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General Toxicity and Antifungal Activity of a New Dental Gel with Essential Oil from *Abies Sibirica L.*

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

DEF 1 Aurelija Noreikaitė
BE 2 Rizvangul Ayupova
CE 3 Elmira Satbayeva
B 3 Aida Seitaliyeva
CD 3 Marzhan Amirkulova
D 3 Guram Pichkhadze
B 4 Ubaidilla Datkhayev
A 1 Edgaras Stankevičius

1 Institute of Physiology and Pharmacology, Lithuanian University of Health Sciences, Kaunas, Lithuania
2 Department of Pharmacist-Technologist, Asfendiyarov Kazakh National Medical University, Almaty, Republic of Kazakhstan
3 Department of Pharmacology, Asfendiyarov Kazakh National Medical University, Almaty, Republic of Kazakhstan
4 Institute of Pharmacy, Asfendiyarov Kazakh National Medical University, Almaty, Republic of Kazakhstan

Corresponding Author: Aurelija Noreikaitė, e-mail: aurelija.noreikaite@lsmuni.lt
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Background: The aim of this study was to analyze the antifungal activity and the general toxicity of a new dental gel containing essential oil from the tree *Abies sibirica* L., which grows in the Republic of Kazakhstan.

Material/Methods: The essential oil from *Abies sibirica* L. was obtained by microwave heating method using the STARTE Microwave Extraction System. Adjutants used to prepare the oil were carbomer 974P, glycerin, polysorbate 80, xylitol, triethanolamine, and purified water, all allowed for medical usage. The antifungal activity of the essential oil was assessed by monitoring the optical density of *Candida albicans* in a microplate reader. The safety was determined by analyzing the acute and subacute toxicity.

Results: The essential oil obtained by the microwave heating method revealed a higher antifungal activity in comparison with the essential oil obtained by the steam distillation method. No obvious changes were detected in guinea pigs following cutaneous application of the gel. Enteral administration of the essential oil caused minimal functional and histological changes in mice after 4 weeks. The new harmless dental gel containing pine oil from *Abies sibirica* L. was provided for the purposes of this particular clinical research.

Conclusions: The high antifungal activity of the gel is the basis for more in-depth studies on its safety and pharmacological activity.

MeSH Keywords: Abies • Antifungal Agents • Dentifrices

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Background

Drug development in dentistry is of great importance due to the growing number of infectious, inflammatory, and fungal mouth diseases. Oral diseases such as dental caries, periodontal disease, tooth loss, oral mucosal lesions and oropharyngeal cancers, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS)-related oral disease, and orodental trauma continue to be frequent public health problems worldwide. Moreover, they have a major impact on people's daily lives and well-being [1]. According to the World Health Organization, up to 95% of adults and 80% of children suffer from inflammatory periodontal diseases such as gingivitis, stomatitis, and glossitis [2].

Microbial resistance to most commonly used antibiotics such as penicillins, cephalosporins, erythromycin, tetracycline and derivatives, as well as metronidazole is well known and documented [3]. Synthetic drugs also have a number of disadvantages, such as hypersensitization and high risk of resistance to pathogenic and saprophytic microflora. The major advantages of phytomedicines used in dentistry are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far [4]. However, antimicrobic and antifungal properties of a variety of medicinal plants used in folk medicine have not been investigated yet.

Abies sibirica L. (Siberian fir) is one of the dominant tree species in European Russia, west and east of the Siberian taiga. It has been used for therapy and prophylaxis in formal and folk medicine since ancient times [5]. Essential oil and metabolites of *Abies* plants are reported to have a range of bioactivities, such as anti-inflammatory, antihypertensive, and cytotoxic effects [6].

Taking into account the above listed factors and that this type of phytomedicine from *Abies sibirica L.* is a source of drugs with anti-inflammatory, antimicrobial, wound healing, regenerating, and antifungal activity [7], a new gel based on raw material from *Abies sibirica L.* was investigated.

The aim of this study was to analyze the antifungal activity and the general toxicity of this new dental gel containing essential oil from *Abies sibirica L.* growing in the Republic of Kazakhstan.

Material and Methods

Preparation of the essential oil

The essential oil from *Abies sibirica L.* was obtained by the microwave heating method on the extractor STARTE Microwave Extraction System. Adjutants used to prepare the oil were carbomer 974P, glycerin, polysorbate 80, xylitol, triethanolamine, and purified water, all allowed for medical usage. Quantitative and qualitative analysis of the essential oil composition was performed by gas-liquid chromatography and testing was performed by test-tube reactions [8–10].

Preparation of the gel

Gel composition has been verified by results of physical and chemical, technological, structural, and mechanical, as well as microbiological studies. The gel consists of the essential oil from *Abies sibirica L.* (0.3 g), carbomer (1.0 g), polysorbate 80 (0.3 g), glycerin (10.0 g), xylitol (20.0 g), triethanolamine (0.34 g), and purified water (up to 100 g).

Carbopol 974P in 1% concentration was used as the gelling agent, polysorbate 80 (0.3%) was used as a stabilizer, and xylitol in concentration of 20% was added to modify the gel taste. The xylitol was used not only as an excipient adjusting the organoleptic properties of the preparation, but its use also enabled significant improvement of the quality parameters and stability [11].

Antifungal susceptibility testing

Candida albicans strains were received from the laboratory of the Department of Infectious Diseases and Microbiology of the Veterinary and Pharmaceutical University in Brno, Czech Republic.

Inoculum test. Antifungal concentrations of the essential oil were diluted in dimethyl sulfoxide (DMSO) and 0.9% saline solution, and 100-µL aliquots were placed into 96-well flat microplates [12]. The final highest concentration of essential oil solution used was 256 mkg/mL for 5-Flucytosine (5FC) (1 mkg/mL) and *Candida albicans*. *Candida albicans* growth was monitored by measuring the optical density at 600 nm in a microplate reader (BMG, Reader Labtech, Germany) at 37°C from 0 to 48 h.

The antifungal activity of the essential oil obtained by steam distillation (sample WM) and microwave heating (sample MW) methods was determined by use of a SPECTRO Star Omega device.

Evaluation of toxicity

All manipulations with animals were performed in accordance with the rules of humane treatment of animals, regulated by Federal Law "Animals protection against cruel treatment" from 01.01.1997 and the provision of the European Convention for the Protection of Vertebrate Animals. The study of the general toxic effect of the essential oil from *Abies sibirica L.* was

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performed according to the general procedure described in the "Guide for preclinical study of new pharmacological substances," edited by Habriyev R.U. (Moscow, 2005) [13]. All experiments were approved by the Local Ethics Committee of Asfendiyarov Kazakh National Medical University (Protocol #2, 24.02.2014).

Acute toxicity study

For research purposes, 12 healthy mice of both sexes (18.00–22.00 g) passed 14-day quarantine in the vivarium of the Kazakh National Medical University; later, mice were divided into 2 groups (I-II) of animals. Animals did not receive any food for 2–3 h before and after administration of the essential oil. Group I received the essential oil from *Abies sibirica L.* diluted at 1:10 per os. The quantity of the administrated essential oil depended on the animal body weight, but did not exceed 0.8 mL. Purified water was used as the solvent. Group II served as the control; these mice received distilled water instead of the essential oil at the same volume. The essential oil was administered into the stomach through a special probe with a syringe.

Subacute toxicity study

The subacute toxicity study was performed on 2 types of animals: white mice (enteral administration) and guinea pigs (cutaneous application).

The subacute toxicity study for enteral administration of the essential oil

Mice (n-12) were divided into 2 groups (I–II). These animals did not receive any food for 2–3 h before and after administration of the essential oil. Group I received the essential oil from *Abies sibirica L.* diluted in 1:10 ratio. The essential oil was administered enterally for 4 weeks. The quantity of the administrated essential oil depended on the animal body weight, but did not exceed 0.8 mL. Group II served as the control; these mice received distilled water instead of the essential oil at the same volume for 4 weeks. The essential oil was administered into the stomach through a special probe with a syringe.

The subacute toxicity study by cutaneous application of the gel

This study was performed according to the general procedure described in the "Guide for preclinical study of new pharmacological substances," edited by Habriyev R.U. (Moscow, 2005) [13]. Guinea pigs (n-12; 250.0-300.0 g) were divided into 2 groups (I–II). One day before the experiment, the fur was carefully shaved on symmetrical parts of the sides. The size of the shaved skin surface was 5.8% of the animal body surface area. Gel formulation containing the essential oil was applied daily on the skin of the guinea pigs for 4 weeks.

Sensitization test

Light-colored guinea pigs (n-12) were divided into 2 groups (I–II). A single intradermal injection of sterile 10% essential oil solution was applied with a 0.2-mL tuberculin syringe to the external surface of the ear. The evaluation of the sensitization test was made on day 10. A spatula was used to apply 50.0 mg of the gel to the mucous membrane of the conjunctiva fornix (transitional zone between the bulbar and palpebral conjunctiva). The lacrimal channel at the inner corner of the eye was pressed after 1 min of introducing the essential oil. The state of the conjunctiva at the site of gel introduction was visually observed after 15 min and 24 h.

The ocular irritation potential in rabbits

Rabbits (n-12) were included in the study. They were divided into 2 groups (I–II) of 6 animals. A spatula was used to apply 50.0 mg of the gel to the mucous membrane of the conjunctiva fornix (transitional zone between the bulbar and palpebral conjunctiva). The lacrimal channel at the inner corner of the eye was pressed 1 min after gel application. The state of the conjunctiva at the site of gel implantation was visually observed immediately after application, after 15 min (quick response), and after 24-48 h (hypersensitivity). The damaging effect of the gel on rabbit eye mucosa was assessed by the degree of hyperemia and edema using a point system scale.

Statistical analysis

The results are presented as mean (M) ± standard error (m). The results were assessed using the *t* test [14]. The statistical program package was for Windows 7.0 and statistical analysis was performed using SPSS 15.0.

Results

Effect on fungal activity

Antifungal therapeutic drug monitoring was conducted within 48 h. Antifungal activity of the essential oil samples obtained by the steam distillation method (sample WM) and the microwave heating method (sample MW) were determined with a SPECTRO Star Omega device. The test results were compared between the following groups: sample WM, sample MW, control growth, and positive control –5FC. We found that the essential oil from *Abies sibirica L.*, obtained by the microwave heating method, had the highest antifungal activity. The test results are shown in Figure 1.

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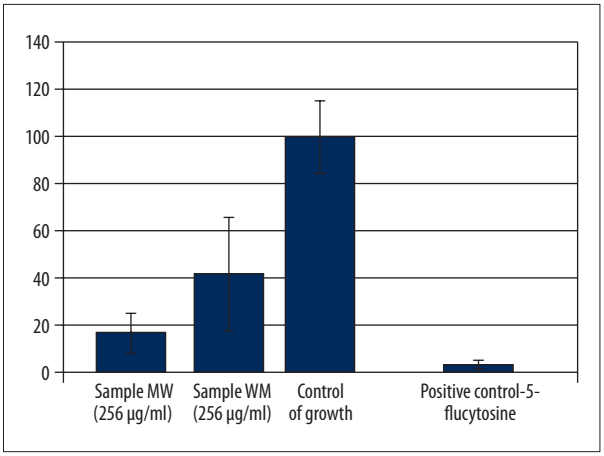

Figure 1. The antifungal activity of the essential oils from *Abies sibirica L.*

Effect on general symptoms of toxicity

In the acute toxicity study, enteral administration of the essential oil did not reveal any noticeable changes in the general behavior of the animals. The animals were active in all the groups. No changes in respiratory, cardiovascular, or central nervous systems were noticed. No deaths or intoxications occurred. Skin and mucous membranes had no alterations.

In the subacute toxicity study, cutaneous application of the gel did not cause any noticeable changes in the general behavior of animals. The motor activity was unchanged, no seizures and/or dystaxia were noted, skeletal muscle tones were normal, and the reaction to various stimuli was unchanged. The state of fur and skin, the size of the pupil, and the position of the tail were unchanged.

Minimal symptoms of subacute toxicity were noticed in animals receiving enteral administration of the essential oil for 4 weeks, including hypoactivity, loss of appetite, and a decrease in water consumption. No deaths occurred.

Effect on changes in body weight

No significant changes occurred in body weight, food intake, or water consumption in the subacute toxicity study following cutaneous application of the gel or in the acute toxicity study following enteral administration of the essential oil. However, a low body weight increase was noticed in the animal group in the subacute toxicity study after enteral administration of the essential oil for 4 weeks compared with the control group (Table 1).

A moderate difference in the animal body weight was noted between the control and experimental groups, and variance in the subjects was identified by univariate analysis of variance (ANOVA) $F(1; 67.21)=195.46$, $p<.001$, $\eta=.951$.

The independent samples *t* test was used to determine the difference between the means of the weight change. There was a significant difference between the mean values of the weight change, $t(12; 10)=13.981$, $P<.001$ [CI: 3.98; 5.49]. The mean change in weight for the experimental group ($M=1.60\pm.30$) was significantly lower than for the control group ($M=6.33\pm.77$). This suggests the same toxic effect of the dental gel with the essential oil from *Abies sibirica L*, when applied enterally.

Table 1. Dynamics of the weight of control and experimental animals.

No.	Week 1	Week 2	Week 3	Week 4
Weight of control animals, g				
1	18.32	20.22	22.98	25.66
2	22.13	25.58	26.73	28.18
3	20.45	22.52	23.99	27.68
4	19.84	21.07	22.67	25.82
5	18.15	20.04	22.79	24.11
6	21.12	23.30	25.13	26.55
Weight of experimental animals, g				
1	22.03	22.35	23.05	23.18
2	20.69	21.72	22.02	22.73
3	21.53	22.12	22.63	23.01
4	18.48	19.02	19.38	20.10
5	18.02	18.13	18.98	19.52
6	20.59	21.48	21.84	22.39

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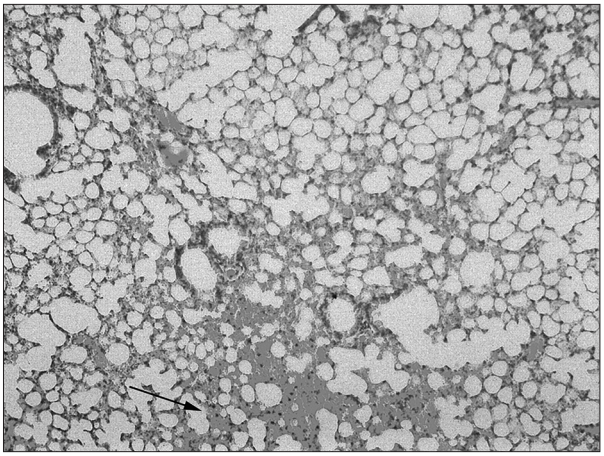

Figure 2. Effect of subacute toxicity of the essential oil from *Abies sibirica L.* on mouse lungs, showing free alveolar cavity, intensively painted bronchial tree epithelium, sporadic sanguineous vessels, and hemorrhages.

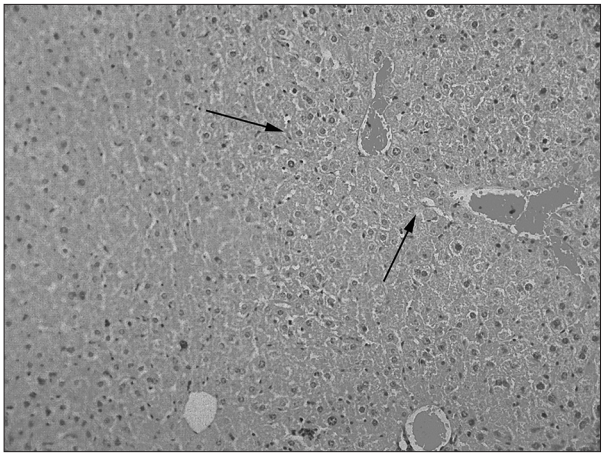

Figure 4. Effect of subacute toxicity of the essential oil from *Abies sibirica L.* on mouse livers showing irregularly stained nuclei of hepatocytes, usual location of struts, and vessels filled with blood.

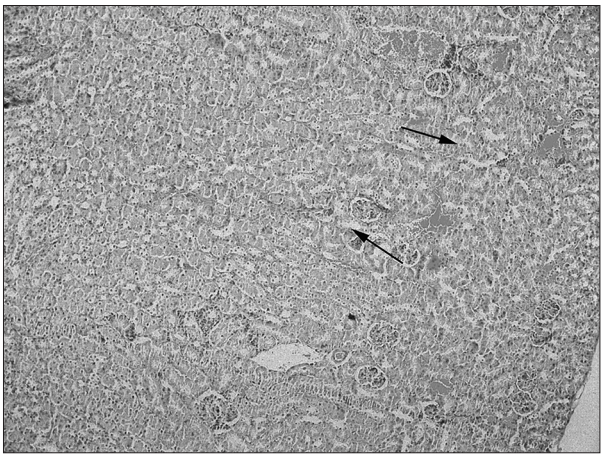

Figure 3. Effect of subacute toxicity of the essential oil from *Abies sibirica L.* on mouse kidneys, showing uniformly colored tubule epithelium, well-stained nuclei with maintained normal histological structure, vessels filled with blood, and sporadic foci of hemorrhage.

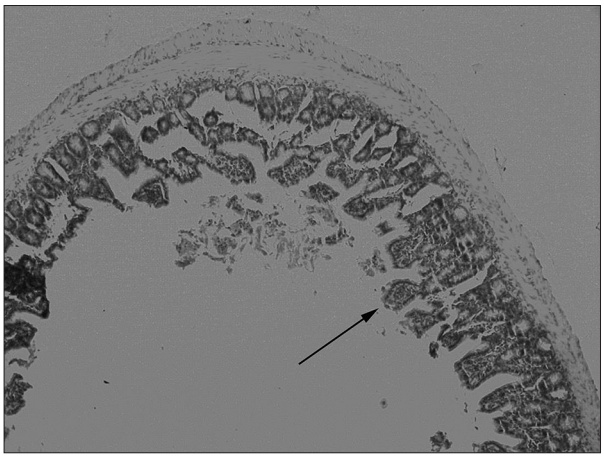

Figure 5. Effect of subacute toxicity of the essential oil from *Abies sibirica L.* on mouse intestine mucosa showing atrophied intestinal mucosa with short villi, small glands, mucosal epithelium without signs of hemorrhages, and evenly colored.

Histopathological examination

No morphological changes of animal internal organs were noticed after the subacute toxicity study following cutaneous application of the gel and after the acute toxicity study following enteral administration of the essential oil. The internal organs of the animals were regularly shaped and had anatomically correct position.

Minimal changes were noticed in the subacute toxicity study after 4 weeks of enteral administration of the essential oil. Internal organs were regularly shaped and had an anatomically correct position, but there was an increased amount of transparent liquid in the abdominal cavity and the cavity of the pleura and mediastinum; the vessels were filled of blood. The results of histological examination are shown in Figures 2–5.

However, histopathological examination of animal internal organs following enteral administration of the essential oil revealed moderate signs of toxic effects: circulatory disorders due to diminished flow of blood and oxygen supply in the lungs, increased vascular permeability, small sporadic hemorrhagic focus, and dystrophy of hepatocytes.

Effect on the sensitization test

No allergic effects were noticed after 15 min, 24 h, or on day 10 of observation. A slight reddening of the tear duct appeared

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immediately after injection and disappeared after 30 min. The mucous membrane of the eye was not swollen, and had usual color and size. There were no hemorrhages or increased lacrimation.

Effect on the ocular irritation potential in rabbits

No signs of irritation such as redness or swelling were noticed on the mucous membranes of the conjunctiva. No changes in the general condition and behavior of the animals were observed.

Discussion

Constituents of *Abies* genus species and their biological activity have been well studied [15–19]. However, there are few data published regarding the use of *Abies sibirica L.* in clinical practice. Previously published data show that pine oil extract contains many biologically active substances [20–22]. Components of this oil have anti-inflammatory, antimicrobial, and antifungal properties [6,23]. These botanical oils used in toothpastes take advantage of these properties and are as effective as conventionally formulated dentifrices in the control of plaque and gingivitis [24], and have beneficial effects on the clinical and microbiological parameters of periodontal inflammation [25]. Moreover, it has been proven that if the dental industry replaces abrasives with vegetable oil in dentifrices, these will be more effective in maintaining oral health and will cause less dental abrasion [26].

In this study, we evaluated a dental gel made with the essential oil from *Abies sibirica L.* and tested its safety for buccal administration. The external application of the essential oil has been suggested in previously performed studies [6,20,21]. The composition of the pine oil from *Abies sibirica L.*, obtained by the microwave heating method, has been studied recently [11]. The present study confirms that antifungal properties of the essential oil, obtained by the microwave heating method, have a higher antifungal activity in comparison with the essential oil obtained by usual methods. The antifungal activity determined by use of a SPECTRO Star Omega device, might be less susceptible to *Candida* strains than obtained by ATB FUNGUS test [27].

Other types of *Pinus* oils and their use in clinical practice have been investigated as well. It was found that *Pinus wallichina* and *Pinus pinaster Ait.* have anti-proliferative activity [28,29]. *Pinus wallichina vs. Abies sibirica* was investigated for its anticancer properties and acknowledged for treatment of diseases caused by reactive oxygen species [29]. Subsequently, properties of other pine oils have been assessed and effects on plasma lipoproteins and atherosclerosis have been identified. Short-term effects have been noticed [30]. However, in the long term, maritime pine oil has no preventive effect on cholesterol-induced aortic lesion development [31]. The toxicity of these oils is little studied due to their use only in folk medicine. The acute toxicity study following enteral administration of the gel from *Abies sibirica L.* oil did not reveal any changes in internal organs, but study results showed some toxic gel effects after long-term use.

Conclusions

Our study results demonstrated that properties of the essential oil from *Abies sibirica L.* depended on the method by which the oil was obtained. The essential oil from *Abies sibirica L.* obtained by the microwave heating method displayed a higher antifungal activity in comparison with the essential oil from *Abies sibirica L.* obtained by the steam distillation method.

Toxicity studies of the essential oil from *Abies sibirica L.* showed absence of acute toxicity signs, but prolonged enteral administration of the essential oil revealed moderate toxic effects. We therefore recommend the use of the essential oil not exceeding 2 weeks.

The high antifungal activity of the gel is the basis for more in-depth studies on the safety and pharmacological activity.

Conflicts of interests

The authors declare no conflicts of interests.

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