Original article

The anti-oxidative, anti-cell proliferative and anti-microbial efficacies of cold-adapted Crepis flexuosa: HPTLC and GC/MS analyses

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A R T I C L E   I N F O

Article history:
Received 14 November 2021
Revised 13 January 2022
Accepted 17 January 2022
Available online 22 January 2022

Keywords:
Crepis flexuosa
Anti-oxidant
Anti-cell proliferative
Anti-microbial, HPTLC, luteolin-7-O-glucoside

A B S T R A C T

The genus Crepis constitutes cold-adapted plant spp., of these some are traditionally used in folk medicine against inflammation or fungal infections without scientific validations. Here, we report the biological activities of Crepis flexuosa total ethanol-extract (CF-EtOH) and its hexane (CF-Hex), ethyl acetate (CF-EtOA), butanol (CF-ButOH), and aqueous (CF-Aqua) fractions. Our in vitro DPPH and ABTS radical-scavenging assays showed CF-EtOH, CF-ButOH and CF-Aqua with maximal, CF-EtOA with moderate, and CF-Hex with mild anti-oxidant activities. When tested on human cancer cell lines, high cytotoxicity was demonstrated by CF-EtOH (IC50: 42.45 μg/ml) and CF-Aqua (IC50: 46.37 μg/ml) on HepG2, followed by CF-Hex (IC50: 63.24 μg/ml) and CF-ButOH (IC50: 65.32 μg/ml) on MCF7 cells. The human primary cell line (HUVEC) had comparatively lower cytotoxicity for the tested samples. Moreover, when assessed for anti-microbial efficacy, CF-ButOH and CF-Aqua exhibited the strongest activity (MIC: 156.25 μg/ml) against S. aureus, E. faecalis and C. albicans. Further, while the developed RP-HPTLC identified the bioactive flavonoid luteolin-7-O-glucoside (17.58 mg/g), GS/MS analysis revealed sixteen compounds in C. flexuosa extract. In conclusion, we for the first time show the promising anti-oxidative, anti-cell proliferative and anti-microbial efficacies of C. flexuosa. This warrants further phytochemical and bio-efficacy studies towards isolations and identifications of active principles.

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1. Introduction

Crepis (family: Asteraceae), a large genus of about 200 species of cold-adapted plants is distributed in the northern hemisphere (Mabberley, 2008). Though most of the species are edible, information on their worldwide usage in traditional medicinal is very limited. In Spanish tradition, C. vesicaria (Arnica) is used for gastric and arterial circulation problems as well as externally applied for wound healing, bruises and inflammations (Gonzalez-Tejero et al., 1995). In African folk medicine, while C. carbonaria is used for increasing the myometrial contractions (Schröder, 1980), C. camerononica (syn. C. newii) is used to treat diarrhea, wounds and fungal infections (Ndom et al., 2006). Also, C. sancta is consumed as laxative and diuretic supplement in Italy (Sansanelli and Tassoni, 2014).

Crepis flexuosa (Lede.) Benth. (syn. Crepis glouca, Barkhausia flexuosa, Youngia flexuosa, Youngia glouca) is a shrub with heavily and nearly leafless, forked branches that terminate in a rounded tuft of very small yellow flower heads. It is distributed in Himalayan ranges (3000–4200 m, a.s.l.) of Central Asia, Tibet, central Nepal, western Pakistan and northern India (Ladakh, Himachal Pradesh, Uttarakhand). In the Himalayan Spiti valley of India, C. flexuosa juice is used to cure jaundice (Singh and Lal, 2008). However, to our best knowledge, the biological activity of C. flexuosa is hitherto not reported. With this background, we for the first time assessed the in vitro therapeutic potential of C. flexuosa.

2. Materials and methods

2.1. Plant material collection and identification

The whole flowering plant of C. flexuosa Benth, locally known as Sili was collected from Shyok-Agham area located between Nubra...
Valley and Pangong (3200 m a.s.l.; Ladakh, India) in July 2018 (Fig. 1). The plant was identified by a local herbalist-Amchi practitioner (voucher specimen no.802018) and further confirmed by Dr. Tariq Husain, a plant taxonomist at National Botanical Research Institute, Lucknow, India.

2.2. Extraction and fractionation

All analytical grade solvents used for extraction and fractionation of C. flexuosa were purchased from Sigma Aldrich (Germany). Of these, n-hexane (Hex) and ethyl acetate (EtOAc) were distilled prior to use whereas 96% ethanol (EtOH) and n-butanol (BuOH) were used as supplied. The details of procedure adopted for extraction and fractionation is shown (Scheme 1).

Briefly, the dried and grounded (61.7 g) aerial of parts of C. flexuosa was extracted with 96% EtOH at RT (3x 200 ml), and filtered (Whatman filter paper no. 1). The solvent was evaporated to dryness at 40 °C in vacuo (Buchi Rotavapor; Model R-215) that yielded a syrupy material of EtOH extract (CF-EtOH; 8.1 g). A portion of CF-EtOH (7.5 g) was subjected to liquid–liquid partition successively with Hex, EtOAc and water-saturated BuOH to obtain Hex-soluble fraction (CF-Hex; 1.3 g), EtOAc-soluble fraction (CF-EtOAc; 0.8 g) and BuOH-soluble fraction (CF-BuOH; 0.8 g), including the remaining water-soluble fraction (CF-Aqua; 4.1 g). Solvents were evaporated to dryness as mentioned above for each sample, and kept at 4 °C until analyzed.

2.3. Determination of in vitro anti-oxidative activity of C. flexuosa

2.3.1. DPPH radical-scavenging activity

The anti-oxidative activities of CF extract (CF-EtOH) and fractions (CF-Hex, CF-EtOAc, CF-BuOH and CF-Aqua) were estimated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method as described elsewhere (Brand-Williams et al., 1995; Hussein et al., 2019). Briefly, 0.5 ml of each test samples (10, 50, 100, 500 and 1000 μg/ml) was mixed with 0.125 ml DPPH and 0.375 ml methanol, and incubated for 0.5 h. Ascorbic acid was used as a positive control and methanol acted as blank. All samples were tested in triplicate and repeated. The optical density (OD; λ.max = 517 nm) was recorded using UV–vis spectrophotometer, and the tested samples’ free radical-scavenging activities were calculated:

Fig. 1. Crepis flexuosa or Sili. (A) Its geographical distribution in high altitude Himalayan ranges (https://indiabiodiversity.org/species/show/259968), and Collection location in Ladakh, India. (B) The whole flowering plant of C. flexuosa.
%Radical-scavenging activity = \frac{(OD_{control} - (OD_{sample}/OD_{control})) \times 100}{}

2.3.2. ABTS cation-scavenging activity

The anti-oxidative activities of CF extract (CF-EtOH) and fractions (CF-Hex, CF-EtOA, CF-ButOH and CF-Aqua) were estimated using 2,2’-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method as described elsewhere (Li et al., 2011) with minor modification. Briefly, aqueous solutions of ABTS (7.0 mM) and potassium persulfate (2.45 mM) were prepared separately, and kept in dark for 12 h. The two solutions were mixed and incubated at RT for 30 min, following refrigeration for 24 h, and further dilutions were made in EtOH. To the solution (50 μg/ml; 1:1), different concentration of each sample (10, 50, 100, 500 and 1000 μg/ml) were pipetted to initiate the reaction until a calibration curve was achieved. While ascorbic acid was used as standard, ABTS solution (50 μg/ml) and EtOH (96%) served as negative and control, respectively. All samples were tested in triplicate and repeated. The OD (λmax = 734 nm) was recorded using UV–vis spectrophotometer, and the anti-oxidant activity was determined:

%Radical-scavenging activity = \frac{(OD_{control} - (OD_{sample}/OD_{control})) \times 100}{}

2.4. Assessment of C. flexuosa fractions on cell viability and proliferation

2.4.1. Human cell culture

The three human cancer cell lines MCF7 (breast), HeLa (cervical) and HepG2 (liver) as well as a primary umbilical vein endothelial cell (HUVEC-16549) were procured (ATCC, USA) and maintained in DMEM culture media, supplemented with bovine serum (10%) and 1x penicillin-streptomycin mix (all from Invitrogen, USA) at 37°C with 5% CO2 supply. The cells (0.5x10^5 /100 μl/well) were seeded in 96-well flat-bottom cell culture plates (Becton-Dickinson Labware), and grown overnight.

2.4.2. Cytotoxicity assay

The C. flexuosa extract (CF-EtOH) and fractions (CF-Hex, CF-EtOA, CF-ButOH and CF-Aqua; 5 mg/ml each) were first dissolved in 100 μg/ml of dimethyl sulfoxide (DMSO; Sigma, Germany) and further diluted in DMEM to make four working concentrations (200, 100, 50 and 25 μg/ml). Overnight grown cells in 96-well plates were treated with each of four doses, including an untreated control (0.1% DMSO) and incubated for 72 h. The anticancer drug dasatinib was used as positive control. The cytotoxicity was evaluated using MTT assay (TACS MTT Cell Proliferation and Viability Assay Kit, USA) according to manufacturer’s guidelines, and the absorbance was recorded (Microplate Reader ELx800; BioTek, USA). All samples were tested in triplicate and repeated. Values of 50% maximal cytotoxicity concentration (CC50) were estimated using the best fit regression curve method in Excel (Microsoft, USA).

2.5. Determination of anti-microbial activity of C. flexuosa

2.5.1. Test microorganisms

Two gram-positive (Staphylococcus aureus; ATCC 25923 and Enterococcus faecalis; ATCC 29212) and two gram-negative (Escherichia coli; ATCC 25922 and Proteus vulgaris; ATCC 8427) bacterial strains as well as one fungal strain (Candida albicans; ATCC 60193) were used in this investigation.

2.5.2. Determination of minimum inhibitory concentrations (MIC)

The MIC for CF extract (CF-EtOH) and fractions (CF-Hex, CF-EtOA, CF-ButOH and CF-Aqua) were assessed using micro-well dilution method as described elsewhere (Mann and Markham, 1998; Sulaiman, 2013) with some modifications. Briefly, duplicated two-fold serial dilutions (2000 through 31.2 mg/ml in DMSO; 5%, v/v) were made using broth media (100 μl/well, final) in 96-well plates, and the microbial suspensions (100 μl; 1x10^6 CFU/ml) were added. Gentamycin and nystatin were used as positive controls. The plates were incubated for 24 h at 37°C for bacterial strains, whereas for 72 h at 25°C fungal strain, respectively. The MIC for each sample was defined as the lowest concentration displaying non-detectable bacterial or fungal growth. For MIC (MBC or MFC for fungus) estimation, 5 μl from the wells showing no microbial growth was spread on agar plates and incubated for 24 or 72 h. Values of MBC and MFC therefore, represented the lowest concentrations of tested samples which did not show any sign of bacterial or fungal growth.

2.6. Development of HPTLC method to analyze luteolin-7-O-glucoside in C. flexuosa

The quantification of luteolin-7-O-glucoside in C. flexuosa extract (CF-EtOH) was carried out on reverse-phase high performance thin layer chromatography (RP-HPTLC; 10 x 10 cm) plate (Merck, Germany). A stock solution of luteolin-7-O-glucoside (Sigma, Germany; 1 mg/ml) was prepared in methanol and further diluted to furnish seven different concentrations (20–140 μg/ml). All samples, including C. flexuosa extract (6 μl) were gently applied on the TLC plate through a micro liter syringe connected with programmed TLC Sampler-4 (CAMAG, Switzerland) with a band size of 6 mm and at a speed of 160 nl/sec to give linearity range between 200 and 1400 ng/band. The plate was developed in a pre-saturated twin-trough glass chamber (Automatic Development Chamber-2, CAMAG; Switzerland) at specific temperature (25 ± 2°C) and humidity (60 ± 5%). The developed plate furnished clear and compact spots of luteolin-7-O-glucoside as well as different phytoconstituents of C. flexuosa, which were quantitatively analyzed at λmax = 254 nm in the absorbance mode.

2.7. GC/MS analysis of C. flexuosa

The chemical constituents of C. flexuosa extract (CF-EtOH) were determined utilizing gas chromatography (GC) and a mass spectrometer (MS) (Turbo mass, PerkinElmer). The temperature program was adjusted to 40°C for a 2 min hold, followed by increasing to 200°C (5°C/min) and held for 2 min. The temperature was further raised to 300°C (5°C/min) and held for another 2 min. The phytochemical composition of CFE was determined by comparing the obtained mass spectra with those of the National Institute of Standard and Technology Spectral Library, the Adams Library (Adams, 2007) and the Wiley GC–MS Library (McLafferty and Stauffer, 1989).

2.8. Statistical analysis

All triplicated data were presented as mean ± standard error, analyzed using one-way analysis of variance and differences between two groups were compared using Student’s t-test (SPSS software; Version 25; IBM, USA). p < 0.05 was considered significant.

3. Results

3.1. Anti-oxidative activities of C. flexuosa extract and fractions

Results of both DPPH and ABTS assays showed dose-dependent scavenging activities by C. flexuosa extract and fractions (Table 1). Of the tested samples, CF-EtOH (ethanol-extract), CF-ButOH
Human cancer cell lines. The estimated CC_{50} (\%)

| Sample  | Concentrations (\mu g/ml) |
|---------|--------------------------|
|         | 10          | 50          | 100         | 500         | 1000        |
| DPPH radical-scavenging activity (%) |          |
| CF-EtOH | 11.6 ± 0.9 | 19.2 ± 1.2  | 33.2 ± 1.8  | 52.1 ± 2.2  | 67.2 ± 2.1  |
| CF-Hex  | 2.4 ± 0.3  | 9.2 ± 3.1   | 18.6 ± 0.8  | 30.6 ± 2.2  | 43.6 ± 1.2  |
| CF-EtOA | 7.3 ± 2.8  | 12.2 ± 0.9  | 25.1 ± 2.2  | 39.2 ± 1.4  | 50.6 ± 2.3  |
| CF-ButOH| 15.6 ± 1.4 | 28.8 ± 2.8  | 38.8 ± 2.4  | 51.3 ± 2.9  | 69.6 ± 2.6  |
| CF-Aqua | 17.5 ± 0.4 | 31.3 ± 0.3  | 41.1 ± 1.2  | 54.3 ± 0.2  | 70.2 ± 1.9  |
| Ascorbic acid | 80.7 ± 2.0 | 85.1 ± 1.3  | 85 ± 1.2    | 88.7 ± 2.4  | 90.7 ± 1.4  |

ABTS cation-scavenging activity (\%)

| Sample  | Concentrations (\mu g/ml) |
|---------|--------------------------|
| CF-EtOH | 10.2 ± 0.7  | 17.1 ± 1.2  | 23.3 ± 1.1  | 45.1 ± 1.5  | 64.1 ± 1.2  |
| CF-Hex  | 2.3 ± 1.9   | 5.2 ± 2.1   | 10.7 ± 1.9  | 21.8 ± 2.1  | 39.3 ± 1.7  |
| CF-EtOA | 5.8 ± 2.3   | 10.2 ± 1.9  | 15.1 ± 1.2  | 26.9 ± 1.6  | 47.9 ± 2.6  |
| CF-ButOH| 10.1 ± 0.9  | 18.3 ± 2.9  | 25.9 ± 1.0  | 47.1 ± 2.4  | 68.4 ± 2.3  |
| CF-Aqua | 11.5 ± 0.6  | 20.4 ± 0.9  | 28.1 ± 2.2  | 51.1 ± 0.9  | 69.1 ± 1.5  |
| Ascorbic acid | 80.7 ± 2.4  | 81.2 ± 2.1  | 84.2 ± 1.9  | 87.2 ± 2.4  | 88.7 ± 2.1  |

Table 1
Radical-scavenging activities of Crepis flexuosa extract and fractions.

| Samples          | Activity | S. aureus (MIC: 156.25 \mu g/ml) | E. faecalis (MIC: 156.25 \mu g/ml) | E. coli (MIC: 156.25 \mu g/ml) | P. vulgaris (MIC: 156.25 \mu g/ml) | C. albicans (MIC: 156.25 \mu g/ml) |
|------------------|----------|----------------------------------|-----------------------------------|---------------------------------|-----------------------------------|----------------------------------|
| CF-EtOH          | MIC 625  | NT                               | NT                                | NT                              | NT                                |
|                  | MBC 1250 | 1250 - NT                         | NT                                | NT                              | NT                                |
| CF-Hex           | MIC 625  | 625 - NT                          | NT                                | NT                              | NT                                |
|                  | MBC 1250 | 1250 - NT                         | NT                                | NT                              | NT                                |
| CF-EtOA          | MIC 625  | 625 - NT                          | NT                                | NT                              | NT                                |
|                  | MBC 1250 | 1250 - NT                         | NT                                | NT                              | NT                                |
| CF-ButOH         | MIC 156.25 | 156.25 - NT                      | 625 - NT                          | NT                              | NT                                |
|                  | MBC 312.5 | 312.5 - NT                        | 1250 - NT                         | NT                              | NT                                |
| CF-Aqua          | MIC 156.25 | 156.25 - NT                      | 625 - NT                          | NT                              | NT                                |
|                  | MBC 312.5 | 312.5 - NT                        | 1250 - NT                         | NT                              | NT                                |
| Gentamycin       | MIC 7.8  | 7.8 - NT                          | 3.9 - NT                          | NT                              | NT                                |
|                  | MBC 15.6 | 15.6 - 7.8                        | 7.8 - NT                          | NT                              | NT                                |
| Nystatin         | MIC 7.8  | 7.8 - NT                          | 3.9 - NT                          | NT                              | NT                                |
|                  | MBC 15.6 | 15.6 - 7.8                        | 7.8 - NT                          | NT                              | NT                                |

3.2. Effects of C. flexuosa extract and fractions on cancer cell viability

The Crepis flexuosa extract and fractions when tested on a panel of human cell lines, showed differential cytotoxicity even at the lowest dose (25 \mu g/ml). Of these, the highest cytotoxicity was demonstrated by the ethanol-extract (IC_{50}: 42.45 \mu g/ml) and aqueous-fraction (IC_{50}: 46.37 \mu g/ml) on liver cancer cells followed by hexane-fraction (IC_{50}: 63.24 \mu g/ml) and butanol-fraction (IC_{50}: 65.32 \mu g/ml). The ethyl acetate-fraction showed no cytotoxicity at 100 \mu g/ml. The cultured HUVEC cells demonstrated comparatively lower cytotoxicity for all tested samples. Notably, while the observed non-cytotoxicity by the ethyl-acetate fraction coincided with its anti-oxidative activity, the high toxicity of hexane-fraction corresponded to its poor anti-oxidant activity.

3.3. Anti-microbial efficacies of C. flexuosa extract and fractions

The Crepis flexuosa extract and fractions showed variable degrees of inhibitory effects on both bacterial and the fungal strains with MIC values in the range of 156.25–1250.0 (Table 3). Of the tested samples, the butanol-extract and aqueous-extract had the best activities against S. aureus and E. faecalis (MIC: 156.25 \mu g/ml) compared to others. On the other hand, the ethyl acetate-fraction and hexane-fraction showed weak activities against S. aureus and E. faecalis and no activity against E. coli and P. vulgaris. Both the butanol-fraction and aqueous-fraction showed strong activities (MFC: 156.5 \mu g/ml) against C. albicans as compared to others (Table 3).

3.4. Quantitative HPTLC analysis of luteolin-7-O-glucoside in C. flexuosa extract

Of the various combinations of mobile-phase solvents tested for the quantitative RP-HPTLC analysis of luteolin-7-O-glucoside in the Crepis flexuosa extract, a combination of acetonitrile-water (40:60; v/v) was found as the most suitable mobile-phase. The piktograms of the developed TLC plate (Fig. 2A) and 3D of all tracks (Fig. 2B) are presented. The developed method furnished intense peaks of luteolin-7-O-glucoside (peak-1; Rf = 0.59) (Fig. 2C) while clearly separating the standard (peak-1) and luteolin-7-O-glucoside from each other.
including different phytoconstituents in the *C. flexuosa* extract (Fig. 2B). The ratio of regression equation (y) and correlation co-efficient (r²) for luteolin-7-O-glucoside was found to be y = 4.8846x - 755.18/0.988. The developed HPTLC method led to the quantification of luteolin-7-O-glucoside (17.58 mg/g) in the dried weight of *C. flexuosa* extract.

### 3.5. Phytochemical composition of *C. flexuosa*

Sixteen compounds were identified in *C. flexuosa* by GC–MS with their retention time, chemical formula, molecular weight, and concentration (Table 4; Fig. 3). The identified compounds in *C. flexuosa* were represented based on their elution order on the

| Compound Name                        | Formula   | MW (g/mol) | RT (min) | Area (%) |
|--------------------------------------|-----------|------------|----------|----------|
| N-Methoxy-N-methylacetamide          | C₂₆H₄₅NO₂ | 103.12     | 3.32     | 27.610   |
| Isoamyl alcohol                      | C₆H₁₂O    | 88.15      | 3.60     | 2.410    |
| Glycerol                             | C₃H₈O₃    | 92.09      | 3.70     | 6.860    |
| Methyl acetate                       | C₃H₆O₂    | 74.08      | 3.83     | 4.090    |
| DL-Glyceraldehyde dimer              | C₆H₁₂O₆   | 180.16     | 5.67     | 4.790    |
| 3,7,7-Trimethyl-1,3,5-cycloheptatriene | C₁₀H₁₄   | 134.22     | 3.70     | 0.700    |
| 1,2,3-Propanetriol monoaacetate      | C₆H₁₀O₄   | 134.13     | 11.08    | 5.120    |
| ihydro-2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-one | C₆H₈O₄     | 144.12     | 11.61    | 6.860    |
| 2-Methoxy-4-vinylphenol              | C₁₀H₁₀O₂  | 156.26     | 11.16    | 1.560    |
| 4-Hydroxymethylbenzaldehyde          | C₆H₁₀O₂   | 136.15     | 14.07    | 1.850    |
| 4-Hydroxyphenylacetic acid           | C₆H₁₀O₃   | 152.15     | 15.66    | 11.930   |
| 2-Decen-1-ol                         | C₁₀H₁₈O   | 156.26     | 19.16    | 0.990    |
| Palmitic acid                        | C₁₆H₃₂O₂  | 256.42     | 20.44    | 3.640    |
| Undecane                             | C₁₁H₂₄    | 156.31     | 25.27    | 1.370    |
| Nonacosanol                          | C₂₉H₅₀O   | 424.8      | 28.13    | 3.500    |
| Octadecane                           | C₁₈H₃₆     | 254.5      | 31.47    | 0.810    |

RRI, relative retention indices calculated against n-alkanes; %, calculated from the flame ionization detector (FID) chromatograms; tr, trace (<0.1%). Identification method: tR, identification based on the retention times (tR) of genuine compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and compared with literature data.
leucotinin and leucotinin-7-0-glucoside have been demonstrated for their anti-oxidative as well as anti-inflammatory salutations (Seelinger et al., 2008; Park and Song, 2013). In line with this, we also report HPTLC based quantification of leucotinin-7-0-glucoside in C. flexuosa. In addition, our GC/MS analysis has identified sixteen compounds in C. flexuosa. Of these, 3,7,7-trimethyl-1,3,5-cycloheptatriene has been reported in cold-adapted Scots pine and pine beetles (Gries et al., 1992).

5. Conclusion

Our data for the first time, demonstrated the promising anti-oxidative, anti-cell proliferative and anti-microbial therapeutic potential of C. flexuosa, including identification of bioactive leucotinin-7-0-glucoside in its extract. This warrants further phytochemical and bio-efficacy studies towards isolations and identifications of active principles.

Declaration of Competing Interest

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

Acknowledgments

This study was financially supported by the Researchers Supporting Project number (RSP-2021/379), King Saud University, Riyadh, Saudi Arabia.

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