ORIGINAL ARTICLET

Phytochemical investigation and technology production of alkaloids in the Kazakh endemic plant *Echinops albicaulis* Kar.Et Kir. (Asteraceae)

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ABSTRACT

Aim: Search the main sources of biologically active substances from raw materials of plant genus *Echinops* L. standardization of extracts from raw materials of plant genus *Echinops* L. **Materials and Methods**: Ultraviolet spectroscopy, one-dimensional paper chromatography, atomic adsorption spectroscopy, gas-liquid chromatography method, concentration and sublimation method, mass spectroscopy, and nuclear magnetic resonance. **Results and Discussion**: Two major alkaloids were isolated from the ethanol extract of *Echinops albicaulis*. The isolated compounds were chemically identified as echinopsin (1)1-methyl-4(1H)-quinolone; 1,4-dihydro-1-methyl-4-oxoquinoline; N-methyl-4quinolone (C10H9NO), echinorin (2)1-methyl-4-methoxyquinolinium (C11H12NO). Compounds FR-100-2 showed antileishmanial activity against *Leishmania donovani* and *Trypanosoma brucei* with IC90 values of 5.33 µg/ml and 1.8 µg/ml, respectively. A technological scheme for the preparation of a drug substance from plants *Echinops* L. **Conclusion**: The study indicates that in endemic plants contain a large amount of alkaloids; this is necessary for the standardization of plants. What are the excipients which can be used for making pill of apple cider vinegar a useful active ingredient as a diet supplement and antiobesity formulation.

Key words: Antileishmanial activity, *Echinops albicaulis,* echinopsin, echinorine alkaloids

INTRODUCTION

A valuable raw material for the production of pharmaceutical preparations is alkaloid containing medicinals plants of Kazakhstan, rich in bioactive compounds, which are in increasing demand in the global market.

Kazakhstan species of plants like *Echinops* L. have not been subjected to systematic investigation, in connection with this study of the chemical composition, development of methods for the identification of potentially biologically active materials, the study of the biological activity, development of new medicines, and herbal remedies is relevant [1,2].

Echinops L. covers about 130 species, mostly confined to the Palearctic temperate latitudes. Of 58 species *Echinops* encountered in the territory of the former USSR, the flora of Kazakhstan indicated 18.[3,4] Of the 58 species of the mordant found on the territory of the former USSR, 18 in the flora of Kazakhstan,[5] according to Abdulina[6] shows 19 species, with the addition of *Echinops fastigiatus* R. Kam.et Tscherneva.

Diversity of the specie is characterized by mountainous areas of Central Asia and Kazakhstan, which likely to be the centers of its origin. In flora of Kazakhstan, this specie is represented by seven endemic species: White-stemmed *Echinops albicaulis* Kar.Et Kir., Kazakh *Echinops kasakorum*

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Received: 10-04-2017
Revised: 03-05-2017
Accepted: 16-05-2017

Pavl., almost bare *Echinops subglaber* Schrenk., rough-scaled *Echinops pubisquameu* Iljin, Zailiyskiy *Echinops transiliensis* Golosk., Talas *Echinops talassicus* Golosk., Zaysan *Echinops saissanicus* (Keller) Bobr.[6] Rare species include *Echinops kazakorum*, *Echinops fastigiatus*, *Echinops saissanicus*.[7]

Biological activity (antifungal, anti-inflammatory, antifertil) has been established for species *Echinops ritro, Echinops ellenbeckii, Echinops longisetus*, and *Echinops echinatus*.[8] Phytogenous, karyological, environmental, and other features of plants were also studied. Taking into consideration the principle of chemotaxonomic relationship, the presence of such compounds and biological activity can be predicted for Kazakhstan representatives of this species.

E. albicaulis Kar.Et Kir. (Asteraceae family) White-stemmed *Echinops*, aksabak laksa [Figure 1] - a perennial herb with a height of 40-80 cm. The root is ligneous and vertical. The stem is single or in the number of 2, at the top sometimes short-branched, angulate, dense, and white-tomentose along the entire length, without glandular pubescence. The leaves are leathery, greenish-gray on the top from loose cobwebby pubescence, corolla is bluish-whitish or white, deeply notched into narrow linear lobes, the tube at the top is weak short-bristly. Achene is ribbed coated with pressed, yellowish-brown tula, closing the crest.[9]

It blooms in late June to early July, fruiting in July. It grows on sands and sandy loams in the northern deserts. The spread in Kazakhstan. It is found in the Aral Sea region, Kyzylorda region, Betpakdala, Murong Kum, Balkhash, Kyzyl Kumah (north). Endemic plant, not edible by farm animals.

MATERIALS AND METHODS

The objects of research were endemic species of plants *Echinops* from Asteraceae family: *E. albicaulis* Kar.Et Kir. (Asteraseae) White-stemmed *Echinops*, aksabak laksa [Figure 1] - were gathered on Malaysary pass belonging to the southwestern spurs of the Embassy of Jungar Alatau, Republic of Kazakhstan, identified population of White-stemmed *E. albicaulis,* grows in hilly-ribbed sand adjacent to the pass, and other part is zhuzgun absinthe plant formation.[10,11]

The whole plant of *E. albicaulis* Kar.Et Kir. of the Compositae Family was collected from Malaysary, Kazakhstan, North of lake (121 km), Khapchagai to the southwestern spurs of the Jungar Alatau.

The plant seeds were collected during the month of July 2015, while aerial parts (leaves/stem) and roots were collected during July and August (flowering time) and were cleaned, dried at room temperature in the shade then pulverized by mechanical mills and weighed, voucher No 9442.

Figure 1: White-stemmed *Echinops albicaulis* Kar.Et Kir., (Asteraceae Dumort Family) aksabak laksa, (named of Kazakh) endemic plant

Chromatographic Separation of Total Alkaloids

Extraction and separation of total alkaloids take place by column chromatography performed using ready-made silica gel plates GF254 nm (20 cm × 20 cm) 0.25 mm thick (MERCK) and aluminum oxide and with three different solvent exhibition systems:

E1 = Benzene:methanol (8:2).[12]

E2 = Chloroform:acetone:diethylamine (5:4:1).[12]

E3 = Toluene:ethyl acetate:diethylamine (70:20:10)[12] and exhibited using the Dragendorff's solution, spraying reagent. Preparative high-performance liquid chromatography (HPLC) was carried out using: Acetonitrile:water (60:40) as a mobile phase. Column: Mediterranea C18, 5 um 15 × 2.12 sm, flow rate: 5 ml/min Injection volume: 1 ml. Detection: Ultraviolet (UV). Detector at a wave length of 210 nm.

Experimental Work

Chemical tests were carry out[13-15] using alcoholic extracts from plants and or their powdered samples using standard procedures to identify the active components.

The test for alkaloids: Reagents Wagner and Mayer.
Test flavonoids: Test lead acetate test and NaOH.
Test terpenoids: NaOH tests.
The test for tannins: The test solution $FeCl_3$.
Tests for steroids: Lieberman-Burchard test.
The test for tannins: The test solution $FeCl_3$.
Saponins test: Foam test.
Tests to anthraquinones: Test Borntrager.
The test for cardiac glycosides: Keller-Kiliani test.

Extraction Method [Figure 2]

Extraction and isolation of constituents The dried and pulverised aerial parts of E. albicaulis were macerated in 80% alcohol (4L x 3) at room temperature. The combined aqueous alcohoiic extract was concentrated under reduced pressure to afford a dark brown viscous residue , which was then added with chloroform to give the chloroform extract. Then add + 5% HCl/EtoAc in batches until complete extraction

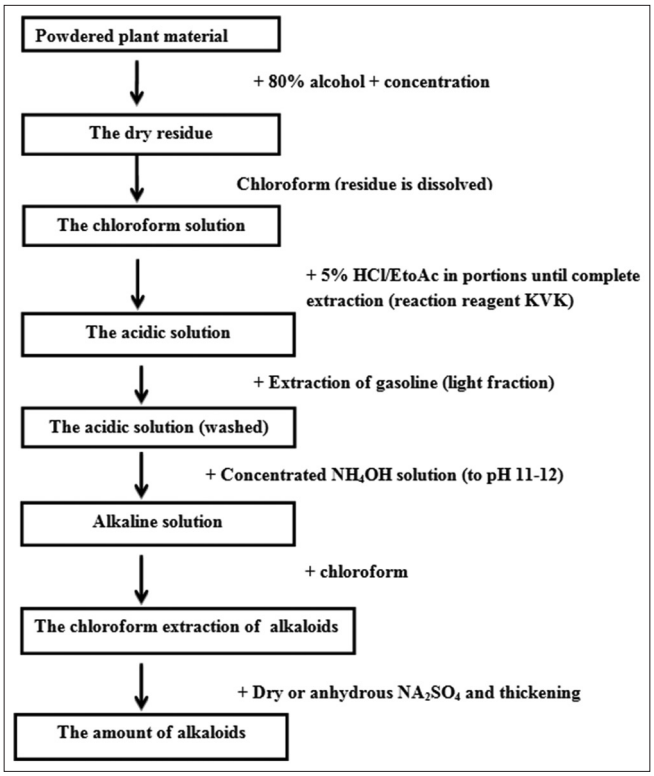

Figure 2: General scheme for the separation of various plant components

(KVK reaction reagent). The resulting acidic solution is extracted with gasoline to a light fraction. The resulting acidic solution is concentrated with NH4OH to pH 11-12. The obtained alkaline solution is added with chloroform, we obtain chloroform extract of alkaloids. The solution is concentrated in a rotary evaporator, the total amount of alkaloids is obtained.

RESULTS AND DISCUSSION

Preliminary qualitative phytochemical analysis: Results of screening and phytochemical analysis are shown in Table 1.

The preliminary screening and phytochemical analysis of extracts showed the presence of alkaloids, flavonoids, steroids, terpenoids, amino acids, carbohydrates, coumarin glycosides, and tannins from different parts of *Echinops* in different percentages, as well as absence of saponin and anthraquinone and all parts of the plant.

Table 2 shows that alkaloids, tannins, and free organic acids predominate in reproductive organs in terms of quantitative content and are equal to underground parts of *E. albicauli*s, whereas flavonoids and amino acids, alkaloids - in the aerial parts of the plants. The root is also not inferior to the quantitative content of amino acids. It is generally known that the phytochemical composition of plant materials mainly depends on the conditions under which the plant was growing. For example, biosynthesis, and consequently, alkaloids,

Table 1: Phytochemical screening of different parts of *Echinops albicaulis*

Plant part	Alkaloids	Flavonoids	Steroids	Carbohydrates	Tannins	Coumarins	Terpenoids	Glucoside	Amino acids	Antraquinoin	Saponins
Seeds	+	+	+	+	-	+	+	+	+	-	-
Arial part	+	+	+	+	+	+	+	+	+	-	-
Roots	+	+	+	+	+	-	+	-	+	-	-

Table 2: Qantitative analysis of plants of the genus *Echinops albicaulis* Kar.Et Kir., %

Names defined parameters of biological active substances	Reproductive organs (seeds, buds) (%)	Aerial part (Stem, leaves) (%)	Root (%)
Alkaloids	6.4	7.52	5.25
Tannins	1.97	1.73	1.33
Flavonoids	1.55	2.17	1.21
Free organic acids	6.02	3.48	4.48
Amino acids	11.91	12.3	10.03
Polysaccharides	11.2	9.6	8.1

amino acids and phenols in plant material are directly related to ambient temperature; the content of polysaccharide and glycosides depends on moisture, content of the mineral components on the soil composition. Consequently, it can be concluded that the phytochemical composition of the plant material varies not only from gathering place but also from the weather conditions in the year of gathering.[13,14]

Preliminary Identification of Various *Echinops* Parts by Thin-layer Chromatography (TLC)

Thin-layer chromatography of fraction 1 (F-1) got from peculiar parts of the *Echinops*, certifies the following: (a) The presence of three distinct alkaloids in fraction-1 (named L1, L2) which is obtained from seeds part and two alkaloids in the same fraction acquired from roots part (L1 and L2) [Figure 3].

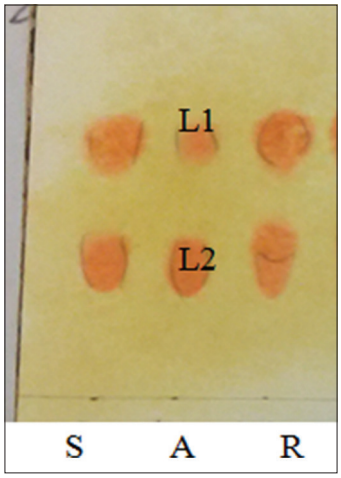

Figure 3: Thin-layer chromatography of fraction one (F-1) for different *Echinops albicaulis* parts (seeds, aerial parts, and roots) using silica gel GF254 nm as adsorbent and S1 as a mobile phase. Detection by Dragendorffs spraying reagent S: Seeds, A: Aerial part, R: Roots

Isolation and Purification of Alkaloids

Two chromatographic analysis were accomodated to isolate in a pure form two alkaloids (named L1, L2) found in the plant which are: Preparative HPLC and preparative TLC since seeds consist of the largest number and highest quantity of the alkaloids so alkaloids fraction obtained from seeds part was used to divide and isolate these compounds in a pure form.

Isolation and Purification of Alkaloids by Preparative HPLC

About 1 g of F-1 gained from plant seeds dissolved in a minimum quantity of chloroform was injected into preparative HPLC [Figure 4].

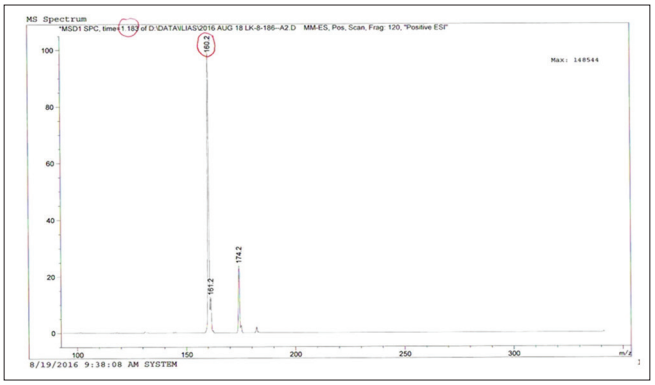

Figure 4: Preparative high-performance liquid chromatography analysis of fraction-1 obtained from aerial part of plant observing two peaks represent two different compounds, one of them (L1) is a major one

Two samples collected from preparative HPLC were weighted and classified to co-TLC. Weight of L1 = 0.085 g and weight of L2 = 0.21 g [Figures 5 and 6].

Isolation and Purification of Alkaloids by Preparative TLC

On a 20 cm × 20 cm glass plates, a slurry of 75 g of silica gel GF 254 suspended in 150 ml of distilled water was handled in 1 mm thickness manually using Jobling Laboratory Division plate coater. The freshly coated plates were left until the transparency of the layer abandones. After 10 min, the plates stacked in a dry rack and heated in vertical position for 1 hour at 110°C with occasional opening of the oven door from time to time to let moisture withdrawal. The completely dried and activated plates were retained in

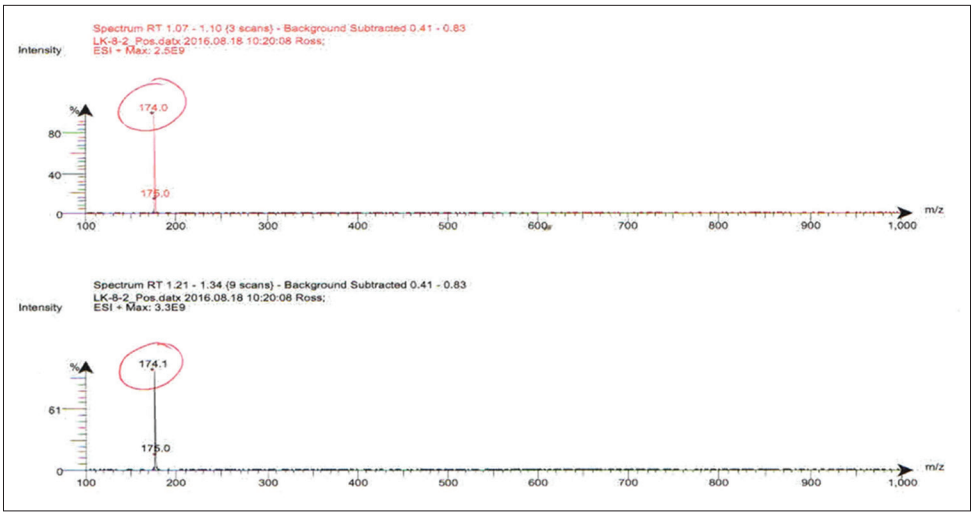

Figure 5: Mass spectrum of fraction-1 obtained from aerial part of plant *Echinops albicaulis*

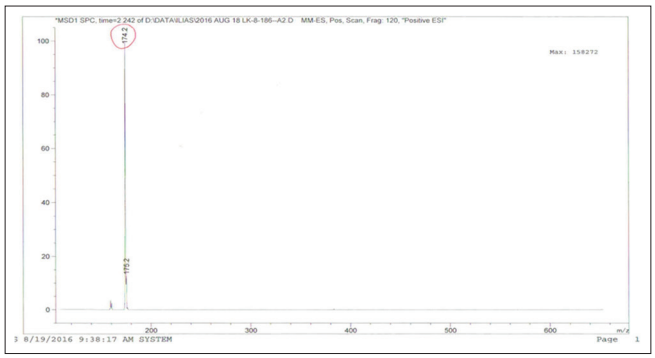

Figure 6: Preparative high-performance liquid chromatography analysis and mass spectrum of fraction-2 obtained from aerial part of plant *Echinops albicaulis*

a dry and moisture free container accommodating adsorbent silica gel.

1 g of F-1 obtained from plant seeds (highest quantity) softened in a minimum quantity of chloroform and implemented on a number of preparative TLC plates using S1a solvent system. The solvent was let to grow to a height of 15 cm from the base line. One major and two minor bands were detected after spraying a side of plates with dragendorffs three band had been scrapped off, ended with chloroform, then filtered. The filtrate evaporated to dryness, *in vacuo* to deliver white crystals, on re-crystallization out of boiling ethylacetate, a fluffy white crystals of L1, L2 were reached.

Two samples collected from preparative TLC were weighted and classified to co-TLC.

Weight of L1 = 0.0420 g, weight of E2 = 0.402 g, from the above results, the quantity of compounds obtained in a (pure form) by preparative HPLC is higher than that obtained by preparative TLC. Classical preparative TLC suffers from several drawbacks, the main disadvantage being the removal of purified substance from the plate and its subsequent extraction from the sorbent, other drawbacks include the length of time required for the separation and degree of purity for the separated compounds, compare with preparative HPLC, which is consider know, the most powerful and versatile method for purification tasks in the pharmaceutical industry.[14]

Despite the fact that among the tools used in the large scale purification of pharmaceuticals, Preparative HPLC is one of the more expensive and solvent-consuming approaches, it yields the highest-purity drug substance. The interest in preparative HPLC will continue to grow because of the increasing uncertainty in the market expectations for product purity. Its nearly linear scalability makes preparative HPLC one of the more viable approaches to compound purification.[15]

Characterization and Identification of the Isolated Alkaloids

(1) Melting point: The isolated compound which is named L2 had a sharp melting point of 160-162°C. (2) Ultra violate spectra: The isolated alkaloid (L2) show UV absorption near 242 nm; (3) Fourier transform infrared spectra. 5-H and C nuclear magnetic resonance (NMR) and 13C NMR spectra (dimethyl sulfoxide [DMSO], 75 MHz): With chemical shifts typical of quinoline rings(28) in the ranges of δC 21.12 (C-2), 24.77 (C-3), 170.13(C-4),126.987 (C-5), 121.825 (C-6), 127.640 (C-7), 114.951 (C-8), 138.26 (C-9), 123.47 (C-10), 30.4 (C-11). 1H NMR (DMSO-d6-, 300 MHz) revealed that E2 compound undergo tautomerism which lead to the appearance of chemical shifts of the hydroxyl group at 10.02 at (C-4), 2.4 (3H, as a singlet of the methyl protons), 2.6 (2H, d, H-2),5.09 (1H,s, H-3), 6.84-7.15 (4H, m, H-5, H-6, H-7, H-8). It is two compounds isolated (for the first time) from Kazakh *E. albicaulis* plant; it seen to be the hydrogenated form of echinopsine (1-Methyl-4(1H)-quinolinone), an alkaloid isolated from 14 species of *Echinops* plant.

1-(4-(4-fluorophenoxy)but-2-yn-1-yl)piperidine (3). Yield: 72%; mp: 104-106°C; 1H NMR (400 MHz, DMSO-d6): 7.13 (2H, dd, ArH), 7.04 (2H, dd, ArH), 4.89 (2H, s, O-CH2), 3.30 (2H, s, CH2-N), 2.81 (2H, dd, pyp), 1.73 (2H, m, pyp), 1.25 (1H, m, pyp). 13C NMR: 156.2, 153.7, 117.1, 116.9, 116.4, 116.2, 85.4, 77.3, 56.5, 51.5, 45.1, 22.6, 21.5; MS, m/z: 248.1451 [MNa]+. C15H19NOF. Calculated: M = 248.1252 [Figures 7-10].

The Biological Activity of the Isolated Alkaloids from *E. albicaulis*

Antimicrobial activity

All organisms are obtained from the American type culture collection (ATCC) (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028 (Ca), *Candida glabrata* ATCC 90030 (Cg), *Candida krusei* ATCC 6258 (Ck), *Cryptococcus neoformans* ATCC 90113 (Cn), and *Aspergillus fumigatus* ATCC 204305 (Af) and the bacteria *Staphylococcus aureus* ATCC 29213 (Sa), methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218 (Ec), *Pseudomonas aeruginosa* ATCC 27853 (Pa), and *Mycobacterium intracellulare* ATCC 23068 (Mi). All organisms are tested using modified versions of the CLSI (formerly NCCLS) methods. For all organisms excluding Mi and Af, optical density is used to monitor growth.[15,16] Media supplemented with 5% Alamar Blue™ (BioSource International, Amarillo, CA) is utilized for growth detection of Mi[17,18] and Af.[19] Samples (dissolved in DMSO) are serially diluted in 20% DMSO/saline and transferred (10 µL) in duplicate to 96-well flat bottom microplates. Inocula are prepared by correcting the OD630 of microbe suspensions in incubation broth [RPMI 1640/0.2% dextrose/0.03% glutamine/MOPS at pH 6.0 (Cellgro) for *Candida* spp., sabouraud dextrose for Cn, cation-adjusted Mueller-Hinton (Difco) at pH 7.3 for *Staphylococcus* spp., Ec, and Pa, 5% Alamar Blue™ (BioSource International, Camarillo, CA) in Middlebrook 7H9 broth with OADC enrichment, pH = 7.0 for Mi, and 5% Alamar Blue™/RPMI 1640 broth (0.2% dextrose, 0.03% glutamine, buffered with 0.165M MOPS at pH 7.0) for Af to afford an assay volume of 200 µL and final target inocula of: *Candida* spp. and Cn: 1.5×10^3, Mi: 2.0×10^6, *Staphylococcus* spp., Ec, Pa: 5.0×10^5 CFU/ml, and Af: 2.7 × 104 CFU/mL. Final sample test concentrations are 1/100th the DMSO stock concentration. Drug controls (Ciprofloxacin [ICN Biomedicals, Ohio] for bacteria and amphotericin B (AMB) [ICN Biomedicals, Ohio] for

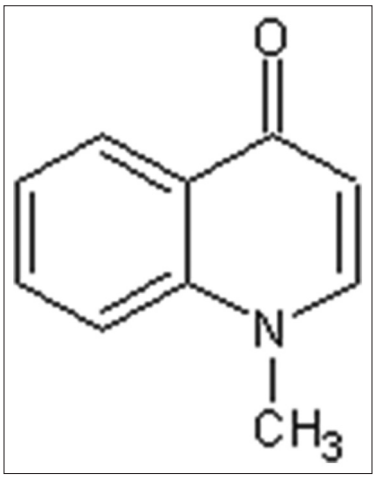

Figure 7: Echinopsine (1-Methyl-4(1H)-quinolinone)

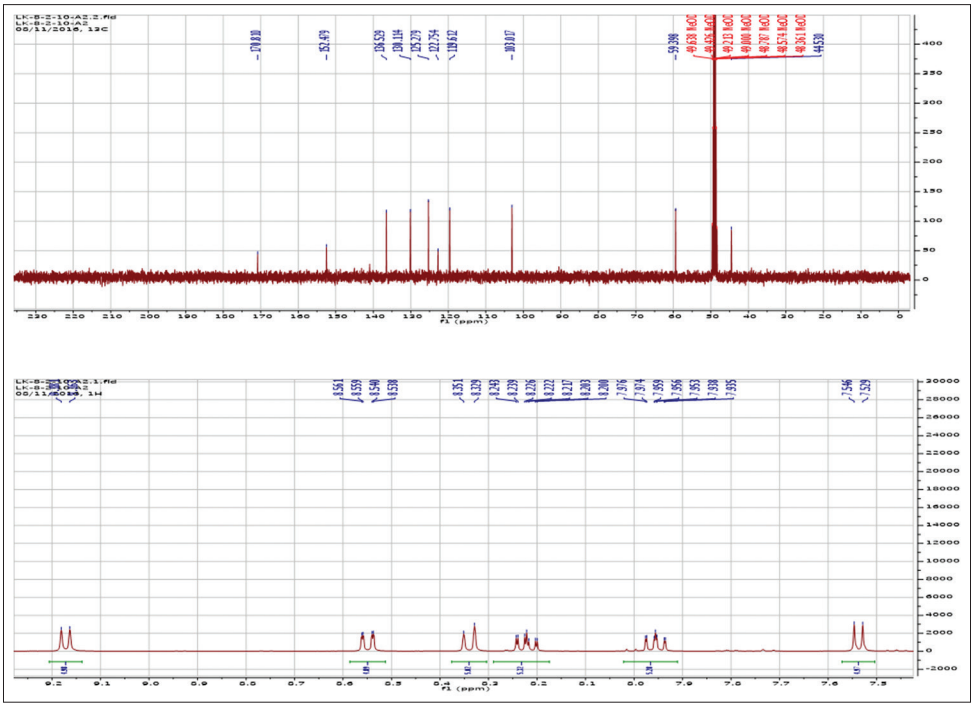

Figure 8: 13C-nuclear magnetic resonance analysis of the isolated L1 compound

fungi) are included in each assay. All organisms are read at either 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (Mi, Af) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) before and after incubation: *Candida* spp. at 35°C for 46-50 h, *Staphylococcus* spp., Ec, and Pa at 35°C for 16-20 h, Cn at 35°C for 70-74 h, Af at 35°C for 46-50 h, and Mi at 37°C and 10% CO_2 for 70-74 h. IC50s (concentrations that afford 50% inhibition relative to controls) are calculated using XLfit 4.2 software (IDBS, Alameda, CA) using fit model 201.

Antileishmanial activity

The products were screened for their ability to inhibit *Trypanosoma brucei* and *L. donovani*, a fly-borne protozoan that causes visceral leishmaniasis. According to the LEM Assay, all samples (2 and 20 mg/mL) are tested in a concentrations of 40, 8.0 and 1.6 µg/mL and IC50 as well as IC90 (test concentration that affords 90% inhibition of the protozoan relative to controls) are reported [Table 1]. All IC50 and IC90 are calculated using the XLFit fit curve fitting software. The drug controls pentamidine and AMB are used as positive controls.

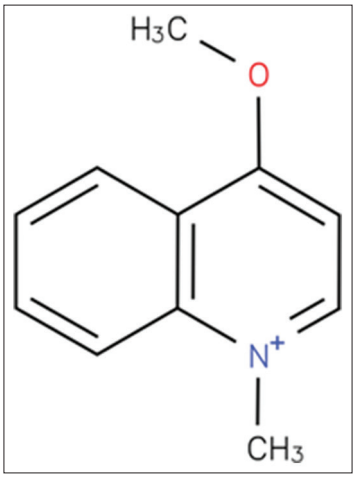

Figure 9: Quinolinium, 4-methoxy-1-methyl

Antimalarial activity and cytotoxicity

The antimalarial activity is determined against chloroquine (CQ) sensitive (D6) and CQ resistant (W2) strains of *Plasmodium falciparum* by measuring plasmodial LDH activity according to the procedure of Makler and Hinrichs.[20] A suspension of red blood cells infected with D6 or W2 strain of *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) is added to the wells of a 96-well plate containing 10 µL of serially diluted samples (plant extracts, column fractions or pure compounds). The plate is incubated at 37°C, for 72 h in a modular incubation chamber with 90% N2, 5% O_2, and 5% CO_2. Parasitic LDH activity is determined by mixing 20 µL of the incubation mixture with 100 µL of the Malstat™ reagent (Flow Inc., Portland, OR) and incubating at room temperature for 30 min. 30 ml of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) is then added and the plate is further incubated in the dark for 1 h. The reaction is then stopped by adding 100 µL of a 5% acetic acid solution and the absorbance is read at 650 nm. Artemisinin and CQ are included as the drug controls. IC50 values are computed from the dose response curves of growth inhibition using XLfit 4.2. The *in vitro* cytotoxicity of samples to mammalian cells was also tested to determine the selectivity index of the antimalarial activity.

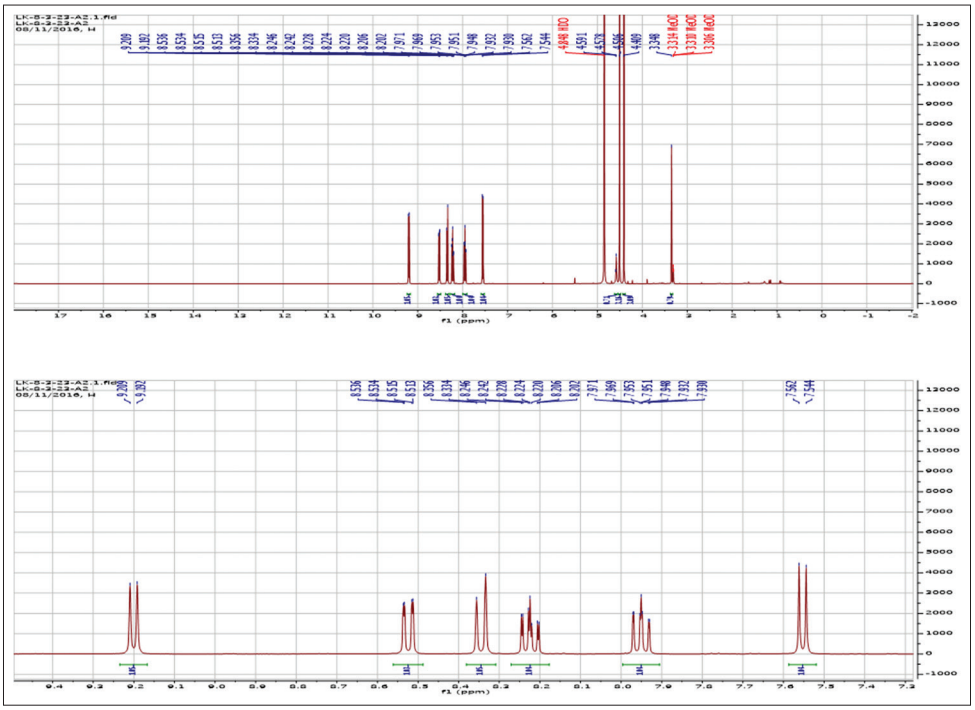

Figure 10: 13C-nuclear magnetic resonance analysis of the isolated L2 compound

The assay is performed in 96-well tissue culture-treated plates. Vero cells (monkey kidney fibroblasts) are seeded to the wells of 96–well plate at a density of 25,000 cells/well and grown for 24 h. Samples at different concentrations are added, and cells are further incubated for 48 h. Cell viability is determined by Neutral Red method.[21] IC50 values are obtained from dose-response curves. Doxorubicin is included as drug control.

CONCLUSION

Phytochemical investigation of a new wild Kazakh plant used traditionally for wound healing and the results revealed the presence of alkaloids, flavonoids, terpenoids, tannins and steroids in the different plant parts and in different percentages, aerial parts contain the highest quantity of flavonoids, while seeds contain the highest amount of alkaloids.

Alkaloids from the endemic species *E. albicaulis* were first isolated. Two chromatographic analysis were performed to isolate in a pure form two alkaloids from aerial part (which contain the highest quantity): Preparative HPLC and preparative TLC, where the quantity of compounds obtained by preparative HPLC was higher than that isolated by preparative TLC.

The advantage of the preparative Mass is to divide the maximum quantity of desired products with the required purity in the minimum time to use it as a standard reference. For the first time, alkaloids were isolated from *E. albicaulis* from various parts of plants. Kazakh endemic plant *E. albicauli*s showed high antimicrobial, antileshmanial, antimalarial, activities. Among all activities only antileishmanial activity is notable. Only Fr 100-2 discovered activity against Leishmania Donovany strains, with an IC90 value of 5.33 µg/ml. Basically, extracts were active against *T. brucei* strains. Their IC90 value ranged between of 1.8 µg/ml (Fr 100-2) to 16.23 µg/ml.

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Source of Support: Nil. **Conflict of Interest:** None declared.