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Original article

The first bioactivity studies of *Acantholimon lycopodioides* from high altitude Karakoram-Himalayan desert

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ABSTRACT

Couple of ethnopharmacological surveys in the Indian Ladakh and Pakistani Shigar valleys has reported the medicinal use of *Acantholimon lycopodioides* against cardiac and gastric disorders that however, remains without scientific rationale or experimental validations. Here, we assess the *in vitro* bio/therapeutic activities of *A. lycopodioides* extracts as well as chloroform, ethyl acetate, n-butanol and aqueous fractions. The *in vitro* β-carotene-linoleic acid bleaching and DPPH radical scavenging methods demonstrated a very high anti-oxidative property of chloroform and ethyl acetate fractions compared to others. Cell viability assay (MTT) on human cervical (HeLa), breast (MDA-MB321) and liver (HepG2) cancer cells revealed their differential cytotoxicity, except the chloroform fraction. Of these, the precipitate exerted highest cytotoxicity on HepG2 cells followed by aqueous fraction on MDA-MB321 cells. Notably, the non-cytotoxicity of chloroform fraction coincided with its highest anti-oxidative activity. Further, the chloroform fraction showed marked hepatoprotection (up to 84%) against 3′7′dichlorofluorescin triggered free radicals induced oxidative damage. Also, the hepatoprotective chloroform fraction mildly activated CYP3A4 in HepG2 cells (dual-luciferase assay). Moreover, the *A. lycopodioides* extracts and fractions showed differential anti-bacterial and anti-fungal activities. Of these, while *S. aureus* was more sensitive to the water-insoluble extract, ethyl acetate fraction showed moderate activity against *E. coli* and *C. albicans*. On the other hand, the chloroform fraction showed promising activity against *S. Aureus*, *C. albicans, P. vulgaris and E. faecalis*. In conclusion, our data for the first time, demonstrated promising anti-oxidative, hepatoprotective, anti-cancer, anti-microbial and CYP3A4 activating salutations of *A. lycopodioides*. This warrants further studies towards isolation and identification of its therapeutically active principles.

1. Introduction

Acantholimon is the second most species-rich (~200) genus in Plumbaginaceae family of cold-adapted xerophytic plants that grow in stony soils or rocks (Kubitzki et al., 1993). *Acantholimon* spp. are pulvinate to densely branched shrubs/subshrubs that bear spike-like inflorescences with rigid acuminate or paniculate leaves (Baker, 1948). Most of them are geographically restricted in mid to high altitude mountains of southeastern Russia, Kazakhstan, Tajikistan, Western Iran, Afghanistan, northwest China, southern Mongolia, western Pakistan and northern India, and to little extent in the Mediterranean region (Moharrek et al., 2019). In the mountainous Wakhan and Pamir in northeastern Afghanistan, *Acantholimon* is an important fuel source (Soelberg and Jäger, 2016). The *Acantholimon* roots decoction is used as a food supplement in some Turkish desserts (Dogan and Akadian, 2005). *Acantholimon* spp. are traditionally used in Iran to treat diabetes and liver disorders where the methanol-extracts of *A. bracteatum* (Nasiri et al., 2016) and *A. gilliati* (Gazor et al., 2017) are shown hepatoprotective in rodent models. In Lebanese traditional medicine, *A. antilibanoticum* roots decoction is used to treat wound and skin injuries (Baydoun et al., 2015). A. acerosum is consumed as tea in Turkey for microbial infection, such as tuberculosis (Ari et al., 2015). Notably, there is no data available on phytochemical analysis or isolation of bioactive compounds from *Acantholimon* spp. Though isolations of few amino acids including a hydroxypipecolic acid from other genera of Plumbaginaceae are reported, they could

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never be detected in leaf extracts of *Acantholimon* spp. (Fowden, 1958).

A. lycopodioides Boiss. (Synonyms: *A. tibeticum, Statice lycopodioides, Armeriastrum lycopodioides*), commonly known as Prickly thrift is distributed in the mid to high altitude Karakoram-Himalayan mountain ranges of India (Ladakh, Zanskar, Dras), Pakistan (Gilgit, Baltistan, Astor, Swat), China (Xinjiang), Afghanistan (Badakshan, Wakhan, Baghlan, Nuristan, Laghman), Tajikistan and Tibet (Hassler, 2019) (Fig. 1A). *A. lycopodioides* has been documented in Himalayan high altitude plants biomass and diversity field studies (Namgail et al., 2012; Khan et al., 2013) and phylo-transcriptomic workflow project (Yang et al., 2017). Notably, only two ethnopharmacological surveys among traditional healers in the Ladakh (India) and Shigar (Pakistan) valleys have reported the medicinal use of *A. lycopodioides* against cardiac (Kala, 2006; Angmo et al., 2012) and gastric (Abbas et al., 2017) disorders, respectively. However, to the best of our knowledge, there is no phytochemical and bioactivity studies on *A. lycopodioides*. With this background information, we have for the first time, studied the *in vitro* biological activities of *A. lycopodioides*.

2. Materials and methods

2.1. Plant material collection and identification

The whole flowering plant of *A. lycopodioides* Bioss, locally known as Longze was collected from Khardong La (18,000 ft or 5570 m a.s.l.), Ladakh valley (India) in July 2018 (Fig. 1B and C). The plant was identified by a Ladakhi herbalist-Amchi practitioner and further confirmed by Dr. Tariq Husain, a plant taxonomist at National Botanical Research Institute, Lucknow, India.

2.2. Extraction and fractionation

The dried and ground aerial parts of *A. lycopodioides* (AL: 42.0 g) were consecutively extracted at room temperature with 85% ethanol till exhausted. The alcoholic extract was concentrated to dryness under reduced pressure at 4 °C, using rotary evaporator to give brown residue (8.6 g). The dried alcoholic extract was further suspended in double-distilled water to give water-insoluble part (AL-A: 992.0 mg) and water-soluble part (AL-B). Extract AL-B was kept overnight furnishing a precipitate (AL-B1: 534.3 mg). The remaining AL-B was fractionated successively with chloroform (AL-B2: 15.2 mg), ethyl acetate (AL-B3: 379.0 mg) and finally with n-butanol (AL-B4: 13.0 mg) along with aqueous mother liquor (AL-B5: 1.9 g) as per the standard procedure.

2.3. In vitro anti-oxidant assays of A. lycopodioides extracts and fractions

2.3.1. DPPH radical scavenging assay

The *A. lycopodioides* extracts and fractions (AL-A, AL-B1, AL-B2, AL-B3, AL-B4 and AL-B5) were prepared in methanol to furnish five different concentrations (10, 50, 100, 500 and 1000 µg/ml, each). The DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was performed as described elsewhere (Brand-Williams et al., 1995). Briefly, the samples were assayed in a final volume of 1 ml (500 µl of sample, 125 µl freshly prepared DPPH and 375 µl methanol). While ascorbic acid (10, 50, 100, 500 and

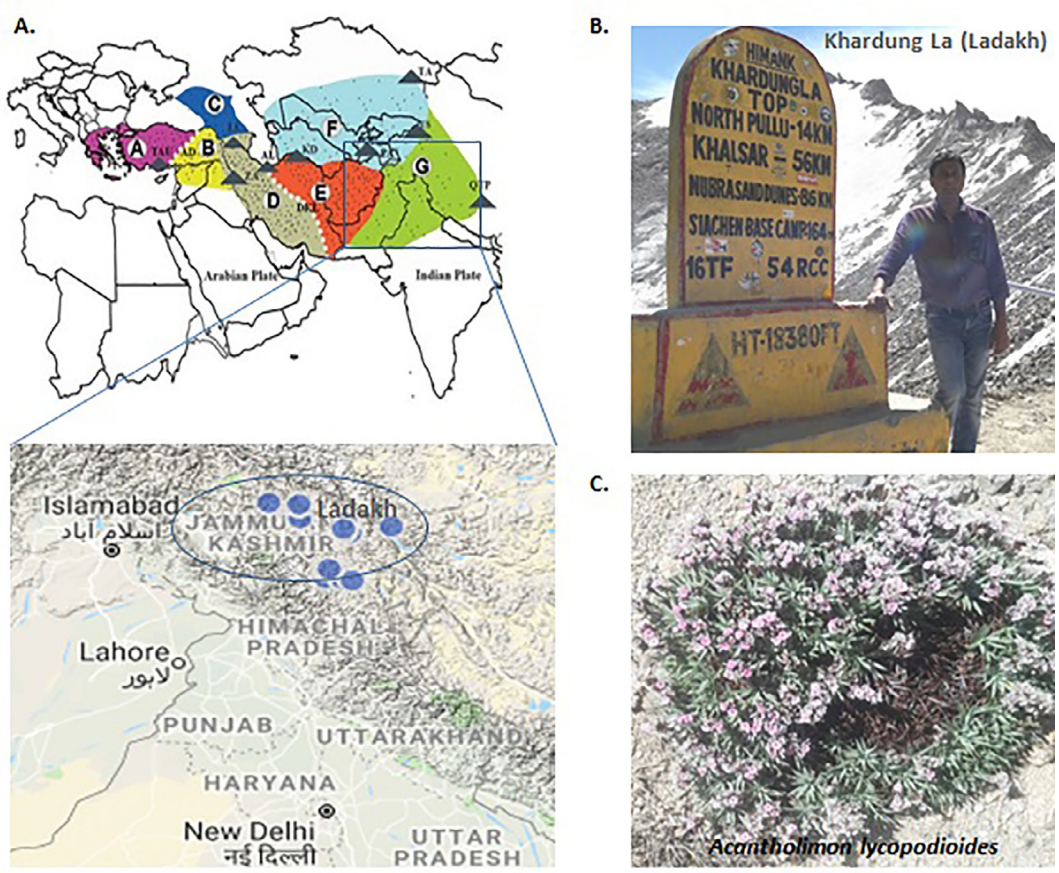

Fig. 1. *Acantholimon lycopodioides*: (A) its geographical distribution in high altitude Karakoram-Himalayan ranges (Moharrek et al., 2019; https://www.gbif.org/occurrence/download?taxon_key=4089167); (B) its collection location in Ladakh, India; and (C) the whole flowering plant.

1000 μg/ml) was used as standard or positive control, 0.2 ml of 80% (v/v) methanol served as the negative control. Following 30 min of incubation at 25 °C, the absorbance (A; λ = 517 nm) was measured using a spectrophotometer (UV mini-1240, Shimadzu, Japan). The radical scavenging activity was calculated for each sample [%Radical scavenging activity = (Ac − As/Ac) × 100; Ac and As are the absorbance of the control and sample, respectively].

2.3.2. β-Carotene–linoleic acid assay

The *A. lycopodioides* extracts and fractions were further evaluated for their anti-lipid peroxidation activities, using β-carotene bleaching method as described elsewhere (Veligoglu et al., 1998) with little modification. Briefly, 1 ml of freshly prepared β-carotene solution in chloroform (0.2 mg/ml) was added to flasks containing 0.02 ml of linoleic acid and 0.2 ml of Tween-20. The chloroform was evaporated at 40 °C using a rotary evaporator and the residue was immediately diluted with 100 ml of distilled water, and mixed to form an emulsion. Rutin (1000 μg/ml) was used as standard or positive control while 0.2 ml of 80% (v/v) methanol served as negative control. A similarly prepared mixture prepared without β-carotene was used as blank. A 5 ml aliquot of the emulsion was added to a tube containing 0.2 ml of a sample (1000 μg/ml) and incubated at 40 °C for 2 h. The absorbance (A; λ = 470 nm) was recorded (UV mini-1240, Shimadzu, Japan) at 15 min intervals and the % anti-lipid peroxidation activity was calculated [$(As_{120} - Ac_{120}/A_0 - Ac_{120}] \times 100$); As_{120} and Ac_{120} are the absorbance of sample and control, respectively at 120 min, and A_0 is the absorbance of control at 0 min.

2.4. Cell culture

Human cancer cell lines MDA-MB231 (breast), HeLa (cervical) and HepG2 (hepatoma) were maintained in DMEM culture media, supplanted with 10% bovine serum and 1x penicillin-streptomycin mix (all from Invitrogen, USA) at 37 °C with 5% CO_2 supply. All cells were seeded ($0.5x10^5$/well) in 96-well flat-bottom cell culture plates (Becton-Dickinson Labware), and grown overnight for the assays.

2.5. Cell viability or toxicity assay of A. lycopodioides extracts and fractions

The *A. lycopodioides* extracts and fractions (AL-A, AL-B1, AL-B2, AL-B3, AL-B4 and AL-B5; 5 mg/ml each) were first dissolved in 100 μg/ml of DMSO (Sigma, Germany) and further diluted in DMEM to make four working doses (200, 100, 50 and 25 μg/ml). Cells were treated with the four doses (in triplicate), including an untreated control and incubated for 48 h. The anti-cancer drug dasatinib and a known cytotoxic plant *Plectranthus cylindraceus* ethyl acetate extract (Amina et al., 2018) were used as positive controls. Cytotoxicity test was performed using MTT assay (TACS MTT Cell Proliferation and Viability Assay Kit, USA) as per manufacturer's instructions, and the absorbance was recorded (Microplate Reader ELx800; BioTek, USA). The 50% cytotoxicity concentration (CC_{50}) values were estimated using the best fit regression curve method in Excel. Experiment was repeated twice to confirm the reproducibility.

2.6. Hepatoprotective assay of the chloroform fraction

HepG2 cells were co-treated (in triplicate) with the non-cytotoxic and anti-oxidative chloroform fraction (AL-B2; 50, 100, 150 and 200 μg/ml), and the cytotoxic agent 2′7′-dichlorofluores cin (DCFH; IC_{50} = 100 μg/mL) (Arbab et al., 2016), including proper controls. At 48 h post-incubation, MTT assay was performed as above and the data were analyzed. Experiment was repeated twice to confirm the reproducibility.

2.7. Hepatic cytochrome P450 (CYP3A4) activation assay of the chloroform fraction

The nuclear pregnane X receptor (PXR) mediated CYP3A4 modulating activity of the hepatoprotective chloroform fraction of *A. lycopodioides* was tested in cultured HepG2 cells, using luciferase reporter assay as described elsewhere (Al-Dosari and Parvez, 2018). Briefly, HepG2 cells were co-transfected (in triplicate) with reporter plasmids pCDG-hPXR and pGL3-CYP3A4-XREM (400 ng each) as well as Renilla-luciferase control plasmid pRL (200 ng), and incubated at 37 °C for 24 h. The co-transfected cells were treated with AL-B2 (50, 100 and 200 μg/ml), including *Dodonea angustifolia* ethanol-extract (50 μg/ml; positive controls) or DMSO (0.1%; negative control), and further incubated for 48 h. The cell lysates were prepared and subjected to luminescence measurement of luciferase expressions (Dual-Luciferase Reporter Assay System; Promega, USA). The data were analyzed and presented as fold-expression of CYP3A4 in relation to the negative control.

2.8. Anti-microbial assays of A. lycopodioides extracts

2.8.1. Test microorganisms

The test microbial strains used were two gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212), two gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Proteus vulgaris* (ATCC 8427), including a fungus *Candia albicans* (ATCC 60193).

2.8.2. Disk diffusion assays

To determine the anti-microbial activities of the *A. lycopodioides* extracts and fractions (AL-A, AL-B1, AL-B2, AL-B3, AL-B4 and AL-B5) against the bacterial and fungal strains, the disc diffusion method was employed as described elsewhere (Aref et al., 2011). Briefly, a total of 100 μl of exponential growth-phase suspensions of bacteria (10^7 CFU/ml) and fungus (10^6 CFU/ml) were spread on Mueller-Hinton agar and Sabouraud dextrose agar containing Petri dishes, respectively. Sample (100 μg/ml each, prepared in DMSO) impregnated filter paper (2 mg/9 mm disk) were placed on the inoculated agar. While Ampicillin (2 mg/disc) and Kanamycin (2 mg/disc) were used as anti-bacterial positive controls, Nystatin (100 μg/disc) served as anti-fungal positive controls. DMSO solvent was included as negative control. Following 24 h incubation at 37 °C for bacteria and 48 h incubation at 30 °C for fungus (yeast), the anti-microbial activities were evaluated by measuring the colony inhibition zones (mm).

2.9. Statistical analysis

The statistical analysis of the triplicated samples (mean ± SD) was carried out by one-way analysis of variance (ANOVA) followed by Dunnet's test.

3. Results

3.1. Anti-oxidative salutations of A. lycopodioides extracts and fractions

The dose-dependent anti-oxidative activities of *A. lycopodioides* extracts and fractions were evaluated using two different *in vitro* methods. In the β-carotene-bleaching assay, they showed variable degrees of inhibition of lipid-peroxidation AL-B2:73.6 > AL-B3:70.1 > AL-A:65.2 > AL-B4:61.2 > AL-B5:48.1 > AL-B1:41.7 at the

Table 1
In vitro anti-oxidant activities *A. lycopodioides* (AL) extracts and fraction.

	*Anti-lipid peroxidation (%)	**Radical Scavenging (%)				
Extracts & fraction (μg/ml)	1000	10	50	100	500	1000
AL-A	65.2 ± 2.8	12.7 ± 0.9	20.3 ± 1.3	34.3 ± 1.9	53.3 ± 2.1	69.7 ± 2.3
AL-B1	41.7 ± 1.1	2.3 ± 0.3	10.2 ± 3.1	19.7 ± 0.7	31.7 ± 2.1	44.7 ± 1.3
AL-B2	73.6 ± 1.4	23.5 ± 0.4	32.3 ± 0.3	45.1 ± 1.2	60.3 ± 0.2	75.2 ± 1.9
AL-B3	70.1 ± 2.2	17.7 ± 1	24.5 ± 1.8	39.2 ± 2.3	57.3 ± 0.7	73.1 ± 1.2
AL-B4	61.2 ± 1.3	11.2 ± 3.6	19.1 ± 4.4	31.5 ± 3.4	47.5 ± 2	63. 6 ± 2.1
AL-B5	48.1 ± 1.7	7.3 ± 2.8	13.2 ± 0.9	27.1 ± 2.2	41.2 ± 1.4	51. 6 ± 2.3
Ascorbic acid	NT	80.7 ± 2.5	85.1 ± 1.3	85 ± 0.6	88.7 ± 2.1	90.7 ± 1.4
Rutin	89.3	NT	NT	NT	NT	NT

A. lycopodioides (AL) extracts & fractions: water-insoluble (AL-A), precipitate (AL-B1), chloroform (AL-B2), ethyl acetate (AL-B3), n-butanol (AL-B4), aqueous (AL-B5). Anti-oxidant assays: *β-carotene bleaching, **DPPH radical scavenging. NT: not tested. Values: means ± SD of three determinants.

highest concentration of 1000 μg/ml (Table 1). On the other hand, the DPPH method demonstrated comparable radical scavenging activity for all samples (Table 1). Taken together, the highest anti-oxidative activity was observed for AL-B2 (chloroform fraction) followed by AL-B3 (ethyl acetate fraction) as compared to the lowest activity of AL-B1 (precipitate).

3.2. Cytotoxic effects of A. lycopodioides extracts and fractions on cancer cells

The *A. lycopodioides* extracts and fractions (AL-A, AL-B1, AL-B2, AL-B3, AL-B4 and AL-B5) tested for anti-oxidant activities were further assessed for their effects on cancer cell viability. All extracts showed differential cytotoxicity, except AL-B2 (chloroform fraction) even at the highest dose of 200 μg/ml (Table 2). Of these, the highest cytotoxicity was demonstrated by AL-B1 (precipitate; CC_{50}: 52.14 μg/ml) on liver cancer cells followed by AL-B5 (aqueous fraction; CC_{50}: 83.28 μg/ml) on breast cancer cells as compared to others. Notably, while the observed non-cytotoxicity by AL-B2 (chloroform fraction) coincided with its highest anti-oxidative activity, the very high toxicity by AL-B1 (precipitate) and AL-B5 (aqueous fraction) corresponded to their poor anti-oxidant properties.

3.3. Hepatoprotective efficacy of A. lycopodioides non-toxic chloroform fraction

The non-toxic and highly anti-oxidative chloroform fraction (AL-B2) showed hepatoprotection against ~50.5% DCFH triggered oxidative damage in HepG2 cells (Fig. 2). While the 50 and 100 μg/ml doses of AL-2 had insignificant effects on attenuating DCFH, ~67.5% and ~84% cells were markedly protected by 150 and 200 μg/ml doses, respectively (Fig. 2).

Table 2
The estimated CC_{50} (μg/ml) values of *A. lycopodioides* extracts and fractions on cultured human cancer cell lines.

Extracts & fractions	MBD-MB321 (breast)	HeLa (cervical)	HepG2 (hepatoma)
AL-A	134.48	138.32	136.45
AL-B1	55.48	54.33	52.14
AL-B2	376.54	362.23	378.62
AL-B3	157.53	154.33	155.26
AL-B4	168.25	166.32	167.43
AL-B5	83.28	87.43	85.25
PCEAE	150.11	155.48	154.33
DOX	15.41	17.11	8.01

A. lycopodioides (AL) extracts & fractions: AL-A (water insoluble), AL-B1 (precipitate),-B2 (chloroform), AL-B3 (ethyl acetate), AL-B4 (n-butanol), AL-B5 (aqueous). Positive controls: PCEAE (*Plectranthus cylindraceus* ethyl acetate extract) and DOX (Doxorubicin).

3.4. Mild activation of hepatic CYP3A4 by A. lycopodioides

Our reporter gene assay of HepG2 cell lysates showed mild activation of hepatic CYP3A4 by *A. lycopodioides* non-toxic, anti-oxidative and hepatoprotective chroloform fraction (100 and 200 μg/ml) as compared to *D. angustifolia* (Fig. 3).

3.5. Anti-microbial efficacies of A. lycopodioides extracts and fractions

The *A. lycopodioides* extracts and fractions (AL-A, AL-B1, AL-B2, AL-B3, AL-B4 and AL-B5) showed differential anti-microbial activities against two gram-positive bacteria (*S. aureus* and *E. faecalis*), two gram-negative bacteria (*E. coli* and *p. vulgaris*) and a fungus *C. albicans* (Table 3). Of these, while *S. aureus* was more sensitive to AL-A (water-insoluble extract), AL-B3 (ethyl acetate fraction) showed moderate activity against *E. coli* and *C. albicans*. On the other hand, AL-B2 (chloroform fraction) showed promising activities against *S. Aureus*, *C. albicans, P. vulgaris and E. faecalis* with an inhibition zone of 15, 18, 9 and 13 mm, respectively. The estimated Ampicillin inhibition zone was 28 mm for *S. aureus* and *E. faecalis*, and 31 mm for *E. coli* whereas the Kanamycin inhibition zones were 26 mm for *S. aureus* and *E. coli*, and 30 mm for *P. vulgaris*. The observed Nystatin inhibition zone was 23 mm for *C. albicans*.

4. Discussion

Plumbaginaceae is one of the top 20% of angiosperm species-rich diverse families, with a worldwide distribution, predominantly in temperate regions of the Northern Hemisphere (Christenhusz and Byng, 2016). Of these, *Limonium*, *Acantholimon* and *Armeria*, all in subfamily Limonioideae, are the most species-rich genera comprising approximately 85–90% of all species in the family (Koutroumpa et al., 2018). The main generic diversity of the family is centered in the mountains of Central Asia in the Irano-Turanian phytogeographic region (Kubitzki et al., 1993), where many genera, including *Acantholimon* Boiss. are endemic. *Acantholimon* species are cold-desert flowering shrubs, distributed from southeastern Europe to central Asia, and of these, some are used in folk or traditional medicine. In Ladakh (Jammu & Kashmir, India), about 60% of the population is traditionally dependent on 'Amchi' or 'Sowa-rigpa' medicine (Kala, 2005), officially recognized by the Central Council of Indian Medicine. Only a few ethnopharmacological surveys have reported the traditional use of *A. lycopodioides* in cardiac patients in Ladakh (Kala, 2006; Angmo et al., 2012) and in the treatment of gastric ulcer in Shigar valley, Pakistan (Abbas et al., 2017). However, unlike the heptoprotective salutation of *A. bracteatum* (Nasiri et al., 2016) and *A. gilliati* (Gazor et al., 2017) demonstrated in rodent models, *A. lycopodioides* still remains scientifically or experimentally nonvalidated. In this study therefore, we for the first time, experimentally

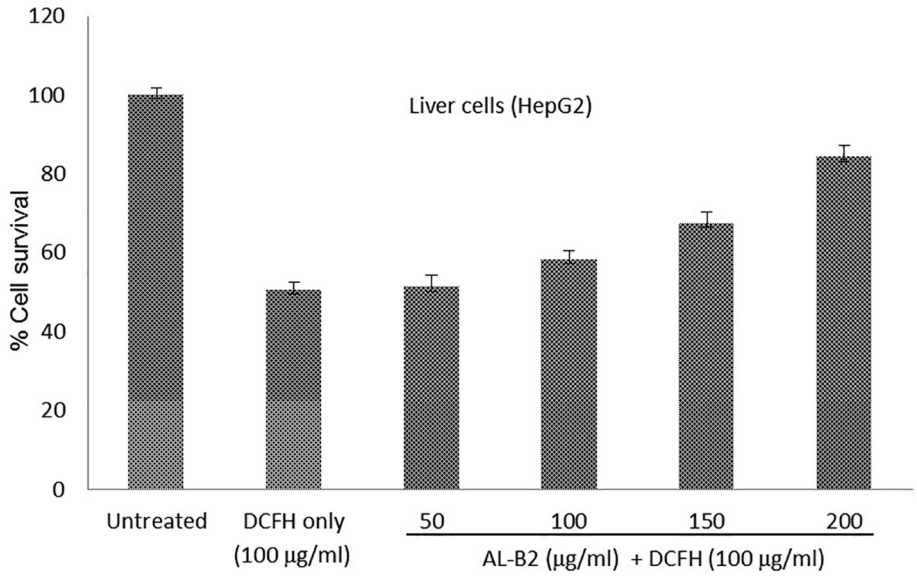

Fig. 2. The MTT assay showing hepatoprotection by the non-toxic and highly anti-oxidant *A. lycopodioides* chloroform fraction (AL-B2) against DCFH-induced oxidative damage in cultured HepG2 cells.

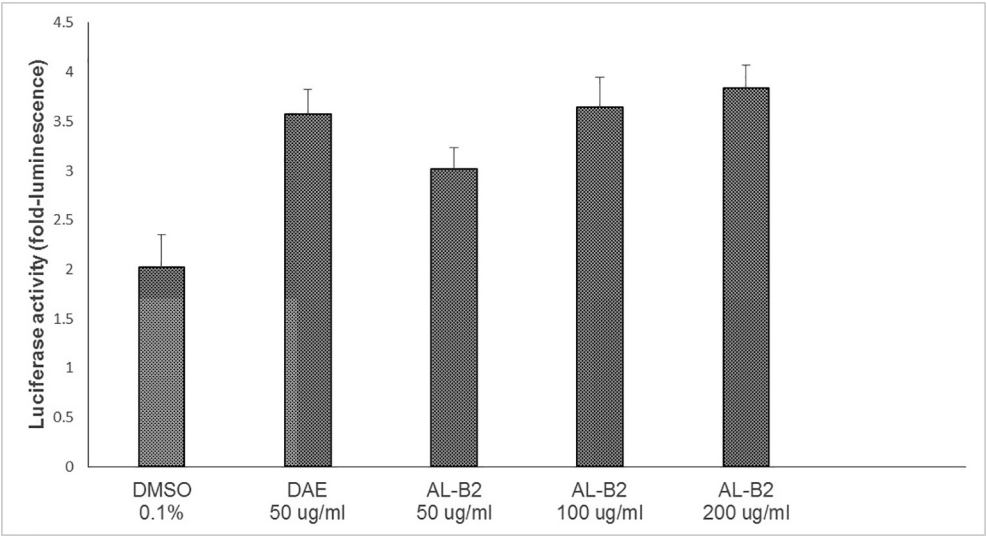

Fig. 3. Reporter gene (Luciferase) assay showing hepatic CYP3A4 activation by the *A. lycopodioides* chloroform fraction (AL-B2) in HepG2 cells. Positive controls: rifampicin (RIF; 10 µM) and *D. angustifolia* ethanol-extract (DAE; 50 µg/ml). Negative control: DMSO (0.1%). Values (Y-axis): means of three determinations.

Table 3
Anti-microbial activities (disc diffusion assay) of *A. lycopodioides* extracts and fractions against gram-positive bacteria (*S. aureus and E. faecalis*), gram-negative bacteria (*E. coli and Protus vulgaris*) and fungus (*C. albicans).*

Extracts & fractions	Estimated zone of inhibition (mm)				
	S. Aureus	*E. faecalis*	*E. coli*	*P. vulgaris*	*C. albicans*
AL-A (insoluble)	14	11	10	9	7
AL-B1 (precipitate)	10	9	–	9	10
AL-B2 (chloroform)	15	13	–	9	18
AL-B3 (ethyl acetate)	12	12	–	9	14
AL-B4 (n-butanol)	11	10	–	–	12
AL-5 (aqueous)	10	10	8	–	10
Ampicillin*	28	28	31	NT	NT
Kanamycin*	26	NT	26	30	NT
Nystatin*	NT	NT	NT	NT	23
DMSO	–	–	–	–	–

A. lycopodioides: AL; *Standards (positive controls); NT: not tested.

explored the poorly or restricted traditional knowledge of the high altitude Ladakhi plant *A. lycopodioides* for its anti-oxidative, anti-cancer and anti-microbial therapeutic salutations.

Employing different *in vitro* methods for anti-oxidant activity is now commonly recommended. The β-carotene bleaching method is used to measure lipid peroxidation property of a sample. In this method, linoleic acid generated free radicals attack unsaturated β-carotene to undergo oxidation resulting in the loss of its orange color. DPPH contains a stable free radical that is widely used to measures the *in vitro* radical scavenging ability of a test sample. In the presence of an anti-oxidant agent which can donate an electron to DPPH where the change in the typical purple color indicating free radical decays is measured spectrophotometrically. In this study, we used both methods to assess the dose-dependent anti-oxidative activities of *A. lycopodioides* extracts and fractions. While in the β-carotene bleaching assay, the extracts showed variable degrees of inhibition of lipid-peroxidation, the DPPH method demonstrated their comparable radical scavenging activities. Overall, the highest anti-oxidant activity was observed for the chloroform fraction followed by the ethyl acetate and other fractions.

All *A. lycopodioides* extracts and fractions assessed for anti-oxidant activities were further tested for their effects, if any, on human cancer cell viability. All extracts showed differential cytotoxicity on cervical, breast and liver cancer cells, except the chloroform fraction that was non-toxic even at the highest tested dose. The highest toxicity was demonstrated by the precipitate on liver cells followed by the aqueous and other fractions on breast cells. Notably, the non-cytotoxicity by the *A. lycopodioides* chloroform fraction coincided with its highest anti-oxidative potential whereas the very high toxicity of the precipitate and other fractions corresponded to their poor anti-oxidant properties.

The accumulation of highly toxic cellular reactive oxygen species (ROS) damage lipids, proteins or nucleic acids and promote oxidative cell or tissue damages (Opara, 2006). DCFH is generally used to estimate *in vitro* free-radicals (Rota et al., 1999) as well as to trigger cytotoxicity in a range of cultured human cells (Arbab et al., 2016; Parvez et al., 2018). Owing to the traditional use *A. lycopodioides* against gastric disorders (Abbas et al., 2017), we therefore, evaluated the hepatoprotective efficacy of its non-toxic and highly anti-oxidative chloroform fraction against DCFH triggered oxidative damage in HepG2 cells. Our data revealed a dose-dependent salutation of the chloroform fraction where the 150 and 200 μg/ml doses protected ~67.5% and ~84% cells, respectively through attenuating DCFH induced free-radicals.

The liver PXR mediated CYP3A4 enzyme activity is primarily involved in the metabolism of a variety of prescription drugs, xenobiotics and bioactive natural compounds (Al-Dosari and Parvez, 2016). In view of the known CYP3A4 activity related drug non-response or organ toxicity (Parvez and Rishi, 2019), a good understanding of the efficacy and safety of a herbal product is inevitable. We therefore, also assessed CYP3A4 modulatory activity of *A. lycopodioides* non-toxic, anti-oxidative and hepatoprotective chloroform fraction in cultured HepG2 cells, using luciferase assay. Our data revealed that *A. lycopodioides* hepatoprotective fraction mildly activated PXR-mediated CYP3A4 gene in relation to drug metabolism and efficacy.

Subsequently, the *A. lycopodioides* extracts and fractions were also evaluated for their anti-microbial activities against two gram-positive bacteria (*S. aureus* and *E. faecalis*) and two gram-negative bacteria (*E. coli* and *p. vulgaris*), including a fungus (*C. albicans*). All tested samples showed differential anti-bacterial and anti-fungal activities. Of these, while *S. aureus* was more sensitive to the water-insoluble extract, the ethyl acetate fraction showed moderate activity against *E. coli* and *C. albicans*. On the other hand, the chloroform fraction showed promising activity against *S. Aureus, P. vulgaris*, *E. faecalis* and *C. albicans*.

5. Conclusion

Our data for the first time, demonstrated the promising anti-oxidative, hepatoprotective, anti-cancer, anti-microbial, and CYP450 modulating activities of *A. lycopodioides*. This warrants its further phytochemical and biological studies towards isolations and identifications of active principles of therapeutic values.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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