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High throughput screening of natural products for anti-mitotic effects in MDA-MB-231 human breast carcinoma cells

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Abstract

Some of the most effective anti-mitotic microtubule-binding agents, such as paclitaxel (*Taxus brevifolia*) were originally discovered through robust NCI botanical screenings. In this study, a high-through microarray format was utilized to screen 897 aqueous extracts of commonly used natural products (0.00015–0.5 mg/ml) relative to paclitaxel for anti-mitotic effects (independent of toxicity) on proliferation of MDA-MB-231 cells. The data obtained showed that less than 1.34 % tested showed inhibitory growth (IG₅₀) properties <0.0183 mg/ml. The most potent anti-mitotics (independent of toxicity) were Mandrake root (*Podophyllum peltatum*), Truja Twigs (*Thuja occidentalis*), Colorado desert mistletoe (*Phoradendron flavescens*), Tou Gu Cao 透骨草 *Speranskia* Herb (*Speranskia tuberculata*), Bentonite Clay, Bunge Root (*Pulsatilla chinensis*), Brucea Fruit (*Brucea javanica*), Madder Root (*Rubia tinctorum*), Gallnut of Chinese Sumac (*Melaphis chinensis*), Elecampane Root (*Inula Helenium*), Yuan Zhi 遠志 Root (*Polygala tenuifolia*), Pagoda Tree Fruit (*Melia Toosendan*), Stone Root (*Collinsonia Canadensis*) and others such as American Witchhazel, Arjun and Bladderwrack. The strongest tumoricidal herbs identified from amongst the subset evaluated for anti-mitotic properties were wild yam (*Dioscorea villosa*), beth-root (*Trillium Pendulum*) and alkanet-root (*Lithospermum canescens*). Additional data was obtained on a lesser-recognized herb: (*Speranskia tuberculata*) which showed growth inhibition on BT-474 (human ductal breast carcinoma) and Ishikawa (human endometrial adenocarcinoma) cells with ability to block replicative DNA synthesis leading to G2 arrest in MDA-MB-231 cells. In conclusion, these findings present relative potency of natural anti-mitotic resources effective against human breast carcinoma MDA-MB-231 cell division.

Keywords

Plant; Screening; Cancer; High throughput; Herbs

Introduction

Over 50 years ago, the National Cancer Institute set up the Cancer Chemotherapy National Service Center (CCNSC) for large scale *in vitro* screenings of synthetic drugs and plants, to elucidate substances with anticancer properties, one of which was the bark of *Taxus brevifolia* (Leiter et al., 1962, Leiter et al., 1961). By the late 1960s “taxol” was purified

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from the bark (Wani et al., 1971), found to halt proliferation by holding microtubules static, thereby blocking β -tubulin polymerization and dynamic treadmilling (Binder, 2013). By the early 1980s, production of taxol required large quantities of natural bark, necessitating the need and eventual development of synthetic taxol, subsequently made soluble, then commercialized for treatment of malignancies (Yared and Tkaczuk, 2012). Today, paclitaxel, semisynthetic taxane docetaxel and nanoparticle albumin-bound paclitaxel (nab paclitaxel; Abraxane®) are approved drugs for treatment of breast cancer (Urquhart, 2013). The modification of taxane drugs is a continued area of research, with aims to produce molecules of greater efficacy, with reduced side effects (e.g. nausea, pain, alopecia, myalgia, hypersensitivity reactions) (Urquhart, 2013), greater solubility (Yared and Tkaczuk, 2012) and transport capacity (Fitzgerald et al., 2012, Gu et al., 2012) and minimizing progressive drug resistance known to be associated with over-expression of III β -tubulin (English et al., 2013), HER-2 (Murray et al., 2012), ninein-like protein (Zhao et al., 2012). SEPT9 (Chacko et al., 2012) or drug-efflux pumps Abcb1 (Froidevaux-Klipfel et al., 2011).

With one of the major treatments for breast cancer today, having emerged from the efforts of botanical screenings, there is a need to continue high throughput evaluation of plants to identify new microtubule-binding agents (MBA) and anti-mitotic agents, which could possibly augment efficacy or reduce limitations associated with current FDA approved drugs. In this work, we screened 897 commercially sold and utilized natural extracts of aqueous solubility under uniform culture conditions, to elucidate and rank potential anti-proliferative propensity relative to paclitaxel. Moreover, we differentiate true anti-mitotic herbs (which block cell division- independent of toxicity in a similar fashion to paclitaxel) vs. herbs that are toxic and thereby indirectly halt proliferation.

Materials and Methods

Hanks Balanced Salt Solution, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), absolute ethanol, 96 well plates, general reagents and supplies were all purchased from Sigma-Aldrich Co. (St. Louis, MO) and VWR International (Radnor, PA). Imaging probes were supplied by Life Technologies (Grand Island, NY), natural products were provided by Frontier Natural Products Co-op (Norway, IA), Monterey Bay Spice Company (Watsonville, CA), Mountain Rose Herbs (Eugene, OR), Mayway Traditional Chinese Herbs (Oakland, California), Kalyx Natural Marketplace (Camden, NY), Futureceuticals (Momence, IL), organic fruit vegetable markets and Florida Food Products Inc. (Eustis, FL).

Extraction

Natural product extracts were macerated, diced, chopped and powered. In some cases portion of plants, including roots, flowers, leaves, seeds, rinds etc were separated into individual extracts. Each product (250mg) was weighed, placed in 5mls of absolute ethanol and homogenized. Ethanol extracts were placed on a rocker shaker for 24 hours and then stored in airtight containers at -20°C in the dark. Small aliquots were taken from storage extracts and diluted in to sterile HBSS + 5 mM (N-[2-hydroxyethylpiperazine]-N'-[2-ethanesulfonic acid]) (HEPES) adjusted to a pH of 7.4. Sterility was maintained by the

extract ethanol and conducting all dilution processes by use of sterile (autoclaved or UV irradiated) tips, plates, and culture tubes for handling.

High Throughput Design

A rapid method for screening potential anti-proliferative agents was adopted based on the design of PCR microarray gene amplification. Briefly, 96 well plates contained a low cell plating density [4000 cells/ well] to which compounds of equal concentration were added and growth monitored over a 72 hour cell incubation period. Each plate contained 8 untreated controls and a 1st Tier study was established at relatively low concentration starting point (0.1 mg/ml) for all extracts so to screen out weak or non-effective compounds. Any compounds that inhibited cell proliferation below 67% of controls at 0.1 mg/ml, were re-evaluated at dilution ranges comprised of a minimum of six concentrations between 0.00015 and 0.5 mg/ml (0.00015, 0.0015, 0.003, 0.007, 0.0150, 0.027, 0.05, 0.07, 0.1, 0.143, 0.150, 0.5). Extracts were ranked for potency, and IG_{50} s were calculated by regression analysis. This method was rapid, reproducible and validated by a subsequent dose dependent tier evaluation process.

Cell Culture

MDA-MB-231 (ATCC® HTB-26™) human breast cancer cells were obtained from ATCC (Manassas, VA). MDA-MB-231 cells were cultured in ATCC-formulated Leibovitz's L-15 Medium [Catalog No. 30-2008], supplemented with 10% FBS and penicillin/streptomycin (100 U /0.1 mg/ml). After confluence, MDA-MB-231, BT-474 (ATCC® HTB-20™) ductal carcinoma and Ishikawa human endometrial adenocarcinoma (Sigma-Aldrich, St Louis, MO) were sub-cultured and grown in DMEM containing phenol red, 10% FBS, 4 mM L-glutamine, 20μM sodium pyruvate and penicillin/streptomycin (100 U /0.1 mg/ml). Culture conditions were maintained [37°C in 5% CO₂/atmosphere] and every 2–5 days, the medium was replaced and cells sub-cultured. For experiments, plating medium consisted of DMEM, 1% FBS (cell cycle synchronization), 5% (proliferation studies) + penicillin/streptomycin (100 U/0.1 mg/ml), 25 mM glucose, 2 mM sodium pyruvate and 3 mM L-glutamine. A stock solution for each experimental compound was prepared in HBSS + 5 mM (N-[2-hydroxyethylpiperazine]-N'-[2-ethanesulfonic acid]) (HEPES), adjusted to a pH of 7.4.

Cell Viability

Viable cell count was quantified using resazurin (Alamar Blue) indicator dye (Evans et al., 2001). A working solution of resazurin was prepared in sterile PBS - phenol red (0.5 mg/ml) and added (15% v/v) to each sample. Samples were returned to the incubator for 6–8 hr, and reduction of the dye by viable cells (to resorufin, a fluorescent compound) was quantitatively analyzed using a microplate fluorometer, Model 7620, version 5.02 (Cambridge Technologies Inc, Watertown, MA) with settings at [550/580], [excitation/emission].

Thymidine incorporation (TdR)

Replicative DNA synthesis was assessed by radio-labeled incorporation of [³H] thymidine (TdR). Briefly, cells were plated in 24-well plates, containing HBSS (control) or treatment

then supplemented with 1.5 μ Ci/ml TdR. After a 72 hour incubation period, the cells were scraped, centrifuged, pelleted and washed 4x with phosphate-buffered saline (PBS). A final rinse was achieved by cell redistribution in PBS and filtering through a 0.2 μ m microcentrifuge filter, which was then placed in scintillation cocktail and vortexed for 30 seconds. [3 H] Radioactivity was determined using a Beckman LS 6500 liquid scintillation counter.

Cell Cycle Phase Determination

Cells were plated in 24 well plates at 0.2×10^6 cells/ml and cultured in low serum medium for 24 hours to synchronize the cell cycle. After 24 hrs, low serum medium was removed and experimental medium (DMEM + 5% FBS and 25mM glucose) was added prior to treatment with experimental extracts. After 24 hrs, cells were trypsinized, centrifuged and washed 2x with assay buffer (Item No. 10009349, *Cayman Chemical*), re-suspended to a density of 10^6 cells / ml in a cell suspension, fixed and stored at -20°C . After 48 hours, fixative was removed, and the pellet was placed in 0.5 ml of staining solution containing PI (Item No. 10009957) and RNase A (Item No. 10009959). The distribution of DNA in all cell cycle phases was assayed by flow cytometer (BD Biosciences, San Jose, CA) in replicates. The proportion of cells in each stage was performed within 2 hour by using a FACS Calibur flow cytometer. In each sample, a total of 10,000 individual events from the gated subpopulation were analyzed separately. CellQuest software was used for acquisition and analysis of the data, and the percentage of cells in each phase was determined by using ModFit LT 3.3 Software.

Cell Imaging

Phase contrast, Alexa Fluor® 488 phalloidin and propidium iodide (PI) fluorescent dyes were used to corroborate proliferation indirectly by DNA / cytoskeleton F-actin. Briefly, stock solutions for fluorescent dyes were prepared by dissolving 5 mg / 1 ml acetone or ethanol. Stock solutions were diluted in HBSS and added to cells with final dye concentrations at 5 μ g/ml (PI) and 6.6 μ M (Phalloidin) and added to fixed, permeabilized cells. Samples were analyzed photographically using a Nikon TE 900 inverted microscope, Nikon PCM 2000 confocal microscope and data acquisition using C-imaging systems PCI-Simple software (Compix Inc. Cranberry Township, PA, USA).

Data Analysis

Statistical analysis was performed using Graph Pad Prism (version 3.0; Graph Pad Software Inc. San Diego, CA, USA) with significance of difference between the groups assessed using a one-way ANOVA, followed by Tukey post hoc means comparison test, or Student's t test. IG₅₀s were determined by regression analysis using Origin Software (Origin Lab, Northampton, MA).

Results

1. Screening

An initial screening of 897 natural product extracts was conducted to evaluate efficacy of natural products to halt cell division (block proliferation) of MDA-MB-231 cells (over 72

hours) at a final working concentration of 0.1 mg/ml in (Figure 1). Herbs demonstrating a growth inhibitory $IG_{67} < 0.1$ mg/ml, were re-screened through subsequent tier evaluations. The initial screening eliminated 87.29% as being weak or not effective, where remaining compounds were re-evaluated at dilution ranges comprised of a minimum of six concentrations between 0.00015 – 0.5 mg/ml (0.00015, 0.0015, 0.003, 0.007, 0.0150, 0.027, 0.05, 0.07, 0.1, 0.143, 0.150, 0.5) (Figure 2 / Table 1). Extracts were ranked for potency, and IG_{50} s were calculated by regression analysis. The data showed that of 897 evaluated, less than 1.34% had a potency level below 0.0183 mg/ml. Of these, only 6 natural products had an IG_{50} less than 0.0099 mg/ml. Products were classified as Level 1 - $IG_{50} < 0.0183$ mg/ml (Potent), Level 2 - IG_{50} (0.02–0.0499 mg/ml) (Strong), Level 3 - IG_{50} (0.0509–0.0801 mg/ml) (Moderate) and Level 4 - IG_{50} (0.0811–0.1) (Relative Mild). 27 compounds were classified as Level 1–2 demonstrating strong to potent inhibitory effects. Several of the most potent anti-proliferative products were tested in estrogen receptor positive cell lines using the same 72 hr growth format, including the human endometrial carcinoma cell line (Ishikawa) and human breast carcinoma cell line BT-474 (Figure 3A–D).

2. Toxicity vs. Anti-proliferative Properties

Determination of toxic vs. anti-mitototic properties of extracts were established by 1) identifying compounds with inherent dose response anti-proliferation viability dropping below the negative paclitaxel (5 μ M) inhibitor control; 2) a second experiment was conducted to assess cell viability at 24 hours with MDA-MB-231 cells at a higher plating density (50,000 cells/well) at concentrations of 0.238, 0.119, 0.060, 0.030 mg/ml where herbs demonstrating a toxicity below 0.238 mg/ml are denoted with Φ = toxic symbol; and 3) photographic examination of all anti-proliferative compounds in Level 1–2 groups were acquired, some displayed in Figure 4. Figure 4 shows morphological changes associated with viable but static cells with use of various anti-mitotics vs. cell death evident for toxic herbs such as wild yam (*Dioscorea villosa*) and beth root (*Trillium Pendulum*). These findings show that in some cases, the toxicity of the herb could appear as halt of cell proliferation (Φ - anti-proliferative) but this is an indirect result of cell death, not anti-mitotic processes. In contrast, true anti-mitotics including paclitaxel are not cytotoxic, but rather halt proliferation of rapidly dividing cells thereby creating a cytostatic cell count vs., cell growth which can be readily quantified over a 72 hour period.

3. Anti-mitotic effects of *Speranskia Tuberculata*

Due to limited existing research on *Speranskia tuberculata*, this extract was chosen for further evaluation relative to paclitaxel (Figure 5) for effects on replicative DNA synthesis using radio-labeled incorporation of [3 H] thymidine (TdR) (Figure 6) and growth inhibitory properties confirmed by distribution of DNA in cell cycle phases by flow cytometry confirming mitotic G2 arrest corresponding with loss of proliferation (Figure 7).

Discussion

Natural products have been used for centuries as remedies for human diseases including uncontrolled tumor growths. Medicinal uses for plant products today are classified as complementary and alternative medicines and rarely constitute the primary means of

pharmaceutical treatment but rather used adjunct to prescription medicines (Mantle et al., 2000). On the other hand, through out history, natural products have formed the basis of discovery and rationale for synthetic drugs and corresponding analogues with improved efficacy and specificity for biological targets (Ram and Kumari, 2001). Some of the most widely effective chemotherapy agents today, originated from plants including: *Catharanthus roseus* - Madagascar periwinkle [vinblastine and vincristine] (Noble, 1990), *Podophyllum peltatum* [etoposide] (Sinkule, 1984) also shown in the current study, *Taxus brevifolia* [taxol] (Foa et al., 1994), *Camptothecin acuminata* [Camptothecin] (Ardizzoni, 1995), and *Viscum album* [Isorel] (Jurin et al., 1997).

Data presented in this study provide information on relative potency rank amongst 897 commonly used natural products used around the world of diverse ethnic practices, to halt proliferation of human breast cancer cells. There is a distinction between compounds that are toxic to malignant tumor cells and those that block mitosis of rapidly dividing tumor cells independent of toxicity. In this study, we report that the most potent anti-proliferative aqueous extracts, independent of toxicity, were that of Mandrake root / Mayapple (*Podophyllum peltatum*) followed by others such as Truja Twigs (*Thuja occidentalis*), Mistle Toe (*Phoradendron flavescens*), Tou Gu Cao 透骨草 *Speranskia* Herb (*Speranskia tuberculata*), Bentonite Clay, Bunge Root (*Pulsatilla chinensis*), Brucea Fruit (*Brucea javanica*), Gallnut of Chinese Sumac (*Melaphis chinensis*), Elecampane Root (*Inula Helenium*), Yuan Zhi 远志 Root (*Polygala tenuifolia*), Persian Silk Tree Flower (*Albizia julibrissin*) and Pagoda Tree Fruit (*Melia Toosendan*). Many of the plant extracts such as wild yam (*Dioscorea villosa*), beth-root (*Trillium Pendulum*) and alkanet-root (*Lithospermum canescens*) were extremely toxic to tumor cells, and thereby are likely to halt cell division due to toxicity alone. In other words, dead cells do not divide. Some of anti proliferative natural products listed in this report, are newly presented, and some of these have been previously reported in the literature.

The most potent extract was: **Mayapple (*Podophyllum peltatum*)**: also historically used by American Indians as an anti-parasitic or for the treatment of skin growths and warts. American Settlers started using parts of this plant to treat other illnesses such as typhoid, cholera, and dysentery. Today, *Podophyllum peltatum* is known to contain podophyllotoxin, which is a precursor for synthesis of antiproliferative chemotherapy drugs such as etoposide, teniposide and etopophos (Graeowska and Ross, 1976, Lata et al., 2009) used for small cell lung carcinoma, testicular cancer and lymphoma (Botta et al., 2001). Etoposide is well studied, and believed to block cell division at G2 phase of the cell cycle (Vogelzang et al., 1982) primarily by inhibiting DNA topo-isomerase II and inducing double strand breaks in DNA. (Damayanthi and Lown, 1998, Fujikawa-Yamamoto et al., 2012). Additional anti-tumor properties include ability to induce apoptosis, reduce vascular endothelial growth factor /vascular permeability, increase expression of endogenous angiogenesis inhibitors such as endostatin or attenuate tumor growth in hormone propelled tumor models (Panigrahy et al., 2010) (Liu and Yamauchi, 2010) (Day et al., 2009). Limitations for the use of etoposide were known to involve the lack of solubility, necessitating the development of compounds such as etoposide phosphate which is a water-soluble prodrug of etoposide (Schacter, 1996). Etoposide's dose-limiting toxicity involves several side effects often

including leukopenia, alopecia, nausea and vomiting (Sinkule, 1984). The results in this paper, corroborate this herb to be amongst the most potent of anti-proliferative extracts evaluated.

Truja Twigs (*Thuja occidentalis*): extracts were also used by native Indians as a remedy for treating moles and tumors, urinary infections and rheumatic disorders (Naser et al., 2005). There are few scientific reports about use of Truja twigs and cancer, however of those that do exist, the evidence suggests a thujone-rich fraction is responsible for anti-proliferative effects in malignant cell lines such as melanoma A375 (Biswas et al., 2011) also effective in attenuating the growth of cancer cells in diverse models including DMBA-induced mammary carcinogenesis (Ojeswi et al., 2010). The findings presented in this work, corroborate Truja twigs extract to serve as a potent anti-proliferative herb, warranting further investigation as to isolated components of the aqueous extract responsible for blocking cell division.

Although, there are few to no studies on **Mistle Toe (*Phoradendron flavescens*):** extensive studies have been conducted worldwide on a related herb called L white-berry mistletoe (*Viscum album* L.) from the same Class [Magnoliopsida – Dicotyledons], Subclass [Rosidae], Order [Santalales], Family [Viscaceae – Christmas Mistletoe family] with variation in genus and species. While some of these reportedly include *Phoradendron macrophyllum* and *P. serotinum*, one of the most studied is *Viscum album* L. also referred to as European mistletoe or Iscador. Chemical extracts or a lectin rich Iscador extract can inhibit growth of tumor types such as TC-1 tumors (Alonso-Castro et al., 2012), glioblastoma (Podlech et al., 2012) and HepG2 cells *in vitro* (Choi et al., 2012). In human clinical trials, adjuvant use of Iscador with traditional chemotherapy such as gemcitabine provides greater overall survival rates in patients with pancreatic carcinoma (Matthes et al., 2010) or lung cancer (Salzer and Havelec, 1978). Similarly, adjunct Iscador with carboplatin for advanced non-small-cell lung cancer can allow lower effective doses of otherwise toxic chemotherapy agents, and reduce deleterious side-effects (Bar-Sela et al., 2013). Similar findings are reported around the world, where standardized mistletoe extract (abnoba VISCUM®) Quercus, aVQ) in patients with gastric cancer used with 5-FU prodrug doxifluridine can with improved quality of life, (Brandenberger et al., 2012, Kim et al., 2012) and reduce chemotherapy side effects (Eisenbraun et al., 2011). Summative analysis of clinical oncology trials using mistletoe therapy consistently report adjunct use as safe with ability to improve quality of life (Melzer et al., 2009) and overall survival (Ostermann et al., 2009).

Speranskia Herb (*Speranskia tuberculata*): is a traditional Chinese medicine (TCM) commonly used for “expelling the wind”, enhancing blood circulation and treatment of the joint pain. There is little to no research on this herb in regard to human disease or potential therapeutic applications, where only two research articles have investigated chemical isolates describing inherent chemical components such as polyoxygenated alkaloids including speranculatines, speranskilatine and speranberculatine A (Shi et al., 2000) 18-hydroxy(-)-manool and beta-sitosterol (Li et al., 2000). There is some suggestion indicating that the speranskia herb contains anti-cancer constituents that themselves display a number of properties against cancer progression such as amentoflavone and beta-sitosterol. Both of these have been reported to induce mitotic arrest (Wang et al., 2006) and apoptosis in

diverse cancer cell lines such as human breast (Pei et al., 2012, Tasyriq et al., 2012), cervical cancer and B16F-10 melanoma cells, correlating to upregulation of Bax and caspase-9, reduction in Bcl-2 or reduction in cyclin and hyperphosphorylated retinoblastoma (Lee et al., 2011). In vivo, similar results were observed on B16F-10 melanoma lung nodules in association with a reduction of MMPs and hypoxia/inflammatory related proteins such as prolyl hydroxylase, VEGF, TNF-alpha, IL-1beta, IL-6, GM-CSF (Guruvayoorappan and Kuttan, 2008a) and angiogenesis (Guruvayoorappan and Kuttan, 2008b). What makes this herb interesting is that it is typically used at a daily dose of 15 to 30 grams, often as tea form, suggesting that it is well tolerated in humans. The potency level of this herb to block proliferation of human breast tumor cells is very strong, as show in the current work and therefore this herb should be evaluated further for its chemotherapeutic properties.

Bunge Root (*Pulsatilla chinensis*): The *Pulsatilla* genus has been historically used in both Korean and Chinese medicinal applications in the treatment of dysentery, malaria, and bacterial infections. A number of studies are now showing that the extracts of *Pusatilla* contain saponins isolated from the root, in part capable of suppressing growth of diverse tumor cells including anaplastic thyroid cancer cells (Park et al., 2012) colon cancer and hepatocellular carcinoma in part due to inducing apoptosis, initiating reduction of VEGF (Park, Jung, 2012) and suppression of the AKT/mTOR pathway (Son et al., 2013) (Hong et al., 2012a). Several saponins isolated from *Pusatilla Koreana* root derived compounds are toxic to diverse cancer cell lines, such as A-549, SK-OV-3, SK-MEL-2, HCT15, human liver 7402 cells (Xu et al., 2012), effective against BDF1 mice bearing Lewis lung carcinoma (Bang et al., 2005, Kim et al., 2004) and tumor growth in xenograft models (Hong et al., 2012b). In animals, *Pulsitilla chinensis* derived saponins are well tolerated in animals with capacity to attenuate growth of human liver tumors in nude mice (Xu, Shu, 2012). Other compounds such as the pentacyclic triterpenes from *Pulsatilla chinensis* (Bunge) are synergistic when combined with doxorubicin preventing chemo-resistance associated with the up-regulation of P-glycoproteins (Zheng et al., 2010).

Brucea Fruit (*Brucea javanica*): has been subject to extensive research proving efficacy in treatment of both tumors and malaria dating back to the 1950s. Today, reports suggest efficacy of *Brucea javanica* oil microemulsion on the attenuation of growth in Hela cells (Yang et al., 2013b) or adjunct to chemotherapy for platinum based drugs resulting in greater efficacy, reduction of side effects and improved quality of life (Wang et al., 2012). Constituents within this herb are well documented and include bruceine D effective on pancreatic cancer cell lines (Panc-1, SW1990, Capan-1/2 cells) some reporting comparable efficacy to that of camptothecin and gemcitabine (Liu et al., 2012). The alcohol extract of the fruit contains diverse quassinoids including brusatol, bruceantin and bruceine A which are itself effective against growth and survival of pancreatic cancers, (Zhao et al., 2011) induce apoptosis associated with elevation of tumor suppressor proteins (Gao et al., 2011) and are believed to promising against pancreatic cancer (Pan et al., 2009) (Lau et al., 2009).

Traditional Chinese Medicine - In this work, we focus on one isolated therapeutic target, without bias as to plant origin, ethnical botanical history or traditional cultural practice, in order to elucidate potential new plants with selective therapeutic value. Many of the herbs, as in the case of *Speranksia* are TCMs, and while here we show potential efficacy against

tumor cell proliferation, its use in TCM is described as having purpose to expel pathogenic wind, relax muscles, dissipate blood stasis for rheumatoid arthritis, spastic muscles, scabies and tinea. What is very interesting is that the logic pattern of TCM is based on historical abstract wisdom, deeply rooted in philosophy with history on effective remedies for human health, not necessarily based on quantitative measure but rather historical applications. (Zhou et al., 2013) However, as world communication condenses and biotechnology advances, there are major strides in bridging this gap between TCM and modern western medicines by evaluating structure-activity relationship, bioinformatics and chemical analysis to establish pharmacological basis for TCMs (Zhang et al., 2013) (Yang et al., 2013a). In contrast, there is a need for modern science to investigate historical and ancient practices using current scientific methodologies (Xu et al., 2013). Perhaps some of the greatest significant wisdom on health and well-being comes from our ancestors, which can now be tested experimentally in a rapidly advancing technological climate.

While discussion of all extracts in this study are outside the scope and breadth limitations for review, the main point of this work was to outline, provide relative potency rank and elucidate potential new natural sources of anti-mitotic compounds. In conclusion, the findings from this research suggest a number of novel natural resources with anti-proliferative capacity, where further investigations are warranted such as bentonite clay and *Speranskia*. Moreover, these findings also corroborate existing work on natural products, pointing to relative rank of potency of aqueous extracts. Future research will be required to evaluate mechanism of action on cell cycle progression and cytoskeletal dynamics involved with mitosis for natural products with anti-proliferative capacity.

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High Throughput Screening of Commonly Sold Natural Product Extracts against Proliferation of human breast cancer cells (MDA-MB-231) at .1 mg/ml

Figure 1.

Tier 1 Level Screen. Inhibitory growth properties of 897 extracts on MDA-MB-231 human breast carcinoma cells (0.1mg/ml) under uniform culture conditions. The data represent 72 hour cell proliferation (% controls) from an initial plating density of (4,000 cells/well) in 96 well plates established with resazurin (Alamar Blue) fluorometric analysis.

High Throughput Screening of Natural Product Extracts against Proliferation of Human Breast Cancer Cells (MDA-MB-231) Classification by Potency Level

Figure 2.

Compartmentalization of efficacy by potency category and rank. Tier 1 level screen eliminated 87.29% of natural products tested as ineffective/weak against proliferation of MDA-MB-231 human breast carcinoma cells. The remaining extracts were re-evaluated at a minimum of six concentrations between 0.00015 and 0.5 mg/ml, n=4. Subsequent IG₅₀s were calculated by regression analysis and products classified as Level 1–4 potency rank based on strength. Products were classified as Level 1 -IG₅₀ < 0.0183 mg/ml (Potent), Level 2 - IG₅₀ (0.02–0.0499 mg/ml) (Strong), Level 3 - IG₅₀ (0.0509–0.0801 mg/ml) (Moderate) and Level 4 - IG₅₀ (0.0811–0.1) (Relative Mild).

Effective Anti-Proliferative Extracts - 72 hour growth inhibition of human breast cancer cells (MDA-MB-231), ER+ Ishikawa endometrial adenocarcinoma ER+ BT-474 Human Breast carcinoma cell line

Figure 3.

A–D Growth inhibitory profile for Tou Gu Cao 透骨草 *Speranskia tuberculata*, Colorado desert Mistletoe (*Phoradendron flavescens*), Bentonite Clay and Truja Twigs (*Thuja occidentalis*) on MDA-MB-231 cells. Additional IG₅₀s are also presented for efficacy on blocking cell division in Estrogen Receptor Positive Cell lines (Ishikawa cells and BT-474 cells). The data represents cell proliferation in MDA-MB-231 Cells (% control) and is presented as the Mean ± S.E.M., n=4. Significance of difference from controls was determined with a one-way ANOVA followed by a Tukey post host test *p<0.05.

Figure 4. Example morphological changes in MBA-MD-231 cells showing anti-proliferative vs. cytotoxic effects at 0.05 mg/ml @ 72 hours of incubation @ 37°C – 5% CO₂ / Atm. Toxicity was also substantiated by a loss of cell viability below paclitaxel growth inhibition controls.

Figure 5.

Anti-proliferative effects of *Speranskia tuberculata* on growth of MDA-MB-231 cells human breast cancer cells as shown by actin cytoskeletal stain with Alexa Fluor® 488 Phalloidin, nuclear staining with propidium iodide and phase contrast imaging (morphology).

Effect of Tou Gu Cao 透骨草 (*Speranskia* Herb) on H3 Thymidine [TdR] Incorporation into DNA in human breast cancer cells (MDA-MB-231)

Figure 6.

Effect of *Speranskia tuberculata* on replicative DNA synthesis through measuring radio-labeled incorporation of [^3H] thymidine (TdR) after 72 hours. The data represents ^3H Isotope (DPM) as % control and is presented as the Mean $\pm$ S.E.M., $n=4$. Significance of difference from controls was determined with a one-way ANOVA followed by a Tukey post host test * $p<0.05$.

Effect of Tou Gu Cao 透骨草 (*Speranskia* Herb) (0.05 mg/ml) on G1, S and G2M Phase in human breast cancer cells (MDA-MB-231)

Figure 7.

The effect of *Speranskia tuberculata* on gated population and cell cycle of MDA-MB 231 cells after synchronization and 24 hour exposure. The data are expressed as % phase count Mean ± S.E.M., from experimental replicates.

Table 1

72 Hr growth inhibitory potency rank by IG₅₀ on MDA-MB-231 human breast carcinoma cells. Extracts were re-evaluated at a minimum of six concentrations between 0.00015 and 0.5 mg/ml, n=4 and IG₅₀ s were calculated by regression analysis with products classified by Level 1–4 potency rank. Level 1–2 compounds were re-evaluated for 24 hour cytotoxicity on MDA-MB-231 cells using (50,000 cells/well) in 96 well plates, so to differentiate (¥ - anti-proliferative) vs. (φ - toxic) properties of the extract.

High Through Put Screening of Natural Products against Proliferation of human breast cancer cells (MDA-MB-231)				
Rank Potency Class	Common Name	Botanical Name	Concentration IG ₅₀	
Level 1 - Potent	¥ Pacitaxel		<0.00014	mg/ml
	¥ Mandrake root	<i>Podophyllum peltatum</i>	<0.001	mg/ml
	φ Wild Yam Root	<i>Dioscorea villosa</i>	0.0001	mg/ml
	φ Beth Root	<i>Trillium Pendulum</i>	0.0011	mg/ml
	¥ Truja Twigs (Arborvitae)	<i>Thuja occidentalis</i>	0.0044	mg/ml
	φ Alkanet Root	<i>Lithospermum canescens</i>	0.0046	mg/ml
	¥ Mistle Toe	<i>Phoradendron flavescens</i>	0.0096	mg/ml
	φ Dragons Blood	<i>Dracaena draco</i>	0.0120	mg/ml
	¥ Speranskia Herb	<i>Speranskia tuberculata</i>	0.0140	mg/ml
	φ Bloodroot	<i>Sanguinaria canadensis</i>	0.0142	mg/ml
	¥ Bentonite Clay	<i>Bentonite Clay</i>	0.0147	mg/ml
	¥ Bunge Root	<i>Pulsatilla chinensis</i>	0.0156	mg/ml
	¥ Brucea Fruit	<i>Brucea javanica</i>	0.0183	mg/ml
Level 2 - Strong	¥ Dyer's Madder Root	<i>Rubia tinctorum</i>	0.0205	mg/ml
	¥ Gallnut of Chinese sumac	<i>Melaphis chinensis</i>	0.0208	mg/ml
	¥ Elecampane Root	<i>Inula Helenium</i>	0.0288	mg/ml
	¥ Yuan Zhi 遠志 Root	<i>Polygona tenuifolia</i>	0.0298	mg/ml
	¥ Pagoda Tree Fruit	<i>Melia Toosendan</i>	0.0314	mg/ml
	¥ Stone Root/Heal-All	<i>Collinsonia canadensis</i>	0.0320	mg/ml
	¥ American witchhazel Root	<i>Hamamelis virginiana</i>	0.0362	mg/ml
	φ Whiteedge morning-glory Seed	<i>Pharbitis nil</i>	0.0374	mg/ml
	¥ Bladderwrack (Seaweed)	<i>Fucus vesiculosus</i>	0.0400	mg/ml

High Through Put Screening of Natural Products against Proliferation of human breast cancer cells (MDA-MB-231)				
Rank Potency Class	Common Name	Botanical Name	Concentration IG ₅₀	
Level 2 (IG50 0.02–0.0499 mg/ml)	¥ Arjun	<i>Terminalia arjuna</i>	0.0408	mg/ml
Level 2 (IG50 0.02–0.0499 mg/ml)	¥ Pomegranate (Granati Peel)	<i>Punica granatum</i>	0.0410	mg/ml
Level 2 (IG50 0.02–0.0499 mg/ml)	φ Gromwell Root	<i>Lithospermum erythrorhizon</i>	0.0414	mg/ml
Level 2 (IG50 0.02–0.0499 mg/ml)	¥ Juniper Berry	<i>Juniper Communus</i>	0.0420	mg/ml
Level 2 (IG50 0.02–0.0499 mg/ml)	¥ Persian Silk Tree Flower	<i>Albizia julibrissin</i>	0.0429	mg/ml
Level 2 (IG50 0.02–0.0499 mg/ml)	φ Galbanum	<i>Ferula Galbaniflua</i>	0.0479	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Black Walnut Hull	<i>Juglans nigra</i>	0.0509	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Cow Soapwort Seed	<i>Vaccaria hispanica</i>	0.0512	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Balm of Gilead	<i>Populus balsamifera</i>	0.0525	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Bearberry	<i>Arctostaphylos uva-ursi</i>	0.0543	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Dan Shen 丹参 Root	<i>Salvia miltiorrhiza</i>	0.0553	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Chang Shan 常山 Root	<i>Dichroa febrifuga</i>	0.0575	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Frankincense	<i>Boswellia carterii</i>	0.0588	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Tasmanian bluegum Leaf	<i>Eucalyptus globulus</i>	0.0590	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Bhumy Amalaki	<i>Phyllanthus niruri</i>	0.0596	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Cranebill Root	<i>Geranium maculatum</i>	0.0602	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Rhodiola Root	<i>Rhodiola kirilowii</i>	0.0647	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Bayberry Root bark	<i>Morella cerifera</i>	0.0654	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	American Wintergreen	<i>Gaultheria procumbens</i>	0.0659	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Pipsissewa	<i>Chimaphila umbellata</i>	0.0662	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Clove Bud	<i>Syzygium aromaticum</i>	0.0683	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Yúx ng cáo 魚腥草	<i>Houttuynia cordata</i>	0.0721	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Japanese Knotweed Root	<i>Polygonum cuspidatum</i>	0.0734	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Blackberry Root	<i>Rubus fruticosus or Rubus villosus</i>	0.0743	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Indian Madder Root	<i>Rubia cordifolia</i>	0.0766	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Copal Resin	<i>Bursera</i>	0.0775	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Pleurisy / Butterfly Milkweed Root	<i>Asclepias tuberosa</i>	0.0784	mg/ml
Level 3 (IG50 0.0509–0.0801mg/ml)	Neem Leaf	<i>Azadirachta indica</i>	0.0794	mg/ml

Level 3 - Moderate

High Through Put Screening of Natural Products against Proliferation of human breast cancer cells (MDA-MB-231)				
Rank Potency Class	Common Name	Botanical Name	Concentration IG ₅₀	
Level 3 (IG50 0.0509–0.0801mg/ml)	Feverfew	<i>Tanacetum parthenium</i>	0.0801	mg/ml
Rank Potency Class	Common Name	Botanical Name	Concentration IG ₅₀	
Level 4 (IG50 0.0721–0.0801mg/ml)	New Jersey tea	<i>Ceanothus americanus</i>	0.0811	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Puff-Ball / Lasiophaera	<i>Lasiophaera fensholtii</i>	0.0819	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Green Clay, French	<i>Green Clay, French</i>	0.0843	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	White Sage	<i>Salvia apiana</i>	0.0864	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	English walnut Seed	<i>Juglans regia</i>	0.0870	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Common myrrh	<i>Commiphora myrrha resin</i>	0.0881	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Eastern Purple Coneflower	<i>Echinacea Purpurea</i>	0.0888	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Great Bumet Root	<i>Sanguisorba officinalis</i>	0.0889	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Butternut Bark	<i>Juglans cinerea</i>	0.0899	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Couch Grass	<i>Elymus repens</i>	0.0935	mg/ml
Level 4 (IG50 0.0721–0.0801mg/ml)	Babul Chiall Bark	<i>Acacia arabica</i>	0.0966	mg/ml

Level 4 - Relative Mild

‡ Antiproliferative

φ_TToxic