Natural product extracts of the Canadian prairie plant, *Thermopsis rhombifolia*, have anti-cancer activity in phenotypic cell-based assays

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Many plant species within the terrestrial ecological zones of Canada have not yet been investigated for anti-cancer activity. We examined the scientific literature describing the endemic flora from the prairie ecological zone and selected the species, *Thermopsis rhombifolia*, locally known as the buffalo bean, for investigation of its anti-cancer potential. We tested it in cell-based assays using phenotypic screens that feature some of the hallmarks of cancer. An ethanolic extract prepared from *T. rhombifolia* was cytotoxic to HT-29 (colon) and SH-SY5Y (brain) cancer cell lines, and showed little cytotoxicity to a normal human cell line (WI-38). In phenotypic assays, we identified activities in the extracts that target cell death, cell cycle and cell adhesion. These data highlight the anti-cancer potential of previously untested plants found in northern ecological zones and the feasibility of using pertinent phenotypic assays to examine the anti-cancer potential of natural product extracts.

**Keywords:** *Thermopsis rhombifolia* (Fabaceae); hallmarks of cancer; HT-29 cells; SH-SY5Y cells; WI-38 cells; phenotypic screening; prairie to pharmacy project

1. Introduction

Cancer is a life-threatening disease worldwide, and in Western nations it remains one of the leading causes of death. To meet the challenge in providing effective cancer therapies for diverse solid tumours, we need to discover new drugs that target a variety of cancer cell pathways. Nearly 50% of the cancer therapeutic agents currently available are either natural products,
or compounds derived from natural products (Newman & Cragg 2012). Plant species are sources of natural product anti-cancer drugs, such as paclitaxel (Wani et al. 1971), also known by its trademark name Taxol®, which is one of the most successful anti-cancer drugs ever developed. It was first isolated from the bark of the plant species Taxus brevifolia (Pacific yew) in a search for anti-cancer compounds in terrestrial plants by the US National Cancer Institute (Cragg 1998). It is estimated that only 15% of the world’s terrestrial plant species have been scientifically examined for pharmaceutically active natural products (Ngo et al. 2013), suggesting that new natural products remain to be discovered.

One of the difficulties in discovering new natural products is selecting terrestrial plant species for testing among the 250,000 to 500,000 species believed to be present on earth (Ngo et al. 2013). A solution is to explore ecological regions that have received little attention in prior expeditions, such as Canada (Uprety et al. 2012). Further information about this potential is required to launch exhaustive bio-prospecting explorations.

Phenotypic screening methods are a valuable approach to investigate natural product extracts for pharmaceutical compounds. They do not require immediate knowledge of the biomolecular target, such as an enzyme; instead these screens focus on identifying compounds that inhibit vital pathways such as the cell cycle (Swinney & Anthony 2011). Knowledge of cellular pathways can then be used in subsequent biology-guided chemical purification and to identify the target biomolecule. Phenotypic screening methods are also valuable when extracts contain two or more cytotoxic chemicals that might not be distinguished by cell viability assays alone.

We have initiated a research programme in which we investigate the anti-cancer properties of plants from the Canadian prairie ecological zone. This zone harbours plant species that are endemic to the prairies and as of yet poorly studied in terms of their anti-cancer properties. One such plant species is Thermopsis rhombifolia, which we investigate here. This plant species is believed to be the source of poisoning in children who had ingested plant parts (McGrath-Hill & Vicas 1997). We find that extracts prepared from T. rhombifolia are cytotoxic to cancer cells and act upon several cancer cellular pathways.

2. Results and discussion
2.1. Description of an endemic Canadian plant species, T. rhombifolia
T. rhombifolia is a herbaceous plant that grows in dense stands to a height of 20–30 cm in undisturbed sites in the prairie ecological zone (Figure S1). It blossoms in May and early June, and was used by local First Nation populations as a seasonal indicator for hunting (Peacock 1992). The legume shape and flower colour give rise to its local common names of buffalo bean or golden bean. The plant has yellow five-petalled flowers at the apex of stems, compound leaves 2–3 cm in length that alternate and legumes containing uniformly shaped seeds 5 mm in length (Figure S1C).

2.2. T. rhombifolia DNA bar code sequences
We amplified DNA from dried leaves using the polymerase chain reaction (PCR) and DNA primers corresponding to the psbA-trnH intergenic spacer and the internal transcribed spacer region (ITS), also known as bar code regions (Kress et al. 2005). The PCR products were sequenced and compared with those present in the NCBI GenBank database by BLAST analysis. Neither of the two bar code regions from T. rhombifolia was present in the NCBI database; however, all the species that shared the greatest percentage identity to our sequences were members of the Thermopsis genus. The aligned sequences of Thermopsis species with the highest shared DNA sequence identity are shown for the psbA-trnH intergenic spacer.
(Figure S2A) and the ITS region (Figure S2B). The psbA-trnH intergenic region of *T. rhombifolia* showed greatest sequence identity (93.3%) to the same region of *Thermopsis chinensis*. The ITS region sequence of *T. rhombifolia* showed greatest sequence identity (99.3%) with the same region of *Thermopsis montana*, and was 98% identical to the sequence from *T. chinensis*. No psbA-trnH sequences were available for *T. montana* in the GenBank database. These sequence data confirm that the *T. rhombifolia* species we harvested is distinct from other *Thermopsis* species that flourish in North America.

2.3. *Extracts prepared from T. rhombifolia are cytotoxic to two cancer cell lines*

We investigated the cytotoxic properties of *T. rhombifolia* by preparing 75% ethanol extraction of leaves (named extract PP-003). Amongst the solvents available for extraction, we selected 75% ethanol because it is effective in extracting a large range of chemical families, is inexpensive and can be safely handled (Gunnar & Bohlin 2009). To compensate for the possibility of incomplete extraction by 75% ethanol, we set the threshold of ‘bioactivity’ to an IC₅₀ of 500 μg/mL in the first biological assays. Human cancer cell lines HT-29 (colon) or SH-SY5Y (brain) were incubated with either 50 μg/mL or with 500 μg/mL of extract PP-003 and compared with cells treated with dimethyl sulphoxide (DMSO) only, and images were taken after 24 h (Figure 1(A)). Extract PP-003-treated cells did not display distinct morphological changes after treatment with 50 μg/mL of PP-003. By contrast, treatment of either cell line with 500 μg/mL for 24 h caused cells to appear granulated and detach from the culture dish, indicating that the PP-003 extract was likely cytotoxic. Cell viability after treatment with PP-003 was then measured by the MTT assay on the two cancer cell lines and a non-cancer cell line WI-38, which is diploid and non-transformed. The IC₅₀ value for extract PP-003 was 220 ± 15 μg/mL.

![Figure 1. Extract PP-003 prepared from *T. rhombifolia* is cytotoxic to cancer cell lines. (A) HT-29 or SH-SY5Y cells were either NT or treated with 50 μg/mL or 500 μg/mL of extract PP-003 and observed by phase-contrast light microscopy 24 h post-treatment. Scale bar = 25 μm. (B) The mean IC₅₀ values and standard error of the mean of extract PP-003-treated HT-29 and SH-SY5Y cells, as determined by the MTT cell viability assay.](image-url)
to HT-29 cells and 183 ± 13 μg/mL to SH-SY5Y cells (Figure 1(B)). The normal cell line WI-38 was relatively insensitive to PP-003. At 1000 μg/mL PP-003 for 96 h, 68 ± 8% of WI-38 cells were still viable and an IC₅₀ value could not be determined. Aqueous extracts of either seeds or leaves and the ethanol extract of seeds had little effect on cell lines when tested at similar concentrations as extract PP-003 (data not shown).

2.4. Extract PP-003 arrests cells in the G1 phase but does not damage DNA

To explore the cytotoxic effects of extract PP-003 in more detail, HT-29 cells were treated with either DMSO or with 200 μg/mL extract PP-003 for 24 h and analysed by flow cytometry (Figure 2). Cells treated with extract PP-003 were arrested in the G1 phase of the cell cycle with 86% of the cells in this phase as compared to 63% of not-treated (NT) cells. Furthermore, only 3% of cells treated with extract PP-003 were in S-phase while 10% were in G2/M-phase, which is consistent with a G1 arrest. By contrast, NT cells showed 16% and 19% of the cell population in S-phase or G2/M-phase, respectively.

We then tested whether the cell cycle arrest induced by treatment with PP-003 was a genotoxic response by using histone γ-H2AX staining and immunofluorescence microscopy. HT-29 cells were either NT or were treated for 24 h with extract PP-003, or with camptothecin (CPT). Cells were fixed, stained with anti-histone γ-H2AX antibodies and observed by microscopy (Figure 3). NT cells and cells treated with extract PP-003 did not show histone γ-H2AX staining, whereas all cells treated with CPT displayed pan nuclear and foci staining, consistent with the response to a genotoxic agent (Cahuzac et al. 2010). These data suggest that cell cycle arrest by extract PP-003 is not caused by damaged DNA.

2.5. Extract PP-003 increases cell attachment

During the course of experiments, we observed that cells became more resistant to trypsin release as they were exposed to higher concentrations of extract PP-003. To investigate this, SH-SY5Y cells were either mock treated, treated with increasing concentrations of extract PP-003 or treated with 5 μM CR8 (Figure 4). After 4 h of treatment, a time that was insufficient to cause death, cells were incubated with a trypsin solution to release them from the culture flask. In the absence of extract PP-003 treatment, all cells were released by trypsin, as expected. Strikingly, however, at concentrations of 100 to 1000 μg/mL of extract PP-003, increasing number of cells

![Figure 2](image_url)

Figure 2. Cells treated with extract PP-003 arrest in the G1 phase of the cell cycle. (A) Exponentially growing (NT) HT-29 cells were prepared for analysis by flow cytometry. DNA content was determined by propidium iodide staining. (B) Cells were treated with 200 μg/mL of extract PP-003 and analysed by flow cytometry at 24 h post-treatment. The arrow points to the S-phase region of the DNA profile. (C) The average percentages of HT-29 cells in G1, S or G2/M phases, and sub-G1 DNA values, in either NT or extract PP-003-treated cells are given.
remained attached to the culture flask with significant differences between the highest and lowest amounts tested ($p < 0.005$) (Figure 4(B)). Cell cycle arrest induced by CR8 treatment (Kubara et al. 2012) did not affect cell release by trypsin, suggesting that cell cycle arrest was not a cause of the insensitivity to trypsin release by the PP-003-treated samples. Mock treated cells (i.e. a negative control in which all steps were performed but without trypsin) remained attached to the culture flask, as expected. Furthermore, a trypsin solution that was collected after exposure to extract PP-003-treated cells could still release cells in a subsequent incubation, indicating that the trypsin solution was not inactivated.

**2.6. Plant species from Canadian ecological zones and their anti-cancer properties**

Several plants from the prairie ecological zone have been investigated in cancer cell line-based assays. In those studies, plants were selected by ethnobotanical information from Plains First Nations in the continental USA (Kindscher et al. 1998), or by the similarities of Canadian plant species to those from China with known medicinal properties (Ramirez-Erosa et al. 2007). *Thermopsis* species were not included in either of those studies and the specific cellular or phenotypic responses to those plant extracts were not recorded. Notwithstanding, those studies reinforce the proposal that plant species from the prairie ecological zone might harbour anti-cancer activities.

**2.7. Anti-cancer properties of T. rhombifolia extracts**

In cancer biology, eight biological processes, known as the ‘hallmarks of cancer’, are believed to be used differently by cancer cells than they are by normal cells (Hanahan & Weinberg 2011). Of these eight processes, six can be investigated at the cellular level: cell proliferation, metabolic pathways, cell death pathways, signalling pathways promoting growth, signalling pathways promoting growth arrest and cell attachment. We found that *T. rhombifolia* harbours activities that may affect cell proliferation, cell death and attachment. The chemicals responsible for PP-003 extract cytotoxicity are not known, but *T. rhombifolia* has been previously shown to produce alkaloids from the quinolizidine chemical family. Molecules from this family include anagyrine,
cytisine and derivatives, thermopsine and lupanine (Cho & Martin 1971). These alkaloids have been shown to be toxic to grazing herbivores such as cattle by gavage studies (Keeler & Baker 1990). It is not known whether any of these compounds cause cell cycle arrest, affect cell adhesion or whether they have any anti-cancer properties. Consistent with the properties of anti-cancer cytotoxic compounds, *T. rhombifolia* plant parts have been found to be toxic by accidental ingestion, where dosing was not controlled. In our experiments, extracts were selective to cancer cells in a dose-dependent manner. The application of the assays used in this study will greatly aid biology-guided fractionation to identify the potential anti-cancer molecules from *T. rhombifolia*.

Figure 4. Extract PP-003 increases adhesion of SH-SY5Y cells. (A) Cells were mock treated, or treated with 0, or 30, 100, 300 and 1000 µg/mL of extract PP-003 for 4 h prior to treatment with a trypsin–EDTA solution. After trypsinisation, images of cells were taken that still adhered to the culture flask. In one sample, cells were treated with 5 µM CR8 in place of extract PP-003. For comparison, cells were examined by microscopy prior to any handling (NT). Scale bar = 100 µm. (B) Cells that adhered to the culture flask after treatments described in A were counted. The values given are the mean percentage of cells still present in the flask relative to the number of cells present prior to trypsin treatment. Bars represent standard error and asterisks denote significant differences $p < 0.005$. 
3. Experimental

3.1. Plant collection and extract preparation

*T. rhombifolia* plants were collected at undisturbed sites near Lethbridge, Alberta, Canada, at GPS coordinates 49.40.888/112.51.690 in June 2013. The species identity was confirmed by Professor John Bain, Director of the University of Lethbridge Herbarium, using botanical criteria ([Moss & Packer 1983](#)). A voucher specimen (#672) has been deposited in the University of Lethbridge Herbarium. Leaves and legumes were collected, rinsed, dried at 40°C and stored in paper bags at room temperature until extracted. Approximately 100 g of dried material was ground with a mortar and pestle. One batch of powder was suspended in water, 10 mg/mL, and boiled for 10 min, and another batch was suspended in 75% ethanol, 10 mg/mL, at room temperature. After overnight incubation, the soluble fractions were filtered and dried under reduced pressure. The dried extracted material was weighed, given a code number and stored at room temperature. For testing in biological assays, samples of dried extracts were dissolved in DMSO, 100 mg/mL.

3.2. Cell culture

Cell lines were obtained from the American Type Culture Collection. HT-29 cells were maintained in RPMI 1640 (Gibco, Burlington, ON, Canada) medium supplemented with 10% decomplemented foetal calf serum (Invitrogen, Burlington, ON, Canada), and 1.6 mM Glutamax (Gibco, 61965-026L). The human neuroblastoma cell line SH-SY5Y was maintained in DMEM with 1.6 mM Glutamax supplemented with 10% decomplemented foetal calf serum. The human lung fibroblast cells WI-38 were maintained in DMEM high glucose (Sigma D6546, Oakville, ON, Canada) supplemented with 10% decomplemented foetal calf serum, 1.6 mM Glutamax and 2 mM non-essential amino acids (Gibco 11140050). Cells were grown at 37°C in 5% CO₂ and media were changed every second or third day. CPT (Sigma) and CR8 (S-CR8, Tocris, Canada) were dissolved in DMSO to a concentration of 10 mM and stored at −20°C until use. DMSO was added to a final concentration of 0.1% as a solvent vehicle control in NT cells, when required.

3.3. DNA sequencing of the psbA-trnH intergenic spacer and the ITS region of the nuclear ribosomal cistron (18S-5.8S-26S) – bar codes

DNA from 20 mg of dry tissue was isolated using the Qiagen DNeasy Plant isolation kit. The genomic regions corresponding to the *psbA-trnH* intergenic spacer (chloroplast) and the ITS region of the nuclear ribosomal cistron (18S-5.8S-26S) were amplified by PCR. Control test samples included primers only, template only and *Packera* species template ([Bain & Golden 2000](#)). The sequences are as follows:

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ITS P1-F (5'-3') GGAAGTAAAAGTCGTAACAAGG
ITS P4-R (5'-3') TCCTCCGCTTATTGATATGC
ITS P2-R (5'-3') GCTGCGTTCTTCATCGATGC
ITS P3-F (5'-3') GCATCGATGAAGAACGCAGC
psbA-F (5'-3') GTTATGCACTGAACGTAATGCTC
trnHR-R (5'-3') CGGCATGGTGATGATCCAAATC
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PCR products were analysed by using an Agilent BioAnalyzer 2100 DNA 7500 chip kit. DNA sequence of both strands of the PCR products was acquired by Macrogen USA (Rockville MD, USA). DNA sequences of PCR products were compared with sequences available in the GenBank database using National Center for Biotechnology Information software (BLAST) and the percentage sequence identity computed.
3.4. Cytotoxicity assays

Cytotoxicity was measured by the microculture tetrazolium assay (MTT; (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Perron-Sierra et al. 2012). Cells were cultivated at 4000–10,000 cells per well on a 96-well plate for 48 h prior to treatment. When possible, results were expressed as an IC₅₀ value, which was the extract concentration that reduced by 50% the absorbance at 590 nm, normalised to DMSO-treated cells. CPT was used as a control compound to validate test conditions. All measurements were done in triplicate and the standard error of the means is shown.

3.5. Light microscopy

HT-29 or SH-SY5Y cells were cultivated at 50,000 cells per well on a six-well plate for 48 h prior to treatment. Images were collected at 24 h after plant extract treatment with an Infinity 1.5 camera powered by Infinity Capture (Lumenera Corporation, Ottawa, ON, Canada) software on an Olympus BX41 inverted microscope.

3.6. Flow cytometry

Total cultures were collected by trypsinisation. Cells were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol (−20°C) for at least 24 h. Samples were incubated for 20 min in wash buffer with 0.02 mg/mL propidium iodide (Invitrogen) and analysed by a FACS Canto II flow cytometer (BD Biosciences, Mississauga, ON, Canada) using BD FACSDiva software. Gating was set using control samples of cells either NT or treated with 25 nM CPT (Kubara et al. 2012). Experiments were repeated twice and the average values are provided.

3.7. Cell adhesion assay

SH-SY5Y cells were plated at a density of 25,000 cells per well in a six-well plate and cultivated for 48 h. Fresh pre-warmed medium was added, and cells were either mock treated (processing them through all steps but substituting trypsin or extracts with equivalent volumes of fresh medium), treated by adding 0, 30, 100, 300 and 1000 μg/mL final concentration of extract PP-003 or treated by adding 5 μM CR8 for 4 h, a time by which cytotoxic effects were not yet observed. The medium was removed, cells were washed twice with PBS and incubated with 0.05% trypsin – 0.48 mM EDTA solution (Gibco #25300-54) for 15 min. Cells were then washed twice with pre-warmed PBS, fresh medium was added and images were taken at positions marked on the six wells. The percentage of cells remaining before and after trypsinisation was determined by counting cells on images before and after processing. Images were taken with an Olympus BX41 inverted microscope equipped with a 4 × objective. Cell adhesion experiments were performed three times. Graphical and statistical analyses were performed using Microsoft Excel 2010 software. Average counts were normalised and means with standard errors were calculated. Significance was determined by applying one-tailed t-tests and setting p values to less than or equal to 0.05.

4. Conclusions

We provide the first analysis of an endemic Canadian plant species, *T. rhombifolia*, in a series of cancer cell assays. The ethanolic extract PP-003 was preferentially cytotoxic to cancer cells. It induced cell cycle arrest in the absence of a genotoxic activity, and caused cells to be strongly adherent. These data are consistent with a known toxicity in humans following ingestion of *T. rhombifolia*, and suggest that this plant species might contain compounds with anti-cancer activity.
Supplementary material

Supplementary material relating to this paper is available online, alongside Figures S1–S2.

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