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Research Article

Biological Activity and Preclinical Study of Toxcicologycal Action of the Essential Oil of Ziziphora Bungeana Juz. from Kazakhstan

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ABSTRACT

Data on study of biological activity preclinical study of toxcicologycal action of the essential oil of *Ziziphora bungeana* Juz. Family of *Lamiaceae* are presented in this article. The raw materials for the researching were collected in summer 2014 at the territory of the Republic of Kazakhstan, at the foothills of the Dzungarian Alatau during the flowering period. Essential oil of plant by hydrodistillation was obtained, the yield is 0.5 %. The component composition of essential oil was studied using GC/MS Clarus-SQ 8 (PerkinElmer). Antimicrobial, antifungal, antileichmanial activities of essential oil were studied. Antibacterial and antifungal activity were identified using the strain of 5 human-pathogenic bacteria (Staphylococcus aureus, St. aureus (MRSa), Escherichia coli, Pseudomonas aeruginosa, Mycobacterium intracellulare) and 5 fungi (*Candida albicans*, *C. glabrata, C. krusei, Aspergillus fumigates, Cryptococcus neoformans*). Antileishmanial activity of the sample was tested for its ability to inhibit *Leishmania donovani*. The affinity of the total extracts and isolated compounds towards cannabinoid and opioid receptors were carried out according to the published method. The data indicate that the oil exhibit moderate levels of binding with Cannabinoid receptor and that more actively was inhibition of CB1 receptors. Results obtained from Radioligand Receptor Binding Assay, indicate that Kappa (26,3% inh.) and Mu (31,9 % inh.) types are the most sensitive receptors, with the higher percent inhibition. Visually, signs of sensitizing effect were not observed on the 10th day of the experiment in 15 minutes and 24 hours after a single subconjunctival administration of the essential oil on mucous membrane of palpebral conjunctiva and guinea pigs; a slight reddening of the tear duct, appeared immediately after injection, disappeared for 30 minutes. The mucous membrane of the eye was not swollen, usual-colored. Hemorrhages were not found; increased lacrimation was not observed. During the observation period the some signs of irritant action (redness, swelling, etc.) on the mucous membranes of the conjunctiva was not observed, which indicates the absence of irritating action. General condition of the animals was without aberration during the whole observation time.

Keywords: *Ziziphora bungeana* Juz., essential oil, GS/MS, antimicrobial, antifungal, anti-leishmanial, toxicology action, sensibility, irritant.

INTRODUCTION

Plants have been provided wide variety of medicines to treat various deceases since being human civilization. The genus *Ziziphora* L. (Lamiaceae) comprises about 30 species wide spread all over Asia, Africa, and Europe. *Ziziphora bungeana* Juz. is distributed mainly in Kazakhstan, China, Central Asia, and Mongolia. It is used for the treatment of hypertension, and cardiovascular diseases[1]. *Z. bungeana* Juz. used as infusion, decoction and maceration for various purposes such as sedative, stomach tonic, common cold, inflammation, coughing, antiseptic, migraine, fever, diarrhea, expectorant, depression, carminative and gastrointestinal diseases[2,3]. Also it is used as a traditional medicine in Kazakhstan and only few bioactive compounds were reported from this plant. This gained our attention to study the chemistry and pharmacology of this plant.

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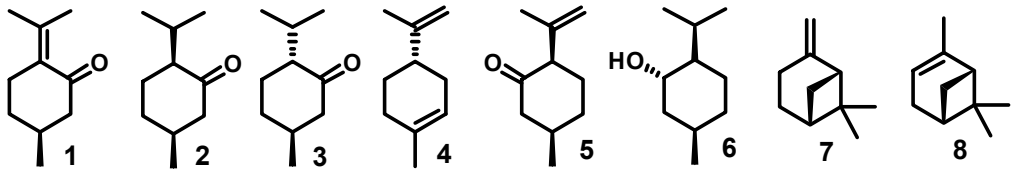

Figure 1**:** Major components of essential oil of *Ziziphora bungeana* Juz.

Table 1: Name of strains.

Bacteria	Fungi
Staphylococcus aureus	*Candida albicans*
Methicillin-resistant *Staphylococcus aureus* (MRSa)	*Candida glabrata*
Escherichia coli	*Candida krusei*
Pseudomonas aeruginosa	*Aspergillus fumigatus*
Mycobacterium intracellulare	*Cryptococcus neoformans*

Table 2: Resut of anti-leishmanial activity of essential oil of *Ziziphora bungeana* Juz.

The name of a plant — a source of essential oil	*Leishmania donovani* 80μg/mL % Inh.
Ziziphora bungeana Juz.	15

MATERIALS AND METHODS

Plant materials

The aerial parts of *Z. bungeana* Juz. were collected in summer 2014 in the flowering stage at the foothills of the Dzungarian Alatau, Republic of Kazakhstan and identified by Dr. Gemejiyeva N.G. (Institute of Botany and Phytointroduction, Science committee-Ministry of Education and Science of the Republic of Kazakhstan). A voucher sample (№01-04/257) has been deposited in the herbarium of the Institute of Botany and Phytointroduction, Science committee-Ministry of Education and Science of the Republic of Kazakhstan.

Essential oil distillation

Essential oil was extracted from the plant sample using a modified Clevenger-type apparatus. The conditions of extraction were: 40 g of air-dried sample, 500 ml distilled water, and 2-2.5 h distillation. After extraction, anhydrous sodium sulfate was used to eliminate water. The extracted oil was placed in a sealed glass tubes and stored at 4°C.

Bioassay procedure

The National Center for Natural Products Research of School of Pharmacy University of Mississippi, USA offers three *in vitro* biological screen anti-leishmanial screen (LEM) antifungal and antibacterial screen (OI) to test a variety of samples. Screens are set up in a tiered process depending upon the sample type submitted (crude extract, column fraction or pure compound). The time to completely analyze a sample also depends upon the sample type, as well as the screen itself (incubation periods, etc.). There are many other factors to consider when determining if your sample is active or worth pursuing. The anti-leishmanial screen (LEM) tests samples for their ability to inhibit *Leishmania donovani*, a fly-borne protozoan that causes visceral leishmaniasis. Crude extracts are initially tested in a Primary Screen at 80μg/mL in duplicate and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing ≥50% inhibition proceed to the Secondary Assay. In the Secondary LEM Assay, all samples (2 and 20mg/mL) are tested at 40, 8.0 and 1.6μg/mL and IC_{50}s as well as IC_{90}s (test concentration that affords 90% inhibition of the protozoan relative to controls) are reported. Samples that have an IC_{50} of <1.6μg/mL in the Secondary LEM assay proceed to the Tertiary Assay where the sample is tested at 40, 8, 1.6, 0.32, 0.064, 0.0128μg/mL and IC_{50}s and IC_{90}s are reported. All IC_{50}s and IC_{90}s are calculated using the XLFit fit curve fitting software. The drug controls pentamidine and amphotericin B are used as positive controls. The antimicrobial screen (OI) tests samples for their ability to inhibit a panel of 5 bacteria and 5 fungi that are pathogenic to humans: In the beginning tested in primary screening for 50 μg/ml twice and growth inhibition percent (% Ing.) was calculated in relation to negative and positive control. Essential oil showing #50 % of inhibition directed to secondary screening. In secondary screening samples dissolved in 20 mg/ml and checked at 50, 10 and 2 μg/ml and IC50s against all 10 strains of microorganisms. Samples dissolved in 2 mg/ml and carried out tests for 20, 4, 0.8 μg/ml and IC50s against all 10 strains of microorganisms. 7 μg/ml on secondary screening directed pure connections which have IC50 $ on tertiary screening. The secondary screening samples were dissolved in 20 mg/ml and tested at 50, 10 and 2 %g/ml and IC50s against all 10 strains of microorganisms. Samples were dissolved in 2 mg/ml, and the test was conducted by 20, 4, 0.8 ug/ml and IC50s against all 10 strains of microorganisms. The antifungal agent — amphotericin B was used as a control, and as an antibacterial — ciprofloxacin. The results of primary screening showed low antimicrobial and antifungal activity of essential oil of *Ziziphora bungeana* Juz.

Cannabinoid Receptor Binding Assay

Reagents. Buffer reagents were purchased from Sigma-Aldrich (St. Louis, MO). All radioligands and MicroScint were purchased from PerkinElmer (Waltham, MA). Non-labeled controls were purchased from Tocris Bioscience (Minneapolis, MN). Membrane preparations were made in a Tris-HCl buffer (50nM Tris-HCl), pH 7.4. Dilutions of membrane, radioligand and control/test compounds were

Table 3: Antibacterial and antimicrobial activity of the essential oil of *Z. bungeana* Juz. - *percent inhibitions (% inh.)*.

Name of strains	Test results of inhibition at a concentration of essential oil 50 mg / ml
Staphylococcus aureus	4
Methicillin-resistant *Staphylococcus aureus* (MRSa)	2
Escherichia coli	1
Pseudomonas aeruginosa	7
Mycobacterium intracellulare	0
Candida albicans	38
Candida glabrata	4
Candida krusei	14
Aspergillus fumigatus	0
Cryptococcus neoformans	0

made in a Tris-HCl buffer (50nM Tris-HCl), pH 7.4, for opioid receptors and Tris-EDTA (50mM Tris-HCl, 20mM EDTA, 154mM NaCl and 0.2% fatty-acid BSA), pH 7.4, for cannabinoid receptors.

Cell Culture. HEK293 cells (ATCC, Manassas, VA) were stably transfected via electroporation with full-length human recombinant cDNA (OriGene, Rockville, MD) for cannabinoid receptor subtypes 1 and 2. These cells were maintained at 37°C and 5% CO_2 in a Dulbecco's modified Eagles' medium (DMEM) and F-12 HAM nutrient mixture (50/50), supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 1% penicillin−streptomycin, and G418 antibiotic solutions. HEK293 cells stably transfected with opioid receptor subtypes μ, δ, and κ were used to perform the opioid receptor binding assays. These cells were maintained at 37°C and 5% CO_2 in a DMEM nutrient mixture supplemented with 2 mM L-glutamine, 10% fetal bovine serum, penicillin−streptomycin, and G418 antibiotic solutions. *Membrane Preparation.* The cells were lysed and scraped in cold Tris-HCl, pH 7.4 and then centrifuged at 5,200 x g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in the same buffer and homogenized via Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA) for 30 seconds and then centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was saved and the pellet underwent the suspension and homogenization process 2 more times with the same conditions. The supernatants were combined and centrifuged at 23,300 x g for 40 minutes at 4°C. The pellet was re-suspended in cold Tris-HCl buffer, aliquoted into 2mL vials and stored at -80°C. The total protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) using to manufactures instructions. The isolated membrane was aliquoted into 2mL vials and stored at -80°C. *Radioligand Receptor Binding Assay.* This assay is designed to use a series of controls to determine binding affinity of the test compounds using a 96-well format. 10uM of a positive control [CP55,940 (CB1, CB2), U-69,593 (κ), DPDPE (δ), or DAMGO (μ)] was used to ascertain non-specific binding (NSB) and 1% DMSO in Tris-EDTA buffer was used to ascertain total binding. To eliminate the possibility of contamination in the test compounds, controls or the radioligand, wells with 1% DMSO with no membrane were tested. Each test well contained 50uL of respective radioligand ([3H]-CP-55,940 (CB1, CB2), [3H]-U-69,593 (κ), [3H]-DAMGO (μ) or [3H]-Enkephalin (δ)), 50uL of compound, control or vehicle and 100uL cell membrane. The K_d for ligands for each receptor was established through a membrane evaluation and saturation binding experiment. For the membrane evaluation experiment 1-15ug of membrane was incubated with 1nM of it respective radioligand. 10uM of non-labeled positive control was ascertained for non-specific binding, 1% DMSO in buffer was used to ascertain total binding. Total, specific and non-specific were used to calculate the % binding of the non-labeled control to receptor. The membrane concentration exhibiting good % binding (>50%) and total binding with high signal (thousands of CPM) was used as the optimal membrane concentration for the assay. For the saturation assay, the optimal membrane concentration and 0.5-10nM of its respective radioligand were incubated with 10uM of a non-labeled positive control (NSB), or 1% DMSO in buffer (total binding). Data was analyzed by a non-linear curve fit model using GraphPad Prizm 5.04 software (GraphPad, La Jolla, CA) and the K_d value was calculated. For a general screening, the optimal concentration of membrane and radioligand determined with from the saturation assay, were incubated with 10uM (purified) or 10ug/mL (extracts/fraction) of a test compound. These samples were incubated for 60 minutes at room temperature for opioid receptor or 90 minutes at 37°C with gentle agitation for cannabinoid receptors. The reaction was terminated via rapid filtration with cold Tris-HCl buffer through a UniFilter GF/B (opioids) or GF/C (cannabinoids) 96-well plate pre-soaked with 0.3% BSA. When the filters were dry, MicroScint (Cat # 6013621, PerkinElmer) was applied to each filter and the plates were read on a TopCount NXT HTS Microplate Scintillation Counter (PerkinElmer, Waltham, MA) where the counts per minute (CPM) were recorded. Non-specific binding was subtracted from total binding to find specific binding. Percent inhibition was determined using the following equation: $\%\ inhibition = 100 - \left(\frac{compound.CPM - nonspecific.CPM}{specfic.CPM} * 100\right)$

Opioid Receptor Binding Assay

Mu, Kappa, and Delta Receptor Binding Assay

This assay is designed to use a series of controls to determine binding affinity of the test compounds using a 96-well format. 10uM of a positive control [*nor*-Binaltorphimine dihydrochloride (κ), DPDPE (δ), or DAMGO (μ)] was used to ascertain non-specific binding (NSB) and 1% ethanol or DMSO in Tris-EDTA buffer was used to ascertain total binding. To eliminate the possibility of contamination in the test compounds, controls or the radioligand, wells with 1% ethanol or DMSO with no membrane were tested. Each test well contained 100uL of *nor*-Binaltorphimine dihydrochloride (κ), DPDPE (δ), or

Table 4: Cannabinoid Receptor Binding by the essential oil of *Z. bungeana* Juz. - percent inhibitions.

Sample Name	Concentration Tested	CB1, % Inhibition	CB2, % Inhibition	Delta, % Inhibition	Kappa, % Inhibition	Mu, % Inhibition
Z. bungeana Juz. essential oil	10ug/mL	32,1	20,6	12,0	26,3	31,9

DAMGO (μ), 10uL of compound, control or vehicle and 100uL cell membrane. The K_d for ligands for each receptor was established through a saturation binding experiment. 1-10ug of membrane was incubated with 0.1-2nM of its respective radioligand ([3H]-U-69,593 (kappa), [3H]-DAMGO (Mu) or [3H]-Enkephalin (delta)). 10uM of a positive control was used to ascertain non-specific binding, 1% ethanol or DMSO in buffer was used to ascertain total binding. Data was analyzed by a non-linear curve fit model using GraphPad Prizm 5.04 software (GraphPad, La Jolla, CA) and IC50 and K_i values were calculated.For a general screening, the optimal concentration of membrane and radioligand determined with from the saturation assay, were incubated with 10uM (purified) or 10ug/mL (extracts/fraction) of a test compound. These samples were incubated for 60 minutes at room temperature. The reaction was terminated via rapid filtration with cold Tris-HCl buffer through a UniFilter GF/B 96-well plate pre-soaked with 0.3% BSA. When the filters were dry, 25 uL MicroScint (Cat # 6013621, PerkinElmer) was applied to each filter and the plates were read on a TopCount NXT HTS Microplate Scintillation Counter (PerkinElmer, Waltham, MA) where the counts per minute (CPM) were recorded. Non-specific binding was subtracted from total binding to find specific binding. Percent inhibition was determined using the following equation:$\% \, inhibition = 100 - \left(\frac{compound.CPM - nonspecific.CPM}{specfic.CPM} * 100\right)$

Purified compounds exhibiting 50% or greater inhibition were assayed for IC50 and K_i values. Ki and IC50 were determined by incubating 0.5-20ug of membrane with the radioligand concentration equivalent to the K_d, and 12 concentrations of each test compound from 0.001-300uM. The assay was setup and completed using the same methods used in the general screening process. The counts per minute (CPM) were recorded. K_i and IC50 values were calculated by a non-linear curve fit model using GraphPad Prizm 5.0 software.

EXPERIMENTAL

The volatile oil was prepared from *Z. bungeana* Juz. by hydrodistillation and analyzed by GC-MS. Eight major components (represent 94.41% of the oil) were identified: pulegone (**1**, 58.30%), isomenthone (**2**, 13.49%), menthone (**3**, 6.71%), α-limonene (**4**, 5.79%), isopulegone (**5**, 5.01%), isomenthol (**6**, 2.63%), β-pinene (**7**, 1.44%), and α-pinene (**8**, 1.04%). Pulegone **1**, can be considered a marker of essential oil *Z. bungeana* Juz.[4].

Preclinical study of sensitizing effect and the irritant action of the essential oil

Preclinical studies approved by the local ethical committee of Kazakh National Medical University (A voucher sample №1 from 01/27/2016). One day before the experiment, animal fur was carefully cut out on symmetrical parts of the sides. Size of the handled skin surface was 5.8% of the body of animal. Daily application, the essential oil, was applied on the skin for 4 weeks. The animals were kept in a restrained state during the application of the test substance over the 4-hour exposure.

Sensitizing effect was studied according to the general procedure, described in the "Guide for preclinical study of new pharmacological substances," edited by Habriyev R. U[5]. In order to study the sensitizing effect, light-colored guinea pigs were used. The animals were divided into 2 groups - experimental and control, 6 animals in each group. For studying sensitizing effect, single intradermal injection were carried out with 0.2 ml tuberculin syringe into the outer surface of the skin of the ear of test animals, 10 % solution of the essential oil were pre-dissolved. Identification of the sensitization effect was done on the 10th day with the help of conjunctive samples by subconjunctive administration of the essential oil: in the amount of 50 mg was applied with a spatula in the transition zone of mucous membrane of cconjunctiva and eye bulb of guinea pigs. After introduction of the substance for 1 min., lacrimonasal channel at the inner corner of the eye were pressed. Application of the substance was produced once. Visual observation of the state of the conjunctiva at the site of implantation of dosage forms was performed in 15 minutes and 24 hours. The damaging effect of the essential oil on the mucosa of the rabbit eye was assessed by the degree of hyperemia and edema due to point system:

1 - Slight reddening of the tear duct;

2 - Redness of the tear duct and sclera, directed to the cornea;

3 - Redness of the whole conjunctiva and sclera.

In order to study the irritant action, rabbits (6 animals in each group) were used. The study was held on with the help of conjunctive sample, using subconjunctive administration of the essential oil: in the amount of 50 mg was applied with a spatula in the transition zone of mucous membrane of palpebral conjunctivaand eye bulb of rabbits. After introduction of the substance for 1 min., lacrimonasal channel at the inner corner of the eye were pressed. Application of the substance was produced once. Visual observation of the state of the conjunctiva at the site of implantation dosage forms was performed immediately after administration in 15 minutes and 24-48 hours. The damaging effect of the essential oil on the mucosa of the rabbit eye was assessed by the degree of hyperemia and edema due to point system. All manipulations with animals were performed in accordance with the rules of humane treatment of animals, regulated by Federal Low "About the protection of animals against cruel treatment" from

01.01.1997 and the provisions of the European Convention for the Protection of Vertebrate Animals.

RESULTS AND DISCUSSION

Data on studying of anti-leishmanial activity of essential oil of *Ziziphora bungeana* Juz. was obtained for the first time. Data on the anti-leishmanial activity of essential oil (primary screening) present Table 2. Primary screening showed that *Ziziphora bungeana* Juz. essential oil doesn't possess anti-leishmanial activity. Antibacterial Activity The data indicate that the essential oil from *Z. bungeana* Juz. exhibit varying levels of antimicrobial activity against the microorganisms. As can be seen in Table 1, essential oil was found to have low activity against *C. glabrata*, *C. krusei*, *S. aureus and P. aeruginosa. C. albicans* was the most sensitive microorganism tested, with the highest percent inhibitions value (38). Lowest inhibitory activity of the oil against *MRS* and *E. coli* were also determined. No activity was observed against *Asp. fumigatus, Cr. neformans and M. intracellulare.* So, the oil from *Z. bungeana* Juz. showed the low antibacterial activity compared to the essential oil isolated from *Z. clinopodioides*[4] This results showed some antibacterial activity, indicating that the plant has potential use in phytotherapy. The results obtained in the course of the present study indicate that *Z. bungeana* Juz. seem to be sources for antibacterial drugs, especially against to *C. albicans* and can be usefull for other drugs due to moderate analgetic activity.

Cannabinoid and Opioid Receptors Binding Assay

Table 4 summarizes the Cannabinoid Receptor inhibition by essential oil from *Z. bungeana*. The data indicate that the oil exhibit moderate levels of binding with Cannabinoid receptor and that more actively was inhibition of CB1 receptors. Results obtained from Radioligand Receptor Binding Assay, indicate that Kappa (26,3% inh.***)*** and Mu (31,9 % inh.) types are the most sensitive receptors, with the higher percent inhibition. With phytochemical studies, *Ziziphora* species was found to be rich in particular for Pulegone[6] and also reported its strong antioxidant activity[7] antidepressant effects[8], immunomodulatory effects[9]. According to literature searched, nothing is known about the Cannabinoid receptor binding properties of *Ziziphora* species. Based on these results, it is possible to conclude that essential oil from *Z. bungeana* have a moderate Cannabinoid receptor binding activity.

CONCLUSIONS

Biological activity

It is experimentally proved that the essential oil of *Ziziphora bungeana* Juz.. does not possess anti-leishmanial activity. Investigations of anti-leishmanial, activity were carried out for the first time. The results obtained in the course of the present study indicate that *Z. bungeana* Juz. seem to be sources for antibacterial drugs, especially against to *C. albicans* and can be usefull for other drugs due to moderate analgetic activity. The data indicate that the oil exhibit moderate levels of binding with Cannabinoid receptor and that more actively was inhibition of CB1 receptors. Results obtained from Radioligand Receptor Binding Assay, indicate that Kappa and Mu types are the most sensitive receptors, with the higher percent inhibition.

Sensitizing effect

Visually, signs of sensitizing effect were not observed on the 10th day of the experiment in 15 minutes and 24 hours after a single subconjunctival administration of the essential oil on mucous membrane of palpebral conjunctiva and guinea pigs; a slight reddening of the tear duct, appeared immediately after injection, disappeared for 30 minutes. The mucous membrane of the eye was not swollen, usual-colored. Hemorrhages were not found; increased lacrimation was not observed.

Irritant action

Assessment of irritant effects during subconjunctival administration of the essential oil was visually performed. Reactions were taken into account immediately after applying the substance in 15 minutes (quick response) and in 24-48 hours (hypersensitivity) delayed-type. During the observation period the some signs of irritant action (redness, swelling, etc.) on the mucous membranes of the conjunctiva was not observed, which indicates the absence of irritating action. General condition of the animals was without aberration during the whole observation time.

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