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Extracts of *Centaurea bornmuelleri* and *Centaurea huber-morathii* inhibit the growth of colon cancer cells *in vitro*

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SUMMARY

Plants from the genus *Centaurea* (*C.*) (Family: Asteraceae *alt.* Compositae), widely distributed in Asia, Europe and North America, have traditionally been used in the treatment of various ailments. As a part of our on-going studies on the plants from the genus *C.* for their phytochemistry and biological activities, extracts of the seeds of Turkish endemic *C.* species, *C. bornmuelleri* and *C. huber-morathii,* were tested for their cytotoxicity towards the CaCo2 colon cancer cell line as well as for the toxicity towards the brine shrimps, using the MTT and the brine shrimp lethality assays, respectively. Among the extracts, the MeOH extract of these plants showed significant toxicity towards the brine shrimps (LD_{50} = 55.2 × 10^{-2} and 42.4 × 10^{-2} mg/ml, respectively). The MeOH extract of both *C.* species also inhibited the growth of CaCo2 colon cancer cells in the MTT assay (IC_{50} = 29.9 and 33.0 g/ml, respectively). As the most prominent activities in both assays were observed with the MeOH extracts, it can be assumed that the compound(s) responsible for these activities are polar in nature.

Key words: Asteraceae; Compositae; *Centaurea bornmuelleri*; *Centaurea huber-morathii*; MTT assay; Brine shrimp lethality assay; Colon cancer; CaCo2

INTRODUCTION

There are more than 500 species within the genus *Centaurea* (*C.*) L. of the family Asteraceae (*alt.* Compositae), which are hardy, herbaceous, perennial and annual plants, distributed in many parts of Asia, Europe and North America (Clapham *et al.*, 1952; GRIN database, 2006). Several *C.* species have traditionally been used in the treatment of a number of ailments and possess different types of pharmacological properties (Grieve, 2002; Phytochemical and Ethnobotanical Database, 2005; Sarker *et al.*, 2005). We have recently reported the anti-colon cancer activity of the extracts and the isolated compounds from *C. schischkinii* and *C. montana* (Shoeb *et al.*, 2005, 2006). The antibacterial and antioxidant activities of the extracts of *C. bornmuelleri* and *C. huber-morathii* have also been reported recently (Sarker *et al.*, 2005).

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As a part of our on-going studies on the plants from the genus *C.* for their phytochemistry and biological activities (Sarker *et al.* 1997a,b, 1998a-c, 2001, 2005; Cooper *et al*., 2002; Ribeiro *et al*., 2002; Ferguson *et al*., 2003; Kumarasamy *et al*., 2003a,b; Middleton *et al*., 2003; Shoeb *et al*., 2004a,b, 2005), we now report on the toxicity towards brine shrimp and anti-colon cancer activity of the extracts of the seeds of *C. bornmuelleri* and *C. huber-morathii.*

MATERIALS AND METHODS

Plant materials

The seeds of *C. bornmuelleri* Hausskn. Ex. Bornm and *C. huber-morathii* Wagenitz were collected from West and East Anatolia, Turkey, during September-October 2002. Voucher specimens (PH800004 and PH800001, respectively) have been deposited in the herbaria of Plant and Soil Science Department, University of Aberdeen, Scotland (ABD) and Canakkale Onsekiz Mart University (COMU).

Extraction

Ground seeds (~100 g) of *C. bornmuelleri* and *C. huber-morathii* were Soxhlet-extracted sequentially using solvents (1.1 liter each) of increasing polarity, *n*-hexane, dichloromethane (DCM) and methanol (MeOH). Solvent was evaporated from the extracts using a rotary evaporator at a temperature not exceeding 50 ^{o}C.

Preparation of the extract solutions for bioassays

The DCM and MeOH extracts (0.025 g) were dissolved in 5 ml DMSO to obtain stock solutions of 5 mg/ml concentrations.

The brine shrimp lethality assay (BSL)

Brine shrimp eggs were purchased from Water Life, Middlesex, UK. The bioassay was conducted following the procedure published previously (Meyer *et al.*, 1982). LD_{50}s were determined from the 24 h counts using the Probit analysis method (Finney, 1971). Percentage mortalities were adjusted relative to the natural mortality rate of the control, following Abbots formula P = (Pi – C)/ (1 – C), where P denotes the observed nonzero mortality rate and C represents the mortality rate of the control.

Materials and chemicals

CaCo2, human colon cancer cell line was obtained from the European collection of cell cultures. Earle's minimum essential medium, Earle's balanced salt solution, trypsin, l-glutamine, non-essential amino acids, penicillin and streptomycin were purchased from Sigma, UK. Foetal calf serum (FCS) was purchased from Biosera, UK. The 96 well plates were purchased from Sero-Wel, Bibbly Sterilin Ltd., Stones, Staffs, UK.

Cryopreservation

Cells were cryopreserved to ensure the continuous supply of a cell line during this study. Healthy and viable cells 4 - 10 × 10^6 per ampoule were used for this purpose. Freezing medium was prepared from the growth medium containing 10% (v/v) sterile dimethyl sulphoxide. Cell pellets dissolved in 1 ml freezing medium were poured into a sterile plastic screw-top cryotubes and immediately cooled at a rate of between 1 - 5°C per min. Normally the cryotubes were placed in the -20°C freezer for 3 - 4 h and transferred to the -80°C for approximately 16 h (overnight). After freezing, the ampoules were transferred to liquid nitrogen.

Thawing of frozen cells

The ampoule was removed from storage and unscrewed the cap ¼ turn to release any residual nitrogen. After 1 - 2 min (to let gas escape) it was placed in the water bath at 37°C. Special care was taken in order to prevent water entering the ampoule and contaminating the cells. When ampoules were fully thawed, they were taken to the laminar flow hood. Then the ampoule contents were transferred into a 30 ml sterile universal. The

growth medium (10 ml) were added slowly to the universal, mixed and centrifuged at the lowest speed to pellet the cells at 70 - 100 × *g*. After decanting the supernatant, the pelleted cells were resuspended in fresh medium and transferred to a culture flask.

Cell counting

Cells were counted by a haemocytometer and a particle size distributor. Similar results were obtained in both cases and constant cell density was maintained for all cell culture work in this study.

Cell viability assay

CaCo2 cells were maintained in Earle's minimum essential medium (Sigma), supplemented with 10% (v/v) foetal calf serum, 2 mM *l*-glutamine, 1% (v/v) non-essential amino acids, 100 IU/ml penicillin and 100 μg/ml streptomycin. Exponentially growing cells were seeded on 96-well plates at 1×10^4 cells per well and incubated for 24 h before the addition of drugs. Stock solution of the test compounds was initially dissolved in DMSO or H_2O and further diluted with serum free medium. Following a 24 hours incubation at 37°C, 5% CO_2, 100 μl of various concentration of test extracts were added in each well in triplicates and cells were further incubated for 72 h. After 72 h of incubation at 37°C, the medium was removed, and 100 μl of MTT reagent (1 mg/ml) in serum free medium was added to each well. The plates were incubated at 37°C for 4 h. At the end of the incubation period, the supernatants were removed and, pure DMSO (200 μl) was added to each well and plates were shaken gently for 15 min. The metabolised MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm using a micro plate reader (Dynex Technologies, USA). The IC_{50} values are defined as the drug concentrations required to reduce the absorbance by 50% of the control values. They are calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation y = 50 (50% value).

RESULTS AND DISCUSSION

The BSL assay has been used routinely in the primary screening of the crude extracts as well as the isolated compounds to assess the toxicity towards brine shrimps, which could also provide an indication of possible cytotoxic properties of the test materials (Ferrigni *et al.,* 1984). It has been established that the cytotoxic compounds usually show good activity in the BSL assay, and this assay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and cost-effectiveness (McLaughlin, 1991). The MeOH and DCM extracts of *C. bornmuelleri* and *C. huber-morathii* were assessed in this assay. Owing to a high degree of lipophilicity, the *n*-hexane extract could not be tested. While the DCM extracts of both species showed low levels of toxicity ($LD_{50} = 12.2 \times 10^{-1}$ and 26.1×10^{-1} mg/ml, respectively), the MeOH extracts were significantly toxic ($LD_{50} = 55.2 \times 10^{-2}$ and 42.4×10^{-2} mg/ml, respectively) (Table 1). The LD_{50} value of the positive control, podophyllotoxin, was 2.79×10^{-3} mg/ml.

As the MeOH extracts were the most active extracts in the BSL assay (Table 1), these were also tested in the MTT assay (Mossman, 1983) using the

Table 1. Toxicity towards brine shrimps and anti-colon cancer activity of the extracts of the seeds of *C. bornmuelleri* and *C. huber-morathii*

C. species	LD_{50} (mg/ml) in the BSL assay		IC_{50} (g/ml) in the MTT assay
	DCM	MeOH	MeOH
C. bornmuelleri	12.2×10^{-1}	55.2×10^{-2}	29.8
C. huber-morathii	26.1×10^{-1}	42.4×10^{-2}	33.0

CaCo2 colon cancer cell line for possible cytotoxic activities. The MTT assay is a well-documented cell viability assay and has been modified by several investigators since it was first developed by Mosmann. This assay is based on the transformation of tetrazolium salt, MTT by mitochondrial succinic dehydrogenases in viable cells yielding purple formazan crystals that are not soluble in aqueous solution. The amount of formazan generated by dehydrogenase enzyme is directly proportional to the number of viable cells in culture and can be measured at 560 nm. The MeOH extracts of *C. bornmuelleri* and *C. huber-morathii* exhibited remarkable cytotoxic activities with the IC_{50} values 29.8 and 33.0 mg/ml, respectively. As any crude plant extract may contain several compounds, the amounts of any actual active compound(s) present in the extract could be very low. Thus, it is reasonable to assume that cytotoxic compounds present in these extract could be even more potent than the extracts. This is the first report on the assessment of the extracts of the seeds of *C. bornmuelleri* and *C. huber-morathii* for their toxicity towards brine shrimps, and the cytotoxicity towards the CaCo2 colon cancer cell line *in vitro*.

CONCLUSION

This study has, yet again, established a good correlation between the BSL assay and anti-cancer activity of plant extracts. As the most prominent activities were observed with the MeOH extracts, it can be assumed that the compound (s) responsible for these activities are polar in nature.

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