Journal of Traditional and Complementary Medicine 8 (2018) 89–94

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original Article

The effect of ethanolic extract of *Thymus kotschyanus* on cancer cell growth in vitro and depression-like behavior in the mouse

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ARTICLE INFO

Article history:
Received 4 October 2016
Received in revised form
31 January 2017
Accepted 8 March 2017
Available online 17 April 2017

Keywords:
Thymus kotschyanus
Depression
Lung cancer
Cervical cancer
Mice

ABSTRACT

Cancer and depression are known as two of the most debilitating disease and disorder increasing evidence suggest an urgent need for new therapeutic agents with lower toxicity and high efficacy. Some Thyme species extracts have remarkably been shown to positively affect depression and cancer cells. In the present study, we investigated the effect of *Thymus kotschyanus* on depression and cancer cells. To this end, in experiment 1, NMRI mice were treated orally with the ethanolic extract of *T. kotschyanus* (50, 150 and 250 mg/ml) for seven days and then depression-like behavior was measured by Forced Swim Test (FST) and Tail Suspension Test (TST). In experiment 2, the pharmacological effect of the extract on the lung (A549) and cervical (Hela) cancer cell lines was also evaluated by MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) in various concentration_(10, 5, 2.5, 1.25, 0.63, 0.31, 0.15 and 0.08 mg/ml). The results indicated that *T. kotschyanus* extract treatment (150 and 250 mg/kg) decreased depression-like behavior in the FST and TST tests in adult mice. Moreover, the treatment inhibited cancer cell growth and viability in a dose and time-dependent manner. Collectively these findings suggest that *T. kotschyanus* have antidepressant and anticancer effects.

1. Introduction

In recent years, extensive research have been done on cancer and depression, the two prime causes of mortality and morbidity in people, however there are two main problems: the first is the etiology and the second is the treatment of these diseases.[1] A case study conducted by Onitilo et al. indicated that cancer and depression are two independent risk factors for mortality rate in humans; hence, their effects on all-cause of mortality are additive, not synergistic.[2,3]

Depression is a debilitating chronic disorder, significantly affecting the quality of life of large population worldwide.[4–6] There are several contributing factors including genetic predisposition, epigenetic alterations, neuroendocrine and immunological dysregulations, monoamine neurotransmitter deficiencies in the brain, affecting depression-related behavior.[7–9]

Cervical and lung cancers are the principal causes of cancer-related death.[10,11] Cervical cancer is caused by chronic infection with a range of high-risk Human Papilloma Virus (HPV) leading to an estimated 274,000 death globally every year. Moreover, lung cancer is a highly aggressive, progressive and heterogeneous malignant disease predominantly results from smoking tobacco with few options of treatment.[12–15] For the treatment, chemotherapy with drugs such as Cisplatin and Iressa and psychotherapy with antidepressant drugs like fluoxetine, sertraline, and citalopram are widely used. However, these drugs have several side effects leading to further research for treatment with herbal medicines to reduce adverse side effects.[16–18]

There is a wide geographical distribution of the genus Thymus in Asia, North America, Europe and Africa, Thymus has several species including *Thymus vulgaris*, *Thymus daenensis*, *Thymus schimperi*, *Thymus zygis* and *Thymus kotschyanus* etc.[19–22] *T. kotschyanus* is a dicot herbal plant belonging to Lamiaceae's family. The name of *T. kotschyanus* is gained from Greek “Thymos” meaning power and courage.[23] In traditional medicine thymus species are used as antibacterial, antifungal, antiviral, anti-helminthic, antioxidative, antispasmodic, sedative and diaphoretic drugs.[24–26] Carvacrol and

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

http://dx.doi.org/10.1016/j.jtcme.2017.03.003

Thymol are known as the major phenolic monoterpenes of thyme oil especially in *T. kotschyanus* oil showing anticancer and antidepressant effects.[27]

Insufficient attention has so far been devoted to *T. kotschyanus*. Therefore, the goal of this study was to investigate the potential effects of *T. kotschyanus* on human lung and cervical cancer cell growth in *in vitro* cell culture and depression-like behavior in male mice.

2. Material and method

2.1. Experimental design

A summary of experimental design is shown in Fig. 1. The mice were randomly divided into four groups (six animals per groups), three groups orally received *T. kotschyanus* extract (50, 150 and 250 mg/kg) once daily for seven days and the one received saline as control group. Each animal was tested only once, with ten-day intervals between behavioral tests. In parallel with the behavioral tests, we investigated the effect of extract (10, 5, 2.5, 1.25, 0.63, 0.31, 0.15 and 0.08 mg/ml) on two cancer cell lines (A549 and Hella) for 24 and 48 h.

2.2. Plant extract

Leaves of *T. kotschyanus* (collected from Dizin, North Tehran province of Iran) were dried at room temperature and ground into powder form by a grinder. The ethanolic extract was extracted from the powdered plant with one liter of solvent (70% ethanol), agitated for two days then filtered for twice with Whatman No. 41 filter paper at the room temperature. The extract was evaporated at 30 °C in the rotary evaporator. The evaporation process involved the total removal of ethanol and water was used for the extraction. The yield of the extract was 10 g/200 g powder. The extract was dissolved in DMSO and then diluted with distilled water (DW) to make required concentration for oral administration. The orally administrated dosses are expressed as milligram of dry extract per kilogram of mice body weight. In the cell culture study, before sterilizing with a 0.22 μm pore size syringe filter (Nuc, Denmark), 20 mg of the extract was dissolved in 100 μl DMSO and diluted with RMPI-1640 to make appropriate concentration for cell culture administration.[27,28]

2.3. Animals

Male NMRI mice were used throughout the study. The animals were obtained from Pasture Institute of Iran. Mice were housed under the standard condition with 12/12 light-dark cycle (lights on 08:00–20:00 h) and controlled humidity and temperature (23 ± 1 °C) in all experiments. All animals had access to food and water *ad libitum*. All procedures were approved by the Cellular and Molecular Research Center at the Baqiyatallah University of Medical Sciences.

2.4. Forced Swim Test

Forced Swim Test is widely used for measuring anti-depressant effect in pharmacology. Briefly, mice were gently dropped into the

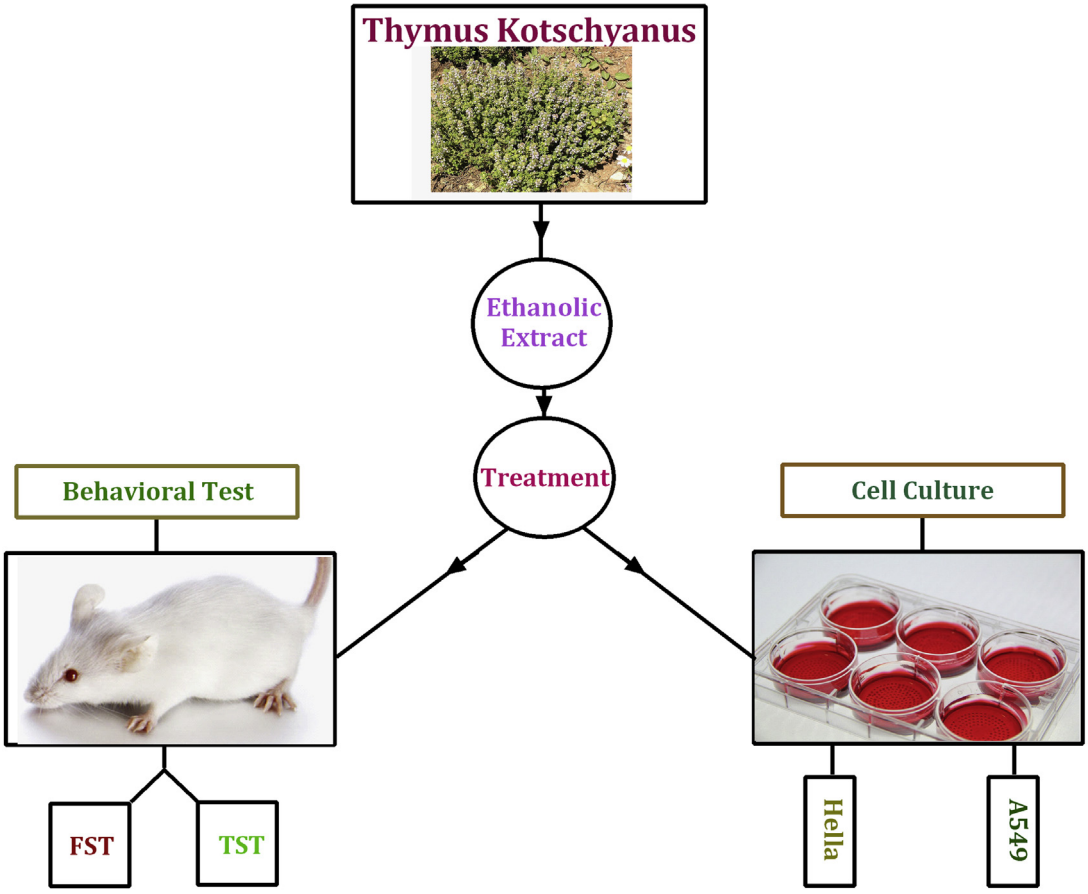

Fig. 1. Experimental design: the effects of *T. kotschyanus* exposure on depression-like behavior and cancer cell growth inhibition in male mice and cancer cell lines respectively. TST and FST performed on the same mice with ten days interval.

transparent glass cylinder, 25 cm height and 10 cm diameter in order to avoid mice touch the bottom of the cylinder. The cylinder filled with water at 25 ± 1 °C to a depth of 15 cm. Moreover water was replaced by fresh one between each test. The total duration of immobility was recorded by a blind observer during the last 4 min of 6 min testing period. The immobility was defined as an absence of any struggling exclude movement requisite for a mouse to keep its head above the water. A decline in immobility time is a demonstration of the anti-depressant-like properties.[29]

2.5. Tail Suspension Test

TST is a valuable behavioral test which is widely used to screen antidepressant-like activity. Briefly, mouse was individually suspended by the tail using a clamp 2 cm from the end. Total immobility was measured during 5 min test period. Moreover, immobility was described as times spent completely immobile posture except those caused by respiration.[30]

2.6. Cell culture

A549 and HeLa cell lines were acquired from the National Cell Bank of Iran (NCBI, Tehran) and were grown in the RPMI 1640 medium (Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), penicillin/streptomycin (100 IU/ml, 100 μg/ml respectively) (Sigma, Germany). The cells were incubated and maintained in a humidified atmosphere at 37 °C and 5% CO_2. Upon reaching 80% confluence, cells were rinsed with phosphate-buffered saline (PBS) then Cells were detached and harvested from 25 cm^2 flasks by using 0.25% Trypsin/EDTA solution (sigma, Germany). Finally, cells were sub-cultured into a 96-well plate according to experiment. The experiment was performed in duplicate.

2.7. MTT assay

The cytotoxicity of the extract was evaluated on HeLa cells as well as A549 cells by using the MTT method. The method is according to the ability of viable cells to produce blue formazan crystals from yellow tetrazolium salt MTT by the mitochondrial dehydrogenase. The cells were plated into 96-well plate (Nunc, Denmark) at a density of 10^4 cells/well/200 μl. The next day the media were replaced with fresh complete medium containing different extract concentrations (10, 5, 2.5, 1.25, 0.63, 0.31, 0.15, 0.08 mg/ml) and 0.2% DMSO (Sigma, USA) as negative control. Then 10 μl of MTT labeling reagent was added to each well, then the cells were administered with the extract for 24 and 48 h. The plates were incubated at 37 °C and 5% CO_2 for 4 h. Afterwards, supernatants were discarded and 100 μl of the Solubilization solution was added to each well and plates were incubated overnight in humidity atmosphere. Finally, cytotoxicity was detected by measuring the absorbance at a wavelength of 570 nm using an ELISA plate reader (Lab System). The percentage of cell cytotoxicity and viability was calculated according to following formula[31]:

%Cytotoxicity = 1 − Mean absorbance of toxicant/Mean absorbance of negative control × 100
%Viability = 100 − %Cytotoxicity

2.8. Statistical analysis

All data were analyzed according to the analysis of variance (ANOVA) followed by Tukey's test using the statistical program SPSS (IBM, Version 20). All values were presented as the means ± SEM and a P value of less than 0.05 was considered statistically significant.

3. Result

3.1. Tail Suspension Test

We determined that the effect of *T. kotschyanus* extract on depression-like behavior in the TST. Fig. 2 illustrates that the extract at dose of 250 mg/kg significantly decreased immobility time [$F_{(3, 20)} = 7.387$, $P < 0.01$] in mice in comparison with control group. These data show that *T. kotschyanus* extract treatment can reduce depression-like behavior in mice.

3.2. Forced Swim Test

The FST was conducted after the TST and its data is presented in Fig. 3. The analysis indicated that the extract treatment at doses of

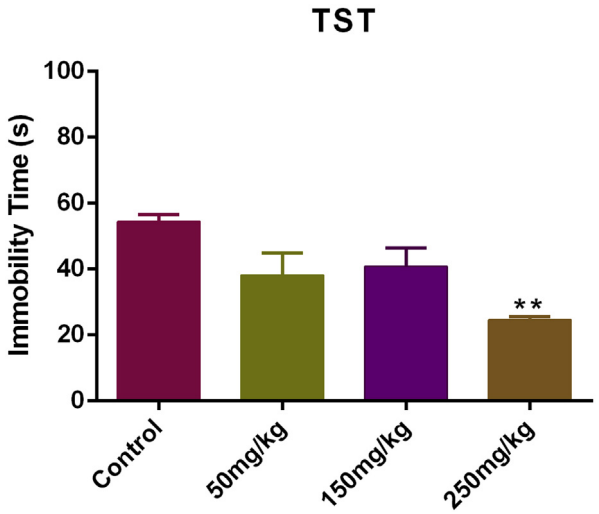

Fig. 2. Effects of *Thymus kotschyanus* extract in male mice in the TST (2) and FST (3). Values are presented as mean ± SEM (N = 6) of total duration of immobility. **P < 0.01 and ***P < 0.001.

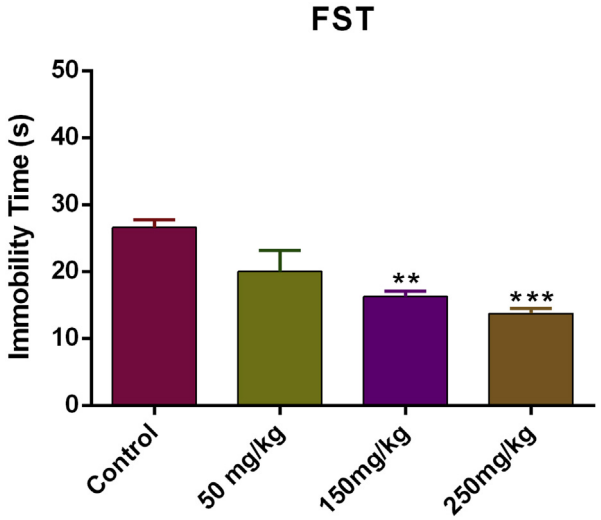

Fig. 3. Effects of *Thymus kotschyanus* extract in male mice in the TST (2) and FST (3). Values are presented as mean ± SEM (N = 6) of total duration of immobility. **P < 0.01 and ***P < 0.001.

150 and 250 mg/kg significantly resulted in decreased the immobility time [$F_{(3, 20)} = 10.137$, $P < 0.001$] in mice, as compared with control group. These results confirmed the TST data, showing that *T. kotschyanus* extract treatment can reduce depression-like behavior in mice.

3.3. Cell viability

The cytotoxic effect of *T. kotschyanus* extract was determined by MTT assay which is depicted in Fig. 4, A–H. After 24 h the extract a dose-dependent reduction in cell viability was found. The long-

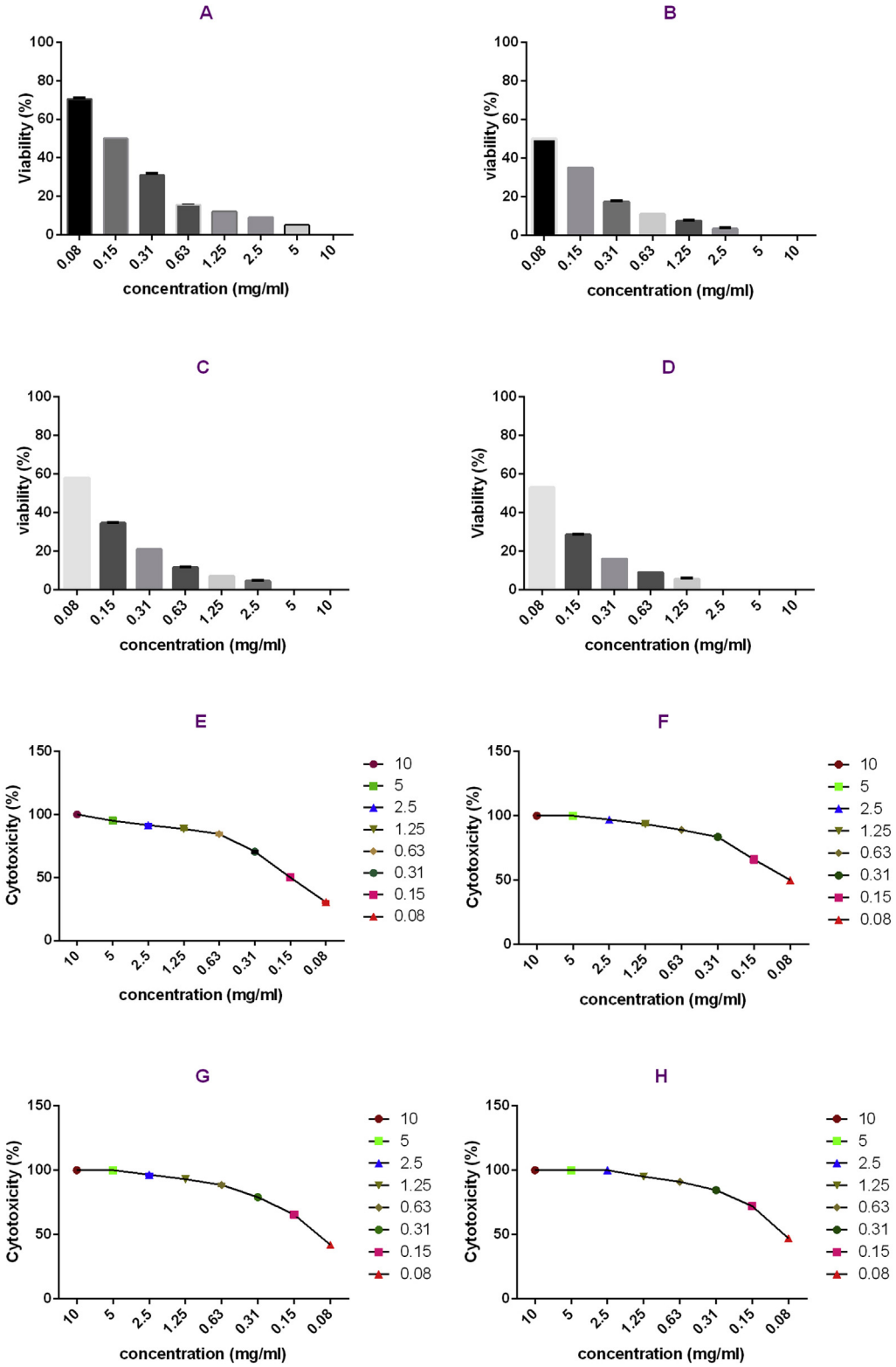

Fig. 4. A–D. Shows the inhibition of A549 and Hela cells growth that was measured by MTT assay (A. viability after 24 h in A549, B. viability after 48 h in A549, C. viability after 24 h in Hela, D. viability after 48 h in Hela). E–H. Shows the cytotoxic effect of the extract of *Thymus kotschyanus* in A549 and Hela cells that were measured using MTT assay (E. 24 h _A549, F. 48 h_ A549, G. 24 h_ Hela, H. 48 h_ Hela).

term extract treatment at higher dose resulted in the maximal cytotoxicity on cells. While A549 (Lung Carcinoma-derived cells) and HeLa (Cervical adenocarcinoma-derived cells) cell lines were treated for 24 h with the extract the viability (%) of HeLa cells was less than A549 cells, the viability of HeLa cells at a concentration of 0.08 mg/ml was more than A549 cells in the same concentration in 48 h treatment. However, the grater viability loss was observed in 48 h treatment including 2.5, 5 and 10 mg/ml for HeLa as well as 5 and 10 mg/ml for A549 cells. The extract at the dose of 10 mg/ml produced the most significant cytotoxicity (in both times) than the other doses.

4. Discussion

Herbal plants are predominantly used because of not only the toxicity and undesirable side effects of synthetic drugs, but also increasing resistance to chemical drugs as well as their high cost.[19,32,33] Among rich variety of medicinal plants containing bioactive substances, Thyme family are well-known as one of the most important spices, and used for culinary purpose[34] in spite of their anti-inflammatory, anti-oxidant, anti-bacterial (in vitro antimicrobial activity against *Salmonella*, *E. coli*, *Listeria*, and *Staphylococcus*) and anxiolytic properties.[35,36] *T. kotschyanus* has been mainly popularly used over centuries in common cold, inflammation, treat headache, vomit and irritation of urinary organs by Iranian.[37]

On the contrary, some useful properties of *T. kotschyanus* have been demonstrated in the recent studies, no studies have so far been specifically evaluated its ethanolic extract effects on both depression and cancer.

4.1. Behavioral effect of T. kotschyanus

We observed a significant decrease in depression-like behavior in mice treated orally with 150 and 250 mg/ml after 7 days compared with control animals. The major components in *T. kotschyanus* are Carvacrol and Thymol.[38,39] A study conducted by Deng et al. indicated that the Thymol administration to mice enhanced serotonin (5-HT) and norepinephrine (NE) in the hippocampus, while negatively regulated the release of pro-inflammatory cytokines, Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Tumor Necrosis-alpha (TNF-α) in this region of the brain.[40] Several parallel studies indicated that the augmentation of pro-inflammatory cytokines levels declines tryptophan, a crucial neurotransmitter precursor in serotonin biosynthesis[41] Moreover, another recent study showed that Carvacrol resulted in reduced immobility time in the TST and FST tests; however, the mechanism appears to be quite different from that by which Thymol leads to decreased depression-like behavior.[42] Interestingly, the anti-depressant effect of Carvacrol, unlike Thymol, could associated to the enhancement of dopamine transmission in the synaptic cleft which occurs in the limbic system.[42] Therefore, it may be concluded that increased level of serotonin or dopamine besides the decrease in pro-inflammatory cytokines affect depression-like behavior in male mice.

4.2. Effect of Thymus kotschyanus on cancer cells

Ideally, besides having fewer side effects and toxicity for normal host tissues, successful anticancer drugs or promising anticancer herbs should inhibit tumor growth through the reduction of cell proliferation and the induction of cell apoptosis.[43] Previous studies have provided conflicting findings about the anticancer activity of *T. kotschyanus* extract. However, our results led to a significant inhibition in A549 and HeLa cell lines growth which may be attributed to the phenolic compounds, in particular, Thymol and Carvacrol; moreover, based on some evidence these substantial substances are also involved in antioxidant properties of *T. kotschyanus*.[44] In this regard, some studies have demonstrated Thymol cell growth inhibition properties.[45,46] The antiproliferative effect of Carvacrol on lung and cervical cancer cell line, chronic myeloid leukemia cells and human metastatic breast cancer cells (MDA-MB231) have been reported.[47–49] The cytotoxicity of *T. kotschyanus* ethanolic extract on A549 and HeLa cell lines has indicated that the herb may exert its inhibitory and killing activity through multiple mechanisms leading to cell death. The possible mechanism seems to be associated with Thymol resulting in apoptosis.[45] Nevertheless, some researchers have reported that the apoptosis induction by Thymol is lower when compared with Carvacrol cell death potential.[47]

Therefore, since *T. kotschyanus* extract was found to decrease the level of depression and inhibit the lung and cervical cancer cell growth, it is possible antidepressant and anticancer effect of *T. kotschyanus* were mediated by Carvacrol and Thymol, the major components of *T. kotschyanus*. Further, studies are needed to find which components could be responsible for these effects.

Conflict of interest

None declared.

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