and the optical density of wells without cells and only culture medium were considered as blank. The percentage of cell viability was calculated using the following formula [5,6]:

\[
\text{Cell viability (\%)} = \frac{\text{Sample A.}}{\text{Control A.}} \times 100
\]

The closer is the obtained value to the IC50 of *Linum usitatissimum* seed aqueous extract, the stronger is the cell viability activity of the material. The graph of the IC50 of the *Linum usitatissimum* seed aqueous extract was produced by drawing the percent inhibition curve versus the *Linum usitatissimum* seed aqueous extract concentration. First, three stock samples with variable concentrations (0-1000 µg/mL) of *Linum*
*Linum usitatissimum* seed aqueous extract were prepared. Then, a serial dilution was prepared from each sample, and IC50 of the above samples was measured separately, following which their mean was calculated [6].

### 2.7 Qualitative Measurement

The obtained results were loaded into the “SPSS-22” program and evaluated by “one-way ANOVA”, accompanied by a “Duncan post-hoc” check ($p \leq 0.01$).

### 3. Results and Discussion

#### 3.1. Antioxidant activity

The antioxidant activity result of the plant extract is tabulated in Table 1. According to the results the extract was rich in phenolic compounds with TPC of 294.8±2.3 mg GAE/g. The value of 53.7±1.2 mgRuE/g was measured for the plant TFC. **These results are less than that of Zhou et al report [21].** The plant extract scavenged the free radical of DPPH with IC50 of 34.2±0.9 μg/mL, which is less than BHT as a positive control. Zhou et al have reported more radical scavenging activity for *Linum usitatissimum* seed extract with 19.3±1.1 μg/mL[21]. However, 49.50 μg/mL was reported in another previous study [22]. The chelating activity of the plant extract was measured for IC50 of 234.5±1.4μg/mL, which was less than Zhou et al study[21].

**Table 1:** Total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity (RSA), and ferrous ion chelating ability (FIC) of *Linum usitatissimum* seeds extract.

<table>
<thead>
<tr>
<th></th>
<th>TPC (mg GAE/g extract)</th>
<th>TFC (mgRuE/g extract)</th>
<th>RSA IC50 (μg/mL)</th>
<th>FIC IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Linum usitatissimum</em> seeds extract</td>
<td>294.8±2.3</td>
<td>53.7±1.2</td>
<td>34.2±0.9</td>
<td>234.5±1.4</td>
</tr>
<tr>
<td>BHT</td>
<td>-</td>
<td>-</td>
<td>23.9±1.2</td>
<td>-</td>
</tr>
<tr>
<td>TOC</td>
<td>-</td>
<td>-</td>
<td>46.2±2.6</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>59.2±1.5</td>
</tr>
<tr>
<td>AscA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1247.2±2.8</td>
</tr>
</tbody>
</table>
Values are presented as means ± SD (n = 3).

3.2. Investigation of anti-human lung cancer effects of *Linum usitatissimum* seed

The MTT assay is a procedure of colorimetric based on reducing and breaking of yellow tetrazolium crystals by the enzyme succinate dehydrogenase to form insoluble purple crystals. In this method, unlike other methods, the steps of washing and collecting cells, which often cause the loss of a number of cells and increase the work error, have been eliminated and all test steps from the beginning of cell culture to reading the results with a photometer are performed on a microplate, so the repeatability, accuracy and sensitivity of the test are high [7-10]. If the test is performed on cells attached to the plate, an appropriate number of cells (about 2,000 cells) must first be cultured in each of the wells. Then we select the control and test wells and add the appropriate amount of mitogen or drug to the test wells and place the plate in the incubator for the required time so that the desired substance affects the cells [11]. At the end of the incubation time, discard the supernatant and add 200 μl of culture medium containing half an mg/mL of MTT solution to each well and put it again in a carbon dioxide incubator for 2 to 4 hours at 37 °C. During incubation, MTT is regenerated by one of the enzymes of the mitochondrial respiratory cycle i.e., succinate dehydrogenase. The regeneration and breakage of this ring produce purple-blue crystals of formazan that are easily detectable under a microscope. At the end, the optical absorption of the resulting solution can be read at 570 nm and the cells number can be calculated using a standard curve. For each cell line, there is a linear relationship between the number of cells and the light absorption of the final solution. Therefore, to examine each cell type, a standard curve related to the same cell line must be drawn and used [10,11].

In the present study, the cytotoxicity of *Linum usitatissimum* seed was explored by studying its interaction with normal (HUVEC), lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines by MTT assay for 48h.

The interactions being expressed as cell viability (%) was observed at different *Linum usitatissimum* seed concentrations (0-1000 μg/mL) with the four cell lines which have been shown in Figure 1. In all the cases the %
cell viability gets reduced with increasing *Linum usitatissimum* seed concentrations. The IC\textsubscript{50} values of *Linum usitatissimum* seed against lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines were found 392, 483, and 564 µg/mL, respectively (Table 2).

The best cytotoxicity results and anti-human lung cancer potentials of our *Linum usitatissimum* seed was observed in the case of the PC-14 cell line.
Fig. 1. The anti-human lung cancer properties (Cell viability (%)) of *Linum usitatissimum* seed (Concentrations of 0-1000 µg/mL) against lung well-differentiated bronchogenic adenocarcinoma (HLC-1: A), lung moderately differentiated adenocarcinoma (LC-2/ad: B), lung poorly differentiated adenocarcinoma (PC-14: C), and HUVEC (D) cell lines.

The numbers indicate the percent of cell viability in the concentrations of 0-1000 µg/mL of *Linum usitatissimum* seed against several human lung cancer cell lines.

### Table 2. The IC50 of *Linum usitatissimum* seed in the anti-human lung cancer test.

<table>
<thead>
<tr>
<th></th>
<th>HLC-1</th>
<th>LC-2/ad</th>
<th>PC-14</th>
<th>HUVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (µg/mL)</td>
<td>564±0⁷</td>
<td>483±0⁶</td>
<td>392±0⁸</td>
<td>-</td>
</tr>
</tbody>
</table>

Oxidation from reactive oxygen species can cause cell membrane disintegration, damage to membrane proteins, and DNA mutation that result is the onset or exacerbation of many diseases such as cancer, liver damage, and cardiovascular disease. Although the body has a defense system, constant exposure to chemicals and contaminants can lead to an increase in the number of free radicals outside the body's defense capacity and irreversible oxidative damage [7-9]. Therefore, antioxidants with the property of removing free radicals play an important role in the prevention or treatment of oxidation-related diseases or free radicals. Extensive molecular cell research on cancer cells has developed a targeted approach to the biochemical prevention of cancers that the goal is to stop or return cells to their pre-cancerous state without any toxic doses through nutrients and drugs. Numerous studies have been performed on using natural compounds as anti-cancer agents in relation to appropriate antioxidant activity [10,11]. It seems the high anti-lung adenocarcinoma properties of *Linum usitatissimum* seed aqueous extract are related to its antioxidant activities.

4. Conclusions
Linum usitatissimum seeds extract was rich in phenolic compounds and a potent herbal product to scavenge free radicals of DPPH. The Linum usitatissimum seed was also assessed in biological applications like radical scavenging and anticancer (adenocarcinoma) activities. The Linum usitatissimum seed exhibited good antioxidant properties, even better than the reference standard molecule. It also showed significant cytotoxic activities against common human lung cancer cell lines i.e., lung poorly differentiated adenocarcinoma (PC-14), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung well-differentiated bronchogenic adenocarcinoma (HLC-1) cell lines.

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