Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: Muhammad Asad Ghufran, Rizwana Aleem Qureshi, Aniqa Batool, Tamara P. Kondratyuk, Jacquelyn M. Guilford, Laura E. Marler, Leng Chee Chang & John M. Pezzuto (2009) Evaluation of selected indigenous medicinal plants from the western Himalayas for cytotoxicity and as potential cancer chemopreventive agents, Pharmaceutical Biology, 47:6, 533-538, DOI: 10.1080/13880200902873847

To link to this article: https://doi.org/10.1080/13880200902873847

Published online: 01 Jun 2009.

Pharmaceutical Biology, 2009; 47(6): 533–538

informa
healthcare

RESEARCH ARTICLE

Evaluation of selected indigenous medicinal plants from the western Himalayas for cytotoxicity and as potential cancer chemopreventive agents

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Abstract

The western Himalayas in the northern areas of Pakistan have significant potential for ethnomedicinal research. In the current study, indigenous informants were interviewed using open-ended questionnaires and a free-listing of knowledge related to native medicinal plants. Information patterns indicated that over 100 local plant species were in frequent medicinal use for a variety of conditions, including inflammation and cancer. Several field surveys were conducted in community forests and meadows, with the aim of exploration, collection, taxonomic identification, and finally, *in vitro* analysis. Organic extracts of eight species were tested for inhibition of nuclear factor-kappaB (NF-κB), activation of retinoic acid X receptor alpha (RXR), induction of quinone reductase (QR), and inhibition of aromatase, along with assessment of cytotoxicity with four human cancer cell lines. *Mellia azedarach*, *Ajuga bractiosa*, *Figonia cretica* and *Swertia chirata* inhibited both tetradecanoylphorbol-13-acetate (TPA) and tumor necrosis factor (TNF) activated NF-κB activity, whereas *Silybum merianum* and *Rumex dentatus* were only active against TNF activation. The lowest IC_{50} values for inhibition of TPA activated NF-κB activity were 0.41 and 0.44 µg/mL for *A. bractiosa* and *S. chirata*, respectively. Extracts from three plant species, *A. bractiosa, R. dentatus* and *R. hastaus*, were active in the RXR assay. Results from the QR assay showed five active samples (with induction ratios >2) belonging to four species: *A. bractiosa, R. dentatus*, *S. merianum* and *S. chirata*. Most of the plant extracts were not cytotoxic (IC_{50} values >20 µg/mL) with HepG2, MCF7, LNCaP and LU cell lines. Only two plants, *R. dentatus* and *R. hastaus*, demonstrated moderate cytotoxic responses (IC_{50} values 5-15 µg/mL) with HepG2, MCF7 or LNCaP cells. None of the plant extracts was found to inhibit aromatase activity. Based on these data, it may be suggested that the plants under investigation contain potential chemopreventive compounds. Additional testing is required. However, the positive responses observed in these bioassays illustrate the high potential of local medicinal plants.

Keywords: *Traditional medication; chemoprevention; ethnobotany; anticancer; medicinal plants; nuclear factor-kappaB; retinoic X receptor; quinone reductase; cytotoxicity*

Introduction

Indigenous people in different parts of the world have a common tendency for dependence on medicinal plants growing in their surrounding areas (Shinwari et al., 2000). Skills have been learned and developed over centuries, which impact their diverse and independent systems of medicine. Some herbal preparations are reported to have enormous potential for the cure of both simple and complex ailments (Salick et al., 1999). The geographic region currently under investigation, situated in the Himalayan and Karakorum mountain ranges, shares a large number of medicinal plants as a result of remarkable flora. Further, the local system of

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(Received 10 June 2008; accepted 12 August 2008)

ISSN 1388-0209 print/ISSN 1744-5116 online © 2009 Informa UK Ltd
DOI: 10.1080/13880200902873847

medicine and cultural use of plants make this area of value for the identification of medicinal species and ethnomedicinal research (Goodman & Ghafoor, 1992). In addition, it is worth noting that some plant-based medicines which are frequently used by the native people to cure simple ailments also protect from more complex diseases (Qureshi & Ghufran, 2005; Pezzuto et al., 2006).

In spite of improved treatment options, many of which are plant-based (Pezzuto et al., 2005; Weinstein, 1991), cancer is still one of the leading causes of death in the world. In addition to being of value for cancer therapy, natural products are known to serve as a useful source of cancer chemopreventive agents (Kondratyuk & Pezzuto, 2004; Sharma et al., 1994; Boone et al., 1990; Schatzkin et al., 1990). In searching for new therapeutic or preventative modalities, it is worth considering various indigenous medicinal preparations that have been reported by native people and herbalists as effective cancer treatments. These medicinal plants also include several foods, seasonings, spices and condiments in everyday use by indigenous communities (Weinstein, 1991). It is possible that regular use of these plants could reduce the incidence of cancer by inhibiting the process of carcinogenesis (Pezzuto et al., 2006; Wattenberg, 1985; Dorai & Aggarwal, 2004).

Following a detailed field survey involving free-listings and questionnaire-based data collection, we selected eight plant species used most frequently in the Himalayas, and tested them for cancer chemopreventive activity with *in vitro* models. Nearly all of the test species were found to be active in one or more of the selected assays. Accordingly, these native remedies may represent sources for the discovery of new chemopreventive agents. Equally important, the results illustrate the value of focused selection based on traditional knowledge.

Materials and methods

Collection of plant material

Plant collection was carried out from the spring of 2004 to the autumn of 2006 in several valleys and villages of an administrative region of Pakistan (called the "northern areas" of Pakistan), situated between 72°32 to 76°56 east longitudes and 35°11 to 37°05 north latitudes (Ghufran et al., 2007). Most of the sampling was performed between the Diamer and Skardu Districts. All of the field studies were conducted in close association with local communities to collect medicinal plants from each community's forest reserves and high altitude pastoral lands. Exploration trips were conducted in different parts of the year in order to collect traditional medicinal plants at their appropriate harvest time. Data concerning the traditional uses of these plant species was collected using semi-structured and open-ended questionnaires, as well as field notes regarding the local traditions for harvest, processing and indigenous use. Informants were also requested to provide a listing of medicinal plants in use in their community, along with the known benefits of each species.

Collection, preservation and taxonomic identification were carried out by comparing each specimen with existing voucher specimens from various herbaria in Pakistan. These samples were then mounted and deposited in the Herbarium of Pakistan (ISL), at the Department of Plant Sciences Quaid-i-Azam University, Islamabad, Pakistan (Accession numbers 124118 to 125010, including the duplicates for each specimen to be used for future analytical work).

In this survey, 135 plant species with reported medicinal value were collected. Data from the questionnaires and free-listings were compiled in order to identify the most promising species for chemoprevention. Based on the frequency, pattern, and methods of using these plants, eight species were selected for testing in cancer chemoprevention and cytotoxicity assays.

Preparation of extracts

Plants were extracted in various solvents and different parts were tested individually for activity (Table 1). Shade-dried plant materials were crushed in small sections (500 g each) and soaked for three days using methanol as the solvent. Plant material was then filtered out and used for further extraction. Three consecutive extractions were collected and the plant residues were discarded.

The solvent was evaporated using a rotary evaporator. Some of the crude extract was retained while the remainder was further partitioned into polar and non-polar fractions using hexane and chloroform. The remaining water fraction was not tested.

Biological assays

Several assays were performed to test the cancer chemopreventive activity of the plant extracts. The *in vitro* assays included nuclear factor-kappaB (NF-κB) (Homhual et al., 2006; Dorai & Aggarwal, 2004), quinone reductase (QR) (Cuendet et al., 2006), aromatase (Strasser-Weippl & Goss, 2005), retinoic X receptor alpha (RXR) (Wu et al., 2002), and assessment of cytotoxicity (Mi et al., 2001). Cytotoxicity was assessed with four different human cancer cell lines, namely, hepatocellular carcinoma ATCC number HB-8065 (HepG2), mammary adenocarcinoma ATCC number HTB-22 (MCF-7), prostate lymph node carcinoma ATCC number CRL-1740,

Table 1. Taxonomic data of plants selected for biological screening.

Sample	Plant name	Family	Source	Extract
1	*Melia azedarach* L.	Meliaceae	leaf	Methanol
2	*Melia azedarach* L.	Meliaceae	leaf	Hexane
3	*Melia azedarach* L.	Meliaceae	leaf	Crude
4	*Hippophae rhamnoides* subsp. *tukrestanica* L.	Elaeagnaceae	fruit	Crude
5	*Ajuga bracteosa* Wallich ex Bentham	Labiatae	leaf	Methanol
6	*Ajuga bracteosa* Wallich ex Bentham	Labiatae	leaf	Hexane
7	*Ajuga bracteosa* Wallich ex Bentham	Labiatae	leaf	Crude
8	*Figonia cretica* L.	Rubiaceae	leaf	Crude
9	*Rumex dentatus klotzschianus* (Meisn.) Rech. f.	Polygonaceae	shoot	Crude
10	*Rumex dentatus klotzschianus* (Meisn.) Rech. f.	Polygonaceae	leaf	Crude
11	*Rumex dentatus klotzschianus* (Meisn.) Rech. f.	Polygonaceae	roots	Crude
12	*Rumex dentatus klotzschianus* (Meisn.) Rech. f.	Polygonaceae	leaf	Hexane
13	*Rumex dentatus klotzschianus* (Meisn.) Rech. f.	Polygonaceae	roots	Hexane
14	*Rumex dentatus klotzschianus* (Meisn.) Rech. f.	Polygonaceae	whole plant	Crude
15	*Rumex hastatus* D. Don.	Polygonaceae	whole plant	Crude
16	*Silybum marianum* (L.) Gaertn.	Astraceae	flower	Crude
17	*Silybum marianum* (L.) Gaertn.	Astraceae	root	Crude
18	*Silybum marianum* (L.) Gaertn.	Astraceae	shoot	Crude
19	*Silybum marianum* (L.) Gaertn.	Astraceae	leaf	Crude
20	*Swertia chirata* (L.) Gaertn.	Gentianaceae	whole plant	Crude

(LNCaP) and lung carcinoma cells (LU) established at the Department of Surgical Oncology, University of Illinois at Chicago. For the screening of potential chemopreventive agents, *in vitro* short-term tests were preferred because they are less time-consuming and more reliable. A brief description of each assay is given below.

NF-κB assay

The 293/NF-κB-Luc cell line, designed for monitoring the activity of the NF-κB (Homhual et al., 2006), was purchased from Panomics (Freemont, CA). Cells were seeded into sterile white-walled 96-well plates at 20×10^3 cells per well in Dulbecco's modified media with 10% fetal bovine serum (FBS). After growing for 48 h to 90% confluency, the medium was replaced with fresh medium containing tetradecanoylphorbol-13-acetate (TPA) (final concentration 100 nM) or tumor necrosis factor (TNF) (final concentration 50 ng/mL) simultaneously with different test extracts (at a final concentration of 20 µg/mL), followed by 6 h incubation. Luciferase activity was determined with a luciferase kit from Promega (Madison, WI) according to the manufacturer's instructions. Briefly, after treatment, the cells were washed with phosphate-buffered saline and 1x Reporter lysis buffer was added before plates were placed in a −80°C freezer. The following day the cells were thawed and assayed for luciferase activity with a LUMIstar Galaxy Luminometer (BMG Labtechnologies, Durham, NC).

RXR ligand binding assay

The LanthaScreen TR-FRET RXR alpha Coactivator Assay from Invitrogen (Carlsbad, CA) was used according to the manufacturer's protocol. Test samples were mixed in a black 384-well plate with a fluorescein labeled PGC1-alpha coactivator peptide, a terbium labeled anti-GST antibody, and the GST-labeled ligand binding domain (LBD) of RXR alpha. After 1 h incubation with shaking at room temperature, plates were read in a Synergy 2 fluorescent plate reader (Biotek, Winooski, VT). Each well was excited at 340 nm and emissions were read at 520 nm (to detect fluorescent resonance energy transfer (FRET)) and 490 nm (to detect terbium background). Binding of test sample to RXR LBD caused a conformational change in the RXR, resulting in binding of both the fluorescein-PGC1-alpha coactivator peptide and the terbium anti-GST antibody. The close proximity of the fluorescein and terbium tags resulted in a time-resolved FRET signal at 520 nm. Assay output was measured as the ratio of 520:495 emissions. Each sample was additionally tested to ensure it did not autofluoresce in the emission ranges used in this assay.

Quinone reductase assay

Quinone reductase was assessed in 96-well plates using Hepa 1c1c7 murine hepatoma cells as previously reported (Song et al., 1999). Briefly, cells were grown to a density of 2×10^4 cells/mL in 200 µL of minimal essential medium (MEM)-α containing 5% antibiotic-antimycotic (Gibco) and 10% fetal bovine serum at 37°C in 5% CO_2 atmosphere. After 24 h preincubation, the media was changed and test compounds were added to a final concentration of 20 µg/mL. For dose dependence, samples were added in five serial three-fold dilutions. The cells were incubated with test samples for an additional 48 h.

Quinone reductase activity was measured as a function of the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimetylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan (Kennelly et al., 1997). Protein content was determined via crystal violet staining of identical plates. Specific activity is defined as nmol of formazan formed per mg protein per min (Prochaska & Santamaria, 1988). The induction ratio (IR) of QR activity represents the specific enzyme activity of agent-treated cells compared with a DMSO-treated control. The concentration to double activity (CD) was determined through a dose-response assay for active substances (IR >2).

Aromatase assay

Aromatase activity was assayed as previously reported (Maiti et al., 2007) with the necessary modifications to perform the test in 384-well plates. The test substance (3.5 µL) was preincubated with 30 µL of NADPH regenerating system (2.6 mM $NADP^+$, 7.6 mM glucose 6-phosphate, 0.8 U/mL glucose 6-phosphate dehydrogenase, 13.9 mM $MgCl_2$, and 1 mg/mL albumin in 50 mM potassium phosphate buffer, pH 7.4) for 10 min. The enzyme and substrate mixture (33 µL of 80 µL/mL enzyme (CYP19, BD Biosciences, San Jose, CA), 0.4 µM dibenzylfluorescein, and 4 mg/mL albumin in 50 mM potassium phosphate, pH 7.4) was added and the plate was incubated for 30 min at 37°C before quenching with 25 µL of 2 N NaOH. After termination of the reaction and shaking for 5 min, the plate was further incubated for 2 h at 37°C. This enhances the ratio of signal to background. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission).

Cytotoxicity analysis

The cytotoxic potential of test substances was determined with HepG2, MCF-7, LNCaP and LU cells were assessed as described previously (You et al., 1995; Mi et al., 2001). Cells were seeded in 96-well plates (10^6 cells/mL), and various concentrations of samples dissolved in 10 µL of 10% DMSO were added to each well. The plates were incubated for 72 h at 37°C and cell viability was determined with sulforhodamine B staining. The IC_{50} values were determined as the concentration of sample required to inhibit the 72 h growth of cells by 50%, relative to a control treated with solvent (0.5% DMSO) only.

Results and discussion

Of 135 plant species with traditional medicinal uses collected, eight were selected for assays to determine cancer chemopreventive activity. These species included *Ajuga bractiosa, Hippophae rhamnoides* subsp. *turkestanica, Figonia cretica, Melia azedarach, Rumex dentatus, Rumex hastaus, Swertia chirata* and *Silybum marianum*. Only those portions of selected species which are used in local medicinal preparations were chosen for extraction. For example, only leaves of *M. azedarach* and *A. bracteosa* are used locally, while all parts of *R. dentatus* and *S. marianum* are used medicinally. Table 1 denotes the origin of each test fraction.

NF-κB is an inducible factor for gene transcription involved in cell survival (Chen et al., 2001), cell adhesion and differentiation, inflammation, and cell and tissue growth (Bharti & Aggarwal, 2002). In healthy cells, NF-κB is present in the cytoplasm and binds to the inhibitory IκB protein, which blocks the nuclear localization sequences of NF-κB (Dorai & Aggarwal, 2004). A number of genes which are involved in tumor cell invasion and angiogenesis have been found to be regulated by NF-κB. Activation of NF-κB supports cell survival and proliferation, while down-regulation of NF-κB sensitizes the cell to apoptosis. There is a need to search for specific inhibitors from new sources like indigenous medicinal plants. Therefore, organic extracts of all species were tested in the NF-κB assay activated by two different activators, TPA or TNF. Extracts of *M. azedarach*, *A. bractiosa*, *F. cretica* and *S. chirata* were found to be active (sample numbers 2, 6, 7, 8 and 20) in inhibiting both TPA and TNF activated NF-κB assays, whereas *S. marianum* and *R. dentatus* (sample numbers 14, 15 and 16) were only active in the TNF activated NF-κB assay (Table 2). Out of the available IC_{50} values, the lowest were 0.41µg/mL and 0.44µg/mL for *A. bractiosa* and *S. chirata*, respectively (Table 2), which validate the higher potential of these plants as candidates for chemoprevention (Dorai & Aggarwal, 2004, Goodman, 1992). As demonstrated previously (Riaz et al., 2007), components of *A. bracteosa* also inhibit lipoxygenase activity.

Retinoids are derivatives of vitamin A which influence cellular proliferation, differentiation, and apoptosis in a retinoid-specific and cell-type specific manner. Retinoids have shown efficacy as anti-cancer drugs that intervene in the carcinogenic process by regulating proliferation and differentiation at several stages (Wu et al., 2002; Conley et al., 1998). Retinoid receptors are nuclear hormone receptor proteins. There are two major classes of retinoid nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Compounds that are specific ligands of RXR have been shown to cause cancer cells to differentiate, which limits their potential to proliferate. RXR specific ligands exhibit less toxicity as compared to ligands of RAR receptors. For this reason, we screened our test samples to determine if they could bind to RXR. Only three plant species, *A. bractiosa, R. dentatus* and *R. hastaus*, were active in the RXR binding assay and therefore were considered significant for future screening. The results were normalized to the negative control (4% DMSO) condition,

Table 2. Activity of plant samples determined with *in vitro* assays for NF-κB, RXR and QR activity.

	NF-κB		RXR		QR	
Sample	IC_{50} μg/mL, TPA	IC_{50} μg/mL, TNF	CRS	EC_{50} μg/mL	IR at 20 μg/mL	CD (μg/mL)
1	>20	>20	1.01	NA	1	>20
2	2.43	8.86	0.89	NA	1.8	>20
3	>20	>20	0.82	NA	1.2	>20
4	>20	>20	1.12	NA	1.7	>20
5	>20	>20	1.04	NA	1.1	>20
6	0.41	12.99	1.24	3.27	2.4	13.9
7	2.44	3.24	1.23	3.83	2.2	17.7
8	5.36	16.9	0.85	NA	1.4	>20
9	>20	>20	1.08	NA	1.2	>20
10	>20	>20	0.99	NA	1.3	>20
11	>20	>20	0.94	NA	1.2	>20
12	>20	>20	1.05	NA	2.4	12.7
13	>20	>20	1.39	1.47	1	>20
14	>20	14.27	1.10	NA	0.1	>20
15	>20	6.66	1.29	3.10	0.4	>20
16	>20	4.9	1.11	NA	1.4	>20
17	>20	>20	1.00	NA	1.5	>20
18	>20	>20	0.82	NA	1.3	>20
19	>20	>20	0.97	NA	2.0	18.9
20	0.44	19.5	1.05	NA	3.7	0.42

IC_{50}, Concentration leading to 50% Inhibition; EC_{50}, Concentration leading to a response halfway between baseline and maximum; CRS, coactivator recruitment score (520sample/490sample)/(520DMSO/490DMSO); IR, induction ratio: specific activity of sample/specific activity of control (DMSO); CD, concentration which doubles activity; NA, not active (>20 μg/mL). The sample numbers correspond to those designated in Table 1.

which yielded a coactivator recruitment score (CRS) of 1. Positive control, 9-*cis*-retinoic acid, gave a CRS of 2.73. Samples with a CRS above 1.2 were tested for dose dependence. Four samples with the highest CRS yielded EC_{50} values in the low μg/mL range. All other samples showed no activity.

Samples were also tested for induction of QR. Quinone reductase is an important phase II enzyme that deactivates reactive and potentially carcinogenic species (Cuendet et al., 2006) by converting quinones to hydroquinones and reducing oxidative cycling. The QR enzyme exhibits a broad specificity, reducing a wide range of hydrophobic quinones of various structures (Benson et al., 1980). The induction of QR often coincides with induction of other phase II enzymes, and is therefore useful in the study of potential chemopreventive agents. Five samples were found to induce QR (with IR values >2). These fractions (6, 7, 12, 19 and 20) represent four species: *A. bractiosa, R. dentatus*, *S. merianum* and *S. chirata*. Fraction 20 (*S. chirata*) is especially noteworthy, with a CD of 0.42 μg/mL.

None of the tested plant extracts developed inhibition of aromatase activity. Importantly, most of the plant extracts were not cytotoxic when tested at 20 μg/mL with HepG2, MCF-7, LNCaP and LU cell lines. Only *R. dentatus* and *R. hastaus* demonstrated cytotoxic effect with HepG2 cells (IC_{50} 12.99 and 15.29 μg/mL), MCF-7 cells (IC_{50} 5.1 and 12.54 μg/mL) and LNCaP cells (IC_{50} 14.86 and 15.53 μg/mL, respectively) while for LU cells there was no cytotoxic effect observed (You et al., 1995). These data provide some insight for the prospective use of the tested plant species in cancer research (Lippman et al., 1990). Studies are still in progress for further screening of active components from these species.

Conclusions

With the exception of *Hippophae rhamnoides* subsp. *tukrestanica*, all of the plant species tested were found to be active in one or more of the assays reported herein. Since approximately 50% (11 samples) of the test substances were active, either the method of extraction or plant part affects the results. Two plants species, *A. bractiosa* and *S. chirata*, were active in NF-κB, RXR, as well as QR assays, and they did not display cytotoxicity, indicating they may represent a promising source of chemopreventive agents that can be identified through the process of bioassay-directed fractionation. In sum, the results of this study demonstrate the chemopreventive potential of local medicinal plants and the merit of working with indigenous communities.

Acknowledgements

The authors are grateful for funding support by the Higher Education Commission Pakistan, under "International Research Support Initiative Program" for field survey. We also acknowledge all the local people, herbalists and field staff for their help during plant collection. This work was supported by Program Project P01 CA48112 USA, awarded by the National Cancer Institute, USA.

This work was presented in part as an abstract at the 31st Annual Conference of the American Society of Ethnobiology, University of Arkansas, Fayetteville, Arkansas, USA, 16–19 May 2008.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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