Evaluation of toxicity and antioxidative effects of *Tussilago farfara* and *Verbascum thapsus* water extracts in zebrafish and in bronchial epithelial cells

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**Abstract:** *Tussilago farfara* (coltsfoot) and *Verbascum thapsus* (mullein) have been used as folk remedies for treating respiratory disorders. The aim of this study was to test the toxicity of the water extracts of *T. farfara* and *V. thapsus* in vivo in zebrafish and in vitro in BEAS 2B epithelial bronchial cells. To the best of our knowledge, this is the first study to investigate the antioxidative properties of *T. farfara* and *V. thapsus* extracts in cell culture. Our results show that the *T. farfara* leaf extract does not produce toxic effects on zebrafish embryos or BEAS 2B cells, and that it has a protective effect in BEAS 2B after induction of oxidative stress. The water extract from *V. thapsus* displayed pronounced toxic effects on zebrafish embryos and BEAS 2B cells and did not exhibit a significant antioxidative effect on BEAS 2B cells exposed to oxidative stress. Our results suggest that the use of *T. farfara* water leaf extract is potentially safe and effective in treating respiratory disorders, whereas the use of *V. thapsus* needs further investigation.

**Keywords:** *Tussilago farfara* (coltsfoot); *Verbascum thapsus* (mullein); BEAS 2B cells; zebrafish; asthma treatment

**INTRODUCTION**

The use of herbs for medicinal and pharmaceutical purposes is a longstanding practice and has increased in the last few years [1,2]. Since plants are a source of numerous bioactive compounds, the toxicological effects and potential beneficial properties of plant extracts are intensively investigated [3]. Among the many plants that have been used in traditional medicine are *Tussilago farfara* and *Verbascum thapsus* (Linn). Both plants are usually used in the form of tea, oil and crude drugs. *T. farfara*, known also as coltsfoot, has long been used as a supplement in the treatment of respiratory disorders such as cough, bronchitis and asthma [4]. It prevents inflammation in airways and some compounds isolated from the flowers have been shown to exert antioxidant activity [5]. Flower and leaf extracts of *V. thapsus*, commonly known as mullein, exhibit demulcent properties and are used in the treatment of various respiratory and gastrointestinal problems [6]. Various extracts of *V. thapsus* have antibacterial, antiviral and antifungal activities [6,7]. The antioxidant activities of extracts prepared from different parts of *V. thapsus* were reported in previous studies [8-10]. Although, *V. thapsus* is suggested to be a rich source of antioxidants, it is not clear which compounds from this plant possess antioxidant activity [11].

Extracts from both plants have been used for treating respiratory disorders where inflammation and oxidative stress have a major role [12]. The respiratory surface is frequently exposed to oxidants through direct exposure to cigarette smoke and air pollutants [13]. In addition, reactive species are released from neutrophils, alveolar macrophages and eosinophils during airway inflammation. The disturbance in the normal redox status is the result of a prolonged production of reactive oxygen species (ROS) or decreased antioxidant defense [14]. ROS are important mediators of cell signaling and are involved in defense against pathogens [12,14]. However, these molecules are highly unstable and easily interact with most cellular mac-
romolecules [14] and consequently, the generated secondary radicals can lead to cell damage and death [12,14]. Enzymatic and nonenzymatic antioxidants are included in the protection of lungs from oxidative stress [15-17]. Supplementation with antioxidants might be useful for maintaining redox homeostasis and disease prevention [12].

We used two model systems to investigate the potential toxicity of the water extracts of *T. farfara* and *V. thapsus* on zebrafish embryos and human bronchial epithelial cell line (BEAS 2B) cells. Zebrafish is one of the standard *in vivo* models for toxicity analysis. Since both plants have been used in the treatment of respiratory disorders, we chose the BEAS 2B as our *in vitro* model. These cells were also used to assess the potential antioxidant properties of the water extracts of *T. farfara* and *V. thapsus*.

**MATERIALS AND METHODS**

**Plant material**

*T. farfara* leaf water extract was prepared from the commercial product “Farfarae folium” obtained from the Herbal Pharmacy of the Institute for the Study of Medicinal Herbs “Josif Pančić”, Belgrade, Serbia. Dried leaves were treated with hot water for 15 min at a ratio of 1:10 (w/v), allowed to cool to room temperature and the extract was dried in an oven at 45°C. The dried extract was resuspended in sterile distilled water to bring to a concentration of 11 µg/mL, and filtered through a filter membrane (pore size 0.22 µm). The obtained filtrate was used in the experiments as the coltsfoot water extract. Fresh flowers of *V. thapsus* were collected in August 2014 on the premises of the Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia (GPS coordinates: 44.7484° N, 20.4934° E). The flowers were rinsed with distilled water, dried on sterile filter paper and transferred to a tube with sterile water. Flowers were homogenized with pestle and extracted for 2 h at room temperature with gentle shaking at a 1:10 plant material to water ratio. The crude extract was centrifuged for 5 min at 6000 x g and the obtained supernatant was dried in a rotary evaporator. The dried supernatant was resuspended in sterile distilled water to a concentration of 36 µg/mL and filtered through a filter membrane (pore size 0.22 µm). The filtrate was used in the experiments as the mullein water extract.

**Zebrafish toxicity assay**

Zebrafish, Tübingen wild-type strain, were maintained and bred according to the Zebrafish Book [18]. The obtained embryos were treated with *T. farfara* and *V. thapsus* water extracts from 6 h post fertilization (hpf), observed at 24 hpf, 48 hpf and 72 hpf, and the mortality and malformations were recorded, according to OECD guidelines [19]. The embryos were treated in 24-well plates, 12 embryos per well in 1 mL of solution or embryo water (5.5 mg/L KCl, 294 mg/L CaCl₂·2H₂O, 123 mg/L MgSO₄·7H₂O and 63 mg/L NaHCO₃). Three replicates were prepared for each dilution (20x, 100x and 1000x (where x denotes fold dilution)), and the experiments were repeated three times.

**Cultivation and treatment of BEAS 2B cells**

BEAS 2B cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 5% fetal bovine serum (FBS), 10 µL penicillin and 10 ng/mL streptomycin. The cells were incubated in a humidified atmosphere at 37°C with 5% CO₂ and were passaged after reaching 70-80% confluence. The cells were seeded the day before treatment into a 6-well plate at a density of 0.2x10⁶. The next day, the medium was aspirated and replaced with fresh medium containing 100x, 500x and 1000x diluted extracts of *T. farfara* or *V. thapsus*. After 24 h, the cells were exposed to 440 μM and 880 μM hydrogen peroxide dilutions for 30 min at 37°C. The experiment was performed in triplicate and it included untreated control cells, cells pretreated with plant extracts with and without exposure to hydrogen peroxide and cells treated only with hydrogen peroxide.

**MTT assay**

The cytotoxic effects of *T. farfara* and *V. thapsus* were evaluated on BEAS 2B cells by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in a 96-well plate at a density of 1x10⁴ the day before treatment. The cells
were treated for 24 h with various dilutions of plant extracts in medium (100x, 500x and 1000x). We did not use a 20x diluted extract since it showed increased mortality and toxicity in the in vivo system. MTT powder was dissolved in phosphate buffered saline (PBS) and diluted in the medium. After the treatment, the medium from each well was replaced with 100 μL of MTT solution and the cells were incubated for 45-60 min at 37°C. The mixture was removed and 100 μL of dimethyl sulfoxide (DMSO) was added into the wells in order to solubilize the formazan crystals. Cell viability was quantified by measuring the absorbance at 550 nm on an automatic Infinite 200 PRO multimode reader (Tecan, Switzerland), and the data were analyzed using Magellan software (Tecan, Switzerland). The percentage of growth inhibition was calculated using the following formula:

\[
\text{percentage of cell survival} = \frac{(A_s - A_c)}{A_c} \times 100,
\]

where As is the absorbance value of the sample and Ac is the absorbance value of the control [20].

**RESULTS**

**V. thapsus** extract displays extensive zebrafish embryo toxicity

To determine the in vivo toxicity of the plant extracts, we used several dilutions (20x, 100x and 1000x) of the water extracts and exposed zebrafish embryos to them. Exposure to the lowest dilution (20x) of *T. farfara* extract caused an increase in embryo mortality after 72 h, with about 30% of the embryos not surviving the treatment (Table 1). Additionally, there were some malformations observed in about 20% of the surviving embryos, such as no tail detachment after 24 h, pericardial edema and bradycardia (Supplementary Fig. S1). However, at the other two dilutions tested (100x and 1000x), there were no changes in embryo survival nor were any malformations observed as compared to the untreated control.

**Table 1. Effects of *T. farfara* and *V. thapsus* water extracts on zebrafish embryo mortality and malformations.**

<table>
<thead>
<tr>
<th>dilution</th>
<th>embryonic mortality</th>
<th>malformation rate</th>
</tr>
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<tbody>
<tr>
<td>control</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td><em>T. farfara</em></td>
<td>20x</td>
<td>29%</td>
</tr>
<tr>
<td></td>
<td>100x</td>
<td>2%</td>
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<tr>
<td></td>
<td>1000x</td>
<td>2%</td>
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<tr>
<td><em>V. thapsus</em></td>
<td>20x</td>
<td>100%</td>
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<td></td>
<td>100x</td>
<td>5%</td>
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<tr>
<td></td>
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</tbody>
</table>

After exposure to 20x dilution of *V. Thapsus* none of the embryos survived the treatment. The other two dilutions did not cause significant differences in embryo survival when compared to the controls. At 100x dilution, numerous malformations were observed in the majority of embryos, such as no tail detachment, impaired development, eye and jaw malformations, pericardial and yolk-sac edema. Only the highest deviation (SD) for continuous variables, and as percentages for categorical variables. To test the normality of parameters, one sample Kolmogorov-Smirnov test was used. Differences between the groups were analyzed by the independent samples Mann-Whitney U test, Kruskal-Wallis test and one-way analysis of variance (ANOVA) with post hoc Tukey’s test. A P value less than 0.05 was considered statistically significant.

**ROS detection**

Dihydroethidium (DHE; Life Technologies, USA), an oxidant-sensitive fluorescent probe, was used for detection of the intracellular superoxide anion by flow cytometry. After pretreatment with plant extracts and induction of oxidative stress, the cells were incubated for 45 min at 37°C in the dark with DHE (10 μM) diluted in medium without FBS. The cells were trypsinized, washed twice and resuspended in PBS. The fluorescence intensity was quantified by CyFlow Space flow cytometer (Partec, Germany) equipped with a blue 488-nm laser for excitation and band pass filter 590/50 for emission detection. A minimum of 10000 events was assayed for each sample. Untreated cells served as the control for assessing the basal level of oxidative stress and stress induced by sample preparation. Analysis was performed on live cells using FloMax software (Partec, Germany).

**Statistical analysis**

Statistical analysis was performed using the Statistical Package for Social Sciences 20.0 (SPSS Inc., USA). Data were expressed as percentages and mean±standard deviation (SD) for continuous variables, and as percentages for categorical variables. To test the normality of parameters, one sample Kolmogorov-Smirnov test was used. Differences between the groups were analyzed by the independent samples Mann-Whitney U test, Kruskal-Wallis test and one-way analysis of variance (ANOVA) with post hoc Tukey’s test. A P value less than 0.05 was considered statistically significant.
dilution used (1000x) showed no significant developmental malformations compared to the untreated control (Fig. 1).

**T. farfara extract does not affect cell viability, V. thapsus extract causes a decrease in cell viability**

The influence of water extracts of *T. farfara* and *V. thapsus* on BEAS 2B cell viability was determined by the MTT assay. After treatment with *T. farfara* extracts, a decrease in cell viability of about 10% was observed at the lowest dilution (100x) (Fig. 2A). After the exposure of cells to *V. thapsus* extracts, the viability of cells was inhibited in a dose-dependent manner (Fig. 2B).

**T. farfara extract exhibits a protective effect on cells after induction of oxidative stress, V. thapsus extract does not**

We investigated the antioxidant activity of *T. farfara* and *V. thapsus* water extracts in BEAS 2B cells. The superoxide anion level was detected by DHE staining and assessed by flow cytometry. Treatment of cells with prepared plant extracts did not cause significant changes in the production of the superoxide anion compared to untreated cells (p=0.940 for *T. farfara* and p=0.833 for *V. thapsus*). Intracellular accumulation of the superoxide anion was induced by treatment with two different hydrogen peroxide concentrations (440 µM and 880 µM) previously reported in literature [21]. The superoxide anion level was slightly increased in cells treated with 440 µM of hydrogen peroxide in comparison with untreated cells, but the difference was not statistically significant (p=0.126). The intensity of DHE fluorescence was significantly increased after treatment with 880 µM of hydrogen peroxide compared to treatment with 440 µM hydrogen peroxide (p=0.002) and compared to untreated cells (p=0.004), which indicated that this concentration induced a significant accumulation of superoxide anion.

Next, we examined if pretreatment with plant extracts altered the production of superoxide anion induced by hydrogen peroxide treatments. After exposure to 440 µM hydrogen peroxide, similar levels of the superoxide anion were observed in cells previously treated with different dilutions of *T. farfara* extract and in cells treated only with hydrogen peroxide (p=0.678).

When oxidative stress was induced by hydrogen peroxide at the higher concentration (880 µM), a decrease in the production of the superoxide anion was observed in cells pretreated with different dilutions of *T. farfara* extract (p=0.004), pointing to the protective effect of this extract (Fig. 3).

A higher intensity of DHE fluorescence was detected in cells pretreated with the *V. thapsus* extract when compared to cells treated with 440 µM of hydro-
gen peroxide, but the difference was not statistically significant (p=0.251), while for the higher hydrogen peroxide concentration, a correlation was not established (p=0.6), indicating no significant effect of this extract (Fig. 4).

DISCUSSION

*T. farfara* and *V. thapsus* extracts prepared in different ways and with different solvents were shown to exhibit antibacterial, antiviral and antifungal properties [6,11,22,23]. Antioxidant and free radical scavenging activities of these plants were also shown using mostly biochemical assays [5,24-26]. The present study was designed to examine the potential toxic and antioxidative effects of the water extracts of *T. farfara* and *V. thapsus* in two model systems, *in vitro* in BEAS 2B epithelial bronchial cells and *in vivo* in zebrafish embryos. The obtained results demonstrated in both model systems that *T. farfara* leaf water extract had low acute toxicity and a significant antioxidative effect when oxidative stress was induced by hydrogen peroxide. *V. thapsus* flower water extract showed high acute toxicity and no antioxidative effects. To the best of our knowledge, this is the first study investigating the toxicity of the water extracts of *T. farfara* and *V. thapsus* in zebrafish embryo model system and their antioxidative properties in cell culture.

*T. farfara* leaf water extract showed limited toxicity in our experimental setup. Additionally, cytotoxicity was not observed in BEAS 2B cells. These results are in agreement with previous results [26,27]. In the latter paper it was shown that the methanol leaf extract of *T. farfara* produced no cytotoxic effect on the human liver cell line Huh7. In addition, the ethyl-acetate fraction of *T. farfara* flower did not exhibit a cytotoxic effects on primary rat cortical cells [28].
Dose-dependent toxic effects of *V. thapsus* water extract were observed in both zebrafish and BEAS 2B cells. The water extract of *V. thapsus* prepared from fresh flowers induced malformations in zebrafish embryos at higher dilutions (100x and 1000x), while at the lower dilution (20x) it had a lethal effect. The toxic effects of leaf and flower extracts or compounds isolated from them have not been investigated thus far on the zebrafish model, but it is known that *V. thapsus* seed can be used as a fish poison [6]. The result described from our study warrants further investigation in order to identify toxic compounds and to further elucidate their potential toxicity in humans. The cytotoxic effects of *V. thapsus* have been reported [7,29,30].

The water extract of the crude plant produced toxic effects in three human liver tumor cell lines at a concentration of 2000 µg/mL, which is much higher than the concentration used in our experiments (0.36 µg/mL). Cell viability was reduced by 31.0% and 69.9% in two hepatitis B virus genome-containing cell lines, HepG2/C3A and HA22T/VGH, respectively, while in the non-hepatitis B virus genome-containing cell line PLC/PRF/5 inhibition of cell growth was by 11.6% [29]. The antiproliferative activity of *V. thapsus* on tumor lung cells A549 was investigated [30]. The authors described significant pro-apoptotic effects of luteolin and 3-O-fucopyranosylsaikogenin F isolated from *V. thapsus* at concentrations of 30 µg/mL and 3 µg/mL, respectively [30]. In our study, cell viability was reduced by 32.4% at the lowest dilution used (0.36 µg/mL). This finding revealed higher sensitivity to the cytotoxic effect of the extract in normal, non-tumor cells originating from the bronchial epithelium, and a possible synergistic effect of the compounds in the extract on cell viability. This result also emphasizes the importance of using different cell types to assess the toxicity of compounds and extracts.

Oxidative stress plays a major role in the development of chronic respiratory diseases such as asthma, which are treated with extracts of *T. farfara* and *V. thapsus*. It was shown that *T. farfara* prevents inflammation in airways by inhibiting the metabolism of arachidonic acid and the generation of various inflammatory mediators, including prostaglandins, leukotrienes and reactive oxygen radicals [28]. Also, the extract inhibits the synthesis of nitric oxide in lipopolysaccharide-stimulated macrophages [31].

Extracts from different parts of *V. thapsus* have been shown to possess an antioxidative potential [9-11]. Previous studies mostly used the 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging assay to study the antioxidative properties of different compounds isolated from *T. farfara* and *V. thapsus*. In our study the influence of *T. farfara* and *V. thapsus* water extracts on the oxidative stress in BEAS 2B cells was examined by flow cytometry. Our results showed no effects of either *T. farfara* or *V. thapsus* water extracts on superoxide anion production. We observed a protective effect of *T. farfara* against oxidative stress induced by hydrogen peroxide. This finding is consistent with previous studies [5,24,32]. Different compounds isolated from flowers showed cytoprotective activities against glucose oxidase-induced oxidative stress in rat fibroblast cells (NIH3T3) and human keratinocyte cells (HaCaT) [33]. Ravipati et al. [32] reported higher free radical scavenging activity of *T. farfara* flower water extracts in comparison to the ethanol extract, which was probably due to the higher content of total phenols and flavonoids in the aqueous extracts. Quercetin and kaempferol are the most common flavonoids present in flavonoid fractions from *T. farfara* flowers and leaves [34]. Kim et al. [5] found that among flavonoids, quercetin glycosides have higher free radical scavenging activity in comparison to quercetin, thus, the antioxidant activity of leaf water extract observed in our study might be due to the presence of these flavonoids. On the other hand, we found no antioxidant activity of *V. thapsus* flower water extracts. The methanol extract of *V. thapsus* leaves was shown to have antioxidant properties *in vitro*, such as nitric oxide scavenging activity, in a concentration-dependent manner. In the same study, the *V. thapsus* extract had an *in vivo* nephroprotective effect and an inhibitory effect on lipid peroxidation in gentamicin-treated rats [9]. This antioxidative property of *V. thapsus* leaves was suggested to be due to the presence of polyphenolic compounds. Also, in a DPPH scavenging activity assay with alcoholic and water extracts of *V. thapsus* leaves, the ethanol extract was found to have a higher antioxidative potential than the water extract [10]. Our results, which apparently oppose most published studies, could be explained by the type of extract, since water extracts of flowers were used.
The present study was the first study to test the toxicity of *T. farfara* and *V. thapsus* extracts in the zebrafish experimental model. We showed that *T. farfara* leaf water extract caused neither increased mortality of zebrafish embryos nor significant changes in BEAS 2B proliferation, which is important and increases our knowledge on the safety of this plant for use in traditional medicine. Our findings further elucidate the action of *T. farfara* function as a natural medicine for treating respiratory disorders, such as asthma, and suggest that its actions are the result of its antioxidative effects on epithelial bronchial cells. On the other hand, water extracts from *V. thapsus* showed pronounced toxic effects on zebrafish embryos and caused decreased proliferation of BEAS 2B cells in our experimental setup. This extract did not show a significant antioxidative effect on BEAS 2B cells exposed to oxidative stress. Identification of the toxic and potentially beneficial compounds in *V. thapsus* extracts and elucidation of their mechanism of action would greatly benefit its safer use as a natural medicine.

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**Author contributions:** SD, ADR and SR designed the study. SD, DK and ADR performed the experiments. SD and ADR wrote the manuscript with support of DK. AN, DR and SR critically reviewed the manuscript. All authors read and approved the final manuscript.

**Conflict of interest disclosure:** The authors declare no conflict of interest.

**REFERENCES**


Supplementary Data

Supplementary data are available at: http://serbiosoc.org.rs/NewUploads/Uploads/Dragicevic%20et%20al_3747_Supplementary%20Fig.%20S1.pdf