Antioxidant, cytotoxicity, and anti-human lung cancer properties of *Linum usitatissimum* seed aqueous extract in *in vitro* conditions: a pre-clinical trial study

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Submitted: 16 April 2021; Accepted: 3 May 2021

Arch Med Sci
DOI: https://doi.org/10.5114/aoms.136340
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Abstract

**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 *in vitro* conditions.

**Material and methods:** 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 evaluate anti-lung cancer properties of the plant.

**Results:** The plant extract scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical with an IC₅₀ of 34.2 ±0.9 µg/ml. The plant was also found to be rich in phenolic compounds with 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 towards the normal cell line. The best anti-human lung cancer properties of *Linum usitatissimum* seed against the above cell lines were observed in the case of the PC-14 cell line. According to the above findings, *Linum usitatissimum* seed may be administered for the treatment of several types of human lung cancer in humans. 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 to be 392, 483, and 564 µg/ml, respectively.

**Conclusions:** It appears the recent formulation can be used as a novel chemotherapeutic supplement in humans.

**Key words:** *Linum usitatissimum* seed, anticancer, cytotoxicity, human lung cancer, chemotherapeutic supplement.
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, Chronic obstructive pulmonary disease (COPD), cystic fibrosis, bronchitis, allergies, tuberous sclerosis, influenza, pneumonia, and asthma are the main diseases of the lung. The common signs of lung adenocarcinoma are dysphagia, shortness of breath, wheezing, coughing up blood, weight loss, chest pain, fatigue, weakness, shoulder pain, cough, and hoarseness. Lung adenocarcinoma symptoms are blurred vision, headaches, seizures, and weakness [1–3]. Of course, 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 therapeutic options for lung adenocarcinoma [4]. Due to the high side effects of chemotherapy, researchers are studying 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 throughout the world, as the ancient civilized nations have a long medical history with world-famous citizens [6]. From ancient times and when man entered the world, he always strove for a better livelihood to meet his needs. In this regard, gaining valuable experiences created only by chance has led to the use of nature 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, and 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 a treatment process almost as long as 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 promote body health [11, 12]. Many plants are used for their antibacterial properties [13, 14]. Due to the current progression in the methodology of herbal extraction, ethnomedicinal plants are extracted in various sorts [15, 16]. One of the medicinal plant compound extraction methods is aqueous extraction [7, 8]. In recent years, interest in aqueous extracts for pharmacological experiments has increased, and it appears that the aqueous extracts have 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 including the United States, Argentina, Uruguay, India, Austria, Hungary, and China. 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 content varies between 42% and 59%. Linum usitatissimum seed dry matter decomposition showed that 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 -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. Experiments measuring phenolic compounds, flavonoids and the percentage of free radical scavenging confirmed the existence of large amounts and high inhibitory power of these compounds in Linum usitatissimum seed oil extract [16, 17]. The presence of phenols, flavonoids, saponins, tannins, terpenoids, proteins, cardiac glycosides, and functional groups in Linum usitatissimum seed was 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 lignans with the highest content in Linum usitatissimum seeds are derivatives of secoisolariciresinol diglucoside (SDG), which is converted to the biologically ac-
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The seeds of \textit{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.

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

**Material and methods**

**Preparation of the plant extract**

The seeds of \textit{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.

**Determination of total phenolic content (TPC)**

The methods of Mohsen Abadi et al. were used 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].

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 Na$_2$CO$_3$ (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 three times. The extract TPC was measured according to mg GAE/g extract (GAE), that is, mg of gallic acid equivalent per gram of dried extract.

**Determination of total flavonoid content (TFC)**

1 ml of AlCl$_3$ in methanol (2%) was poured into 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 in triplicate. A standard curve of rutin was used to calculate the extract TFC in terms of mg RuE/g extract.

**Determination of radical scavenging activity (RSA)**

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 triplicate. The following equation was used to calculate the RSA: RSA% = \((\frac{A_c - A_s}{A_c}) \times 100\). $A_c$ is the control (DPPH solution without extract) absorbance; $A_s$ is the extract absorbance (extract with DPPH solution).

**Ferrous ion chelating ability assay**

200 µl ferrozine (5 mM) was added to 100 µl of FeSO$_4$ (2 mM), 1 ml of the plant extract solution in methanol (80–320 µg/ml), and 2 ml of 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 three times. EDTA and AsCa (ascorbic acid) were used as the positive controls. The following equation was used to express the plant extract FIC: % Inhibition = \((\frac{(A_s - A_i)}{A_s}) \times 100\). $A_i$ is the control (contains FeSO$_4$, ferrozine, and water) absorbance, and $A_s$ is the sample absorbance.

**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 a normal cell line (HUVEC), were used to study the cytotoxicity and anti-human lung cancer potential of the \textit{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 was placed in a CO$_2$ incubator for 2 h 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 min and the cells should be prevented from overheating. The medium was added dropwise to the vial and then its contents were taken out and centrifuged with the medium in 15 ml 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]. The cell lines were cultured 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<sub>2</sub>. Cells began to grow in 75 cm<sup>2</sup> T-flasks in 15 ml medium with an initial number of 1–2 × 10<sup>6</sup> cells. After three days and covering the flask bed with the cell, the adhesive layer on the bottom of the flask was separated enzymatically using trypsin-panver and transferred to a sterile test tube for 10 min 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 blank containing only culture medium. Another column was considered to contain culture medium and healthy cells and other columns were 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 h 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 µg/ml) and 100 µl of each dilution were added in columns to the plate wells (thus, the final concentration of the studied compound in the wells was halved. Therefore, the concentrations were prepared twice as high to reach the final concentration after being added to the well). The cells were incubated for 37 h at 37°C and 5% CO<sub>2</sub> in the atmosphere. After 72 h, 20 µl of MTT solution (5 mg/ml) was added to each well. The plates were incubated for 3 to 4 h and then the residue was removed and 100 µl of DMSO was added to each well to dissolve the resulting formazan. After 10 min, using shaking of 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 a control and the optical density of wells without cells and only culture medium was considered as a blank. The percentage of cell viability was calculated using the following formula [5, 6]:

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

The closer the obtained value is to the IC<sub>50</sub> of Linum usitatissimum seed aqueous extract, the stronger is the cell viability activity of the material. The graph of the IC<sub>50</sub> 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 usitatissimum seed aqueous extract were prepared. Then, a serial dilution was prepared from each sample, and IC<sub>50</sub> of the above samples was measured separately, following which their mean was calculated [6].

Qualitative measurement

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

Results and discussion

Antioxidant activity

The antioxidant activity results of the plant extract are tabulated in Table I. 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 mg RuE/g was measured for the plant TFC. These results are less than those of the Zhou et al. report [21]. The plant extract scavenged the free radical of DPPH with IC<sub>50</sub> of 34.2 ±0.9 µg/ml, which is less than BHT as a positive control. Zhou et al. 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

| Variable       | TPC [mg GAE/g extract] | TFC [mg RuE/g extract] | RSA IC<sub>50</sub> [µg/ml] | FIC IC<sub>50</sub> [µg/ml] |
|----------------|------------------------|------------------------|----------------------------|-----------------------------|
| Linum usitatissimum seed extract | 294.8 ±2.3 | 53.7 ±1.2 | 34.2 ±0.9 | 234.5 ±1.4 |
| BHT            | –                      | –                      | 23.9 ±1.2                  | –                           |
| TOC            | –                      | –                      | 46.2 ±2.6                  | –                           |
| EDTA           | –                      | –                      | –                         | 59.2 ±1.5                   |
| AscA           | –                      | –                      | –                         | 1247.2 ±2.8                 |

Values are presented as means ± SD (n = 3).

Table I. Total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity (RSA), and ferrous ion chelating ability (FIC) of Linum usitatissimum seeds extract
of the plant extract was measured for IC$_{50}$ of 234.5 ±1.4 µg/ml, which was less than in the Zhou et al. study [21].

Investigation of anti-human lung cancer effects of Linum usitatissimum seed

The MTT assay is a colorimetric procedure 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, the supernatant is discard and 200 µl of culture medium containing 0.5 mg/ml of MTT solution is added to each well and it is placed again in a carbon dioxide incubator for 2 to 4 h 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 cell number

Figure 1. 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.
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 48 h. The interactions expressed as cell viability (%) were observed at different *Linum usitatissimum* seed concentrations (0–1000 µg/ml) with the four cell lines which are shown in Figure 1. In all the cases the % cell viability decreased with increasing *Linum usitatissimum* seed concentrations. The IC₅₀ 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 392, 483, and 564 µg/ml, respectively (Table II).

The best cytotoxicity results and anti-human lung cancer potential of our *Linum usitatissimum* seed were observed in the case of the PC-14 cell line. 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 with the goal of stopping or returning 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.

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

|       | HLC-1 | LC-2/ad | PC-14 | HUVEC |
|-------|-------|---------|-------|-------|
| IC₅₀ [µg/ml] | 564 ±0 | 483 ±0 | 392 ±0 | –     |

In conclusion, *Linum usitatissimum* seed extract was found to be 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 such as 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.

**Acknowledgments**

The authors extend their appreciation to the deanship of Scientific Research at King Khalid University, Abha, KSA for supporting this work under grant number R.G.P.2/122/42, and the work was supported by the Taif University Researchers Supporting Project Number (TURSP-2020/99), Taif University, Taif, Saudi Arabia.

**Conflict of interest**

The authors declare no conflict of interest.

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