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

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**A B S T R A C T**

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′,4′-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.

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**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. antilbonoticum* roots decoction is used to treat wound and skin injuries (Baydoun et al., 2015). *A. acerosus* 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 hydroxyproline acid from other genera of Plumbaginaceae are reported, they could not...
never be detected in leaf extracts of Acantholimon spp. (Fowden, 1958).

A. lycopodioides Boiss. (Synonyms: A. tibeticum, Static 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; \( \lambda = 517 \text{ nm} \)) was measured using a spectrophotometer (UV mini-1240, Shimadzu, Japan). The radical scavenging activity was calculated for each sample \( \% \text{Radical scavenging activity} = \frac{(A_{0} - A_{c0})}{A_{0} - A_{c1}} \times 100; A_{c0} \) and \( A_{c1} \) are the absorbance of the control and sample, respectively.

2.3.2. \( \beta \)-Carotene–linoleic acid assay

The \( A. \) lycopodioides extracts and fractions were further evaluated for their anti-lipid peroxidation activities, using \( \beta \)-carotene bleaching method as described elsewhere (Veligoglu et al., 1998) with little modification. Briefly, 1 ml of freshly prepared \( \beta \)-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 \( \beta \)-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; \( \lambda = 470 \text{ nm} \)) was recorded (UV mini-1240, Shimadzu, Japan) at 15 min intervals and the % anti-lipid peroxidation activity was calculated \( \left[ \frac{(A_{120} - A_{c120})}{A_{0} - A_{c120}} \times 100 \right] \). \( A_{120} \) and \( A_{c120} \) 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, supplemented with 10% bovine serum and 1x penicillin-streptomycin mix (all from Invitrogen, USA) at 37 °C with 5% CO2 supply. All cells were seeded (0.5x10⁵/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 \( \text{Plectranthus cylindraceus} \) were used as anti-bacterial positive controls, Nystatin (100 µg/disc) 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).

2.6. 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; IC50 = 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 \( \text{Dodeonea angustifolia} \) 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 \( \text{Staphylococcus aureus} \) (ATCC 25923) and \( \text{Enterococcus faecalis} \) (ATCC 29212), two gram-negative bacteria \( \text{Escherichia coli} \) (ATCC 25922) and \( \text{Proteus vulgaris} \) (ATCC 8427), including a fungus \( \text{Candida albicans} \) (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 (10⁷ CFU/ml) and fungus (10⁵ 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 \( \beta \)-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
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; CC50: 52.14 µg/ml) on liver cancer cells followed by AL-B3 (ethyl acetate fraction) 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).

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 chloroform 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. albincons. 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 (Kubitzi, 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 (Abbasi 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

| Table 1
| In vitro anti-oxidant activities A. lycopodioides (AL) extracts and fraction. |
| --- |
| Extracts & fraction (µg/ml) | 'Anti-lipid peroxidation (%) | 'Radical Scavenging (%) |
|  | 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 | 15.5 ± 0.7 | 31.7 ± 2.1 | 44.7 ± 1.3 |
| AL-B2 | 73.6 ± 1.4 | 23.5 ± 2.3 | 32.3 ± 2.3 | 45.1 ± 2.3 | 60.3 ± 0.2 | 75.2 ± 2.3 |
| AL-B3 | 70.1 ± 2.2 | 17.7 ± 1.1 | 24.5 ± 2.3 | 39.2 ± 2.3 | 57.3 ± 0.7 | 73.1 ± 1.2 |
| AL-B4 | 61.2 ± 1.3 | 11.2 ± 3.6 | 15.1 ± 3.4 | 31.5 ± 3.4 | 47.5 ± 2.2 | 63.6 ± 2.2 |
| AL-B5 | 48.1 ± 1.7 | 7.3 ± 2.3 | 13.2 ± 2.2 | 27.1 ± 2.2 | 41.2 ± 1.4 | 51.6 ± 2.3 |
| Ascorbic acid | 80.7 ± 2.3 | 85.1 ± 1.3 | 85.6 ± 2.1 | 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.

| Table 2
| The estimated CC50 (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 | 138.23 | 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), AL-B2 (chloroform), AL-B3 (ethyl acetate), AL-B4 (n-butanol), AL-B5 (aqueous). Positive controls: PCEAE (Plectranthus cymadicus ethyl acetate extract) and DOX (Doxorubicin).
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 *Pseudomonas vulgaris*) and fungus (*C. albicans*).

| Extracts & fractions | *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 Ladakh 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 measure 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 measure the oxidative activities of *A. lycopodioides* extracts and fractions assessed for anti-oxidant, anti-bacterial, anti-microbial, and hepatoprotective therapeutic salutations.

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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References

Abbas, Z., Khan, S.M., Alam, J., Khan, S.W., Abbasi, A.M., 2017. Medicinal plants used by inhabitants of the Shigar Valley, Baltistan region of Karakorum range- Pakistan. J. Ethnobiol. Ethnomed. 13, 53–58.

Al-Doarsi, M.S., Parvez, M.K., 2018. Novel plant inducers of PXR-dependent cytochrome P4503A4 expression in HepG2 cells. Saudi Pharm. J. 26, 1069–1072.

Amina, M., Alam, P., Parvez, M.K., Al-Musayyeb, N.M., Al-Hwatay, S.A., Al-Rashidi, N. S., Al-Doarsi, M.S., 2018. Isolation and validated HPTLC analysis of four cytotoxic compounds, including a new sesquiterpene from aerial parts of *Plectranthus ciliaris* corynus. Nat. Prod. Res. 32, 804–809.

Angnou, K., Adhikari, B.S., Rawat, G.S., 2012. Changing aspects of traditional healthcare system in western Ladakh, India. J. Ethnopharmacol. 143, 621–630.

Arbab, A.H., Parvez, M.K., Al-Doarsi, M.S., Al-Rehaily, A.J., Ibrahim, K.E., Alam, P., Al-Said, M.S., Rafatullah, S., 2016. Therapeutic efficacy of ethanolic extract of *Aerva Javanica* aerial parts in the amelioration of COX-2-induced hepatotoxicity and oxidative damage in rats. Food Nut. Res. 60, 20864.

Aref, H.L., Karina, B.H.S., Fekih, A., Chemli, R., Mars, M., Aouni, M., Jean Pierre Chaumont, J.P., Said K. 2011. Variation in antimicrobial activity of latex from two varieties of *Ficus carica*. African J. Microbiol. Res. 5, 1361–1367.

Ari, S., Temel, M., Kargöğlu, M., Konuk, M., 2015. Ethnobotanical survey of plants used in Afyonkarahisar-Turkey. J. Ethnobiol. Ethnomed. 11 (84).

Baker, H.G., 1948. Dimorphism and monomorphism in the *Plumbaginaceae*: a survey of the family. Ann. Bot. 12, 207–219.

Baydoun, S., Lamis, C., Helena, D., Nelly, A., 2015. Ethnopharmacological survey of medicinal plants used in traditional medicine by the communities of Mount Hermon, Lebanon. J. Ethnopharmacol. 173, 139–156.

Brand-Williams, W., Cuvelier, M.E., Berset, C., LWT-Food Sci. Technol. 28, 25–30.

Christenhusz, M.J., Byng, J.W., 2016. The number of known plant species in the world and its annual increase. Phytotaxa 261, 201–217.

Dogan, M., Akadun, G., 2005. A new species of *Acradenoma Boiss.* sect. *Glumaria Boiss.* from *Plumbaginaceae* from Elazig, Turkey. Bot. J. Linn Soc. 149, 351–356.

Fowden, L., 1958. Some observations on a hydroxypropionic acid from *Armeria maritima*. Biochem. J. 70, 629–633.

Gazor, R., Asgari, M., Pasdaran, A., Mohammadhosseini, F., Nasiri, E., Roushan, Z.A., 2017. Evaluation of Hepatoprotective Effect of *Acantholimon giliati* aerial plant methanolic extract. Iran. J. Pharm. Res. 16, 135–141.

Hassler, M., 2019. World plants: synomnic checklists of the vascular plants of the world. In: Roskov, Y., Ower, G., Orrell, T., Nicolson, D., Bailly, N., Kirk, P.M., Bourquin, T., DeWalt, R.E., Decock, W., van Nieukerken, E., Zarucchi, J., Peney, L. (Eds.). Species 2000 & ITIS Catalogue of Life, 2019 Annual Checklist.

Kalá, C.P., 2006. Medicinal plants of the high altitude cold desert in India: Diversity, distribution and traditional uses. Int. J. Biodiversity Sci. Manage. 2, 43–56.

Khan, S.M., Page, S.E., Ahmad, H., Harper, D.M., 2013. Sustainable utilization and conservation of plant biodiversity in montane ecosystems: the western Himalayas as a case study. Ann. Bot. 112, 479–501.

Koutroumpa, K., Theodoridis, S., Warren, B.H., Jiménez, A., Clepl, F., Doğan, M., Romeiras, M.M., Santos-Guerra, A., Fernández-Palacios, J.M., Caujapé-Castells, J., Moura, M., Menezes de Sequeira, M., Conti, E., 2018. An expanded molecular phylogeny of *Plumbaginaceae*, with emphasis on *Limonium* (sea lavenders): taxonomic implications and biogeographic considerations. Ecol. Evol. 8, 12397–12424.

Kubitzki, K., Rohwer, J.G., Bittrich, V., 1993. The Families and Genera of Vascular Plants. Springer Verlag, Berlin, Germany, 2, pp. 523–530.
Moharrek, F., Sanmartín, I., Kazempour-Osaloo, S., Nieto Feliner, G., 2019. Morphological innovations and vast extensions of mountain habitats triggered rapid diversification within the species-rich Irano-Turanian genus *Acantholimon* (Plumbaginaceae). Front. Genet. 9, 698.

Namgail, T., Rawat, G.S., Mishra, C., van Wieren, S.E., Prins, H.H.T., 2012. Biomass and diversity of dry alpine plant communities along altitudinal gradients in the Himalayas. J. Plant. Res. 125, 93–101.

Nasiri, E., Naserrad, S., Lashgari, A.P., Gazor, R., Mohammadghasemi, F., Atrkar, Z., 2016. Hepatoprotective effect of *Acantholimon bracteatum* (Girard) Boiss. on formaldehyde-induced liver injury in adult male mice. Res. J. Pharmacogn. 3, 55–61.

Opara, E.C., 2006. Oxidative stress. Dis. Mon. 2, 183–198.

Parvez, M.K., Arbab, A.H., Al-Dosari, M.S., Al-Rehaily, A.J., Alam, P., Ibrahim, K.E., AlSaaid, M.S., Rafatullah, S., 2018. Protective effect of *Atriplex suberecta* extract against oxidative and apoptotic hepatotoxicity. Exp. Ther. Med. 15, 3883–3891.

Parvez, M.K., Rishi, V., 2019. Herb-drug interactions and hepatotoxicity. Curr. Drug Metab. 20, 275–282.

Rota, C., Chignell, C.F., Mason, R.P., 1999. Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescein to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. Free Radic. Biol. Med. 27, 873–881.

Soelberg, J., Jäger, A.K., 2016. Comparative ethnobotany of the Wakhi agropastoralist and the Kyrgyz nomads of Afghanistan. J. Ethnobiol. Ethnomed. 12, 2–7.

Yang, Y., Moore, M.J., Brockington, S.F., Timoneda, A., Feng, T., Marx, H.E., Walker, J.F., Smith, S.A., 2017. An efficient field and laboratory workflow for plant phylotranscriptomic projects. Appl. Plant Sci. 5, e1600128.

**Further Reading**

Kumar, G.P., Gupta, S., Murugan, P.M., et al., 2009. Ethnobotanical Studies of Nubra Valley—A Cold Arid Zone of Himalaya. Ethnobot Leaflets, vol. 6.

Lledó, M.D., Karis, P.O., Crespo, M.B., Fay, M.F., Chase, M.W., 2001. Phylogenetic position and taxonomic status of the genus *Aegialitis* and subfamilies Staticoideae and Plumbaginoideae (Plumbaginaceae): evidence from plastid DNA sequences and morphology. Plant System Evol. 225, 107–124.