

**A NOVEL ANTI-MITOTIC ACTIVITY ON HUMAN CELLS BY  
PULCHELLOID A, A COMPOUND ISOLATED FROM THE PRAIRIE PLANT  
*GAILLARDIA ARISTATA***

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## ABSTRACT

In this thesis we investigate the prairie plant species *Gaillardia aristata* for compounds of biological interest. Through the use of phenotypic screens based upon the hallmarks of cancer, we have characterized the effects of *G. aristata* extracts upon human cell lines, and found that extracts induced mitotic arrest. This arrest was characterized by low levels of pTyr15-Cdk1, and high levels of cyclin B, securin, and pThr320-PP1 $\alpha$ , indicating that cells arrested at metaphase-anaphase transition. Arrested cells exhibited distorted mitotic spindles and damaged DNA. By biology-guided fractionation, we isolated a compound from *G. aristata* plant extracts that inhibits mitosis in human cells. The compound was the sesquiterpene lactone Pulchelloid A, and we are the first to identify its activities upon human cells. Pulchelloid A affects mitosis through a mechanism that could involve the inhibition of the ubiquitin-proteasome pathway. Insight into this mitotic process might be beneficial to future studies in cancer therapies.

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## LIST OF ABBREVIATIONS

3-D	Three-dimensional
6-OAP	6-O-Angeloylplenolin
8 $\beta$ -SC	8 $\beta$ -sarracinoyloxycumambranolide
$\beta$ -TrCP	Beta-transducin repeat containing protein
A431	Human epidermoid carcinoma cell line
A549	Human lung adenocarcinoma cell line
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP	Alkaline phosphatase
APC/C	Anaphase promoting complex/cyclosome
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ATR	ATM and Rad3-related
BC	Before Christ
BSA	Bovine serum albumin
BT-549	Human breast ductal carcinoma cell line
CAK	Cyclin-dependent kinase activating kinase
CAL-27	Human tongue squamous cell carcinoma cell line
Caov-3	Human ovarian adenocarcinoma cell line
CCS	Canadian Cancer Society
Cdc	Cell-division cycle protein
Cdh	Cadherin 1
Cdk	Cyclin-dependent kinase
Chk	Checkpoint kinase
CKI	Cyclin-dependent kinase inhibitor
CO <sub>2</sub>	Carbon dioxide
CPT	Camptothecin
Cul1	Cullin 1
CYP3A	Cytochrome P450, family 3, subfamily A
DAPI	4', 6-diamidino-2-phenylindole
DCM	Dichloromethane
DDR	DNA damage response
DLBCL	Human diffuse large B-cell lymphoma cell line
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DU-145	Human prostate carcinoma cell line
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Emi1	Early mitotic inhibitor 1
ER	Endoplasmic reticulum

EtOH	Ethanol
FaDu	Human squamous cell carcinoma cell line
FBS	Fetal bovine serum
FBXO11	F-box protein 11
FDA	Food and Drug Administration
FE	Flower extract
FR	Fraction
GST	Glutathione S-transferase
G <sub>0</sub>	Resting phase
G <sub>1</sub> phase	Gap 1 phase
G <sub>2</sub> phase	Gap 2 phase
GPS	Global positioning system
H1299	Human lung carcinoma cell line
H <sub>2</sub> O	Water
H460	Human lung carcinoma cell line
H69	Human small cell lung carcinoma cell line
HA22T/VGH	Human hepatocellular carcinoma cell line
HCT116	Human colorectal carcinoma cell line
HECT	Homologous to E6-AP carboxyl terminus
HEK293	Human embryonic kidney cell line
HeLa	Human cervical adenocarcinoma cell line
HEp2	Human larynx epidermoid carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HL60	Human acute promyelocytic leukemia cell line
HN5	Human tongue squamous cell carcinoma cell line
HNSCC	Human head and neck squamous cell carcinoma cell line
HPLC	High performance liquid chromatography
HPV	Human Papilloma virus
HSC-2	Human oral cavity squamous cell carcinoma
HSC-3	Human tongue squamous cell carcinoma cell line
HT-29	Human colorectal adenocarcinoma cell line
IKK	I- $\kappa$ B kinase
I- $\kappa$ B	Inhibitor of NF- $\kappa$ B
IPEGAL	Octylphenoxy polyethoxyethanol
J-Jhan	Human Jurkat-derivative leukemia cell line
Jurkat	Human T-cell leukemia cell line
KBM-5	Human chronic myelogenous leukemia cell line
L1210	Murine lymphocytic leukaemia cell line
L12ADT	8- <i>O</i> -(12-{L-leucinoylamino}dodecanoyl)-8- <i>O</i> -debutanoyl-thapsigargin
LC-1	Dimethylamino-parthenolide
LC-3	Light chain 3
LE	Leaves extract
M phase	Mitotic phase
M059K	Human glioblastoma cell line
MCF-7	Human metastatic breast cancer cell line
MDA-MB-157	Human breast medullary carcinoma cell line

MDA-MB-231	Human breast carcinoma cell line
MDA-MB-436	Human breast adenocarcinoma cell line
ME	Mercaptoethanol
MG132	Benzyl N-[(2S)-4-methyl-1-[[[(2S)-4-methyl-1-[[[(2S)-4-methyl-1-oxopentan-2-yl]amino]-1-oxopentan-2-yl]amino]-1-oxopentan-2-yl]carbamate
MM.1R	Human multiple myeloma cell line
MM.1S	Human immunoglobulin A lambda myeloma cell line
MTOC	Microtubule organizing centers
MTT	3-((4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NB4	Human acute promyelocytic leukemia cell line
NCI	National Cancer Institute
NCI-H1975	Human non-small cell lung carcinoma cell line
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health
NIPA	Nuclear-interacting partner of ALK
NSCLC	Human non-small cell lung cancer cell line
OZ	13-hydroxy-15-oxozaapatlin
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC-3	Human prostate carcinoma cell line
PH3	Phospho-histone H3
PI	Propidium iodide
Plk	Polo-like kinase
PP1	Protein phosphatase 1
PP1 $\alpha$	Protein phosphatase PP1 alpha catalytic subunit
PP2	Protein phosphatase 2
RING	Really interesting new gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI 8226	Human plasmacytoma cell line
S phase	DNA synthesis phase
SAC	Spindle assembly checkpoint
SCC-25	Human tongue squamous cell carcinoma cell line
SCC-4	Human tongue squamous cell carcinoma cell line
SCC-9	Human tongue squamous cell carcinoma cell line
SCF	Skp1/Cul1-F-box complex
SDS	Sodium dodecyl sulphate
SE	Stems extract
SERCA	Sarco/endoplasmic reticulum calcium ATPase pump
Skp1	S-Phase Kinase-Associated Protein 1
Skp2	S-Phase Kinase-Associated Protein 2
SSB	Single-strand break
ssDNA	Single stranded DNA
T24	Human urinary bladder transitional carcinoma cell line
TBS	Tris-buffered saline
TCP	Tubulin carboxypeptidase enzyme

TNF	Tumour necrosis factor
Top1	Topoisomerase I
TTL	Tubulin tyrosine ligase enzyme
U251	Human glioblastoma astrocytoma cell line
U266	Human myeloma cell line
U2OS	Human osteosarcoma cell line
U373	Human glioblastoma astrocytoma cell line
U937	Human histiocytic lymphoma cell line
UACC-62	Human melanoma cell line
Ub	Ubiquitin
UPP	Ubiquitin-proteasome pathway
WHO	World health organization
WI-38	Human non-cancerous lung fibroblastic cell line
W18-Va2	Human SimianVirus 40-transformed buccal mucosa cell line

## CHAPTER 1

### **General introduction**

This thesis is about a compound that inhibits mitosis, which we isolated from the plant, *Gaillardia aristata*. I will describe how we investigated the properties of extracts prepared from *G. aristata* and found that they can inhibit mitosis when applied to human cells. We then used biology-guided fractionation to isolate the compound from the plant extract. The compound is a member of the sesquiterpene lactone family and we are the first to identify several new activities of this compound upon human cells. A better understanding of how human cells undergo mitosis is now possible and may be beneficial to future studies in cancer therapies.

#### *1.1 Cancer*

Over 200 diseases that share similar biological features can be grouped under the name of cancer. Cancer cells arise from a sequential accumulation of mutations, which lead to abnormal proliferation, loss of responsiveness to regulatory signals, and altered metabolism (Seyfried et al., 2010; Hanahan et al., 2011).

Cancer is among the leading cause of morbidity and mortality worldwide with an estimated 70% increase in number of new cases over the next two decades (Stewart et al., 2014; W.H.O., 2015). Current treatment strategies include surgery, radiation therapy and chemotherapy; however, these are often not sufficient to cure cancer (Urruticoechea et al., 2010; Stewart et al., 2014; W.H.O., 2015). One approach to improve patient outcomes is to develop new chemotherapeutic agents. It is postulated that new cancer therapies require a large repertoire of drugs with different mechanisms of action to achieve cytotoxicity and avoid resistance. Currently, most therapeutic

approaches focus on genotoxicity, or a limited number of protein targets. Therefore, cancer represents an unmet medical need and it requires the development of new effective drugs.

### *1.2 The hallmarks of cancer*

Cancer is comprised of a number of molecularly distinct diseases that can occur in the same patient and even in the same tumour (Moffat et al., 2014). These diseases share similar biological properties, known as the “hallmarks of cancer”, which discriminate cancerous cells from normal cells. The hallmarks of cancer were first described by Hanahan and Weinberg in 2000, in which they listed the six biological hallmarks that allow cancer cells to survive, proliferate, and disseminate (Hanahan et al., 2000). These biological hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death. In an updated version of 2011 Hanahan and Weinberg reported two new hallmarks; deregulating cellular energetics and avoiding immune destruction (Hanahan et al., 2011). Cancer cells are defined by these eight hallmarks that are acquired during the course of multistep tumourigenesis. Two enabling characteristics allow cancer cells to acquire these different functions and drive tumour progression: genome instability and mutation, and tumour-promoting inflammation (Hanahan et al., 2011). The hallmarks that are relevant to this thesis are resisting cell death, enabling replicative immortality, and genomic instability because they occur at a cellular level. We investigated the mechanisms by which natural products from the prairie plant *Gaillardia aristata* affect these hallmarks. These chosen hallmarks will be expanded below.

Cancer cells frequently acquire mutations that make them insensitive to cell death (Hanahan et al., 2011). Tumours bypass the activation of cell death process through several mechanisms such as the elimination of damage sensor proteins from the cell death-inducing pathways, by increasing expression of anti-apoptotic regulators or survival signals, by downregulating proapoptotic factors, or by escaping the extrinsic apoptotic pathway (Hanahan et al., 2011). Cancer cells therefore have the ability to survive in conditions that would normally be prohibitive for normal cells.

The second hallmark of cancer that was investigated is enabling replicative immortality. Unrestrained proliferation is the archetypical hallmark of cancer cells (Hanahan et al., 2011; Moffat et al., 2014). To generate macroscopic tumours, cancer cells acquire mass through unlimited replicative potential, in contrast to normal cells that have a limited number of successive divisions (Hanahan et al., 2011). Cancer cells can circumvent barriers to proliferation in various ways, such as avoiding senescence, which is an irreversible non-proliferative but viable state, or by activating proliferation signals by mutation, or inactivating anti-proliferation signals.

The third hallmark of cancer is genome instability and mutation. Genome instability increases the frequency of mutations in whole genome. Tumour cells that accumulate mutations have a greater chance of acquiring selective growth advantage and increasing the fitness of a tumour cell over the surrounding cells (driver mutations) (Bozic et al., 2010; Hanahan et al., 2011). During tumorigenesis, cancer cells acquire and maintain mutations in genes that encode for proteins or regulatory RNAs that are central to genomic maintenance (Hanahan et al., 2011). If these are mutated, cells are more likely to acquire new mutations (Hanahan et al., 2011). The frequent occurrence of specific mutations at particular sites in the genome suggests that genes in those sites are

implicated in neoplastic progression. Thus, defects in genome maintenance and repair confer selective advantages to cancer cells, by accelerating the rate of accumulation of genotypes favourable to the cancer phenotype (Hanahan et al., 2011).

The hallmarks of cancer provide rational points of intervention in the treatment of cancer. By targeting one or more components that make up a hallmark, it might be possible to stop tumour cell replication or survival. Inhibition of cancer might be achieved through the use of agents that cause irreparable amounts of damaged DNA, or arrest cell proliferation, or induce cell death pathways.

### *1.3 Chemotherapeutic agents*

Chemotherapy is one of the major categories of pharmacotherapy to treat cancer patients. Chemotherapeutic drugs are agents that are either non-specific cellular poisons and inhibit processes such as mitosis, or that interfere with specific targets involved in carcinogenesis and tumour growth. Some of the obstacles in current chemotherapy are: acquired resistance, undesirable side effects, or lack of drugs to target a pathway in the hallmarks of cancer. Therefore, there is a need to identify new chemicals that can become new chemotherapeutic drugs. It is noteworthy that more than half of the current chemotherapeutic drugs are natural products or derivatives thereof, indicating that, historically, natural products are a reliable source of compounds with anti-cancer properties (Newman et al., 2016). One strategy to improve chemotherapy options is to discover new natural product chemicals that have anti-cancer properties.

### *1.4 The source: natural products*

The term “natural products” is used to describe chemical substances derived from animals, plants, fungi or microbes. A subclass of natural products are secondary metabolites, which are organic compounds produced by organisms to confer a selective

advantage (such as defense against predators) (Katz et al., 2016). Secondary metabolites are typically not required for primary functions such as growth, reproduction or development of the organism that produces them. They may be produced under conditions such as starvation, growth, or predation (Clardy et al., 2004).

By virtue of their unparalleled structural diversity, natural products have played a key role in organic chemistry and traditional medicine. Natural products have more stereogenic centers and more architectural complexity than synthetic molecules. They typically have desirable pharmacological properties such as cell penetration and a capacity to interact with cell components such as proteins (Clardy et al., 2004; Dobson et al., 2009). In fact, many effective drugs resemble natural occurring metabolites (Gupta et al., 2007; Dobson et al., 2009). Through natural selection, natural compounds may have an optimal structure leading to high potency and specificity. Natural products can bind to a wide range of proteins or other cellular targets (Sukuru et al., 2009; Walsh et al., 2010). This is possible because natural products are synthesized by proteins, and consequently exhibit activities mediated by interactions with proteins (Balamurugan et al., 2005; Drewry et al., 2010). The inherent advantage of natural chemical entities over synthetic chemicals is that the biology, target and molecular mechanism of action may have been optimized through evolution (Swinney et al., 2011). Natural molecules and their semi-synthetic derivatives are the major source of human medicines and constitute a large portion of new chemical entities (Newman et al., 2016). The multiplicity of biochemical synthetic pathways and the abundance of species belonging to the four kingdoms of plants, animals, fungi and bacteria gives rise to a number of compounds that is predicted to be enormous and diverse. Natural products are the best source from which to discover new compounds.

### *1.5 Natural products in plants*

There are many historical records of plants with medicinal properties used against a range of human diseases (Newman et al., 2000). For example, there are written records of medicinal plants that date to 2600 B.C. showing Mesopotamian traditional medicine, which used more than 1000 plant derived substances. Some of these were oils from plant species (*Cupressus* spp., *Papaver* spp., *Glycyrrizha* spp.) that were active against minor diseases such as cough and cold, and are still used in modern medicine (Newman et al., 2000; Chin et al., 2006). The notion of pure compounds as active drugs came in the early 1800s and was corroborated by the isolation of what can be considered the first commercial pure natural product, morphine, by E. Merck in 1826. The production of the first semi-synthetic pure drug based on a natural product, aspirin, was achieved by Bayer in 1899 (Grabley et al., 1999; Newman et al., 2000).

Research of natural products from plants has been guided by ethno-medicinal knowledge (Verpoorte et al., 2005; Cos et al., 2006). An analysis of drugs deriving from natural products showed that out of 122 plant-derived drugs, approximately 80% were related to their ethno-medicinal uses (Fabricant et al., 2001). The ethno-medical approach uses traditional knowledge in the forms of herbalism, folklore and shamanism as a criterion to select candidate plant. Artemisinin is an example of an immensely important anti-malarial drug from a plant that was known in an ancient traditional medicine. Artemisinin was isolated from the Chinese plant *Artemisia annua*, which was recognized as a treatment for alleviating malarial symptoms in the 4<sup>th</sup> century Ge Hong's "*A Handbook of Prescriptions for Emergencies*" book (Shen, 2015). Semi-synthetic derivatives of artemisinin are now used as drugs approved by the F.D.A. (Food and Drug

Administration) and W.H.O. (World Health Organization) as treatments against malaria (W.H.O., 2015). The Nobel prize was awarded to YouYou Tu in 2015 for the modern discovery of the active ingredient artemisinin and its derivative dihydroartemisinin as an anti-malarial compound (Tu, 2011; Shen, 2015; Tu, 2016).

Many plant natural products or their derivatives are powerful anti-cancer drugs (Hartwell, 1982; Graham et al., 2000). A review of anti-cancer agents of natural origin that are currently used in chemotherapy includes the vinca alkaloids, vinblastine and vincristine, from the *Catharanthus roseus* periwinkle; camptothecin from *Camptotheca acuminata*, a Chinese tree, and its synthetic derivative irinotecan; paclitaxel, isolated from the bark of the tree *Taxus brevifolia* (Grabley et al., 1999; Newman et al., 2000). Furthermore, between 1940s and 2014, of the 175 small molecules recognized by the F.D.A. as drug entities, 49% are natural products or derivatives thereof, demonstrating the key role of natural products in the discovery of new chemicals to treat human diseases (Newman et al., 2016).

Historical successes in investigating plant species are used as arguments to continue to research plant extracts for new chemicals of biological or medical interest. In support of this, of the 300,000 to 350,000 existent plant species in the world only 10-15% have been investigated, and they were tested only for a limited set of bioactivities (Hostettmann et al., 2002; Miller, 2011; Ngo et al., 2013). There are practical reasons to explain why our knowledge about the secondary metabolite composition of plants is incomplete. Many plants grow only in remote geographical areas, and the full bioactive potential of a plant is not known unless it is tested in the proper assay. Given that the known natural products come from approximately 60,000 species, there remain many possibilities to discover new compounds in untested plant species.

A contrary argument to studying natural products is the notion that the major classes of compounds have been identified in representative plants. Therefore, examining related species might produce, at best, related compounds with similar activities. This argument is weakened by an analysis of related compounds, such as the highly used anti-cancer drugs paclitaxel (Taxol<sup>®</sup>) and docetaxel. Paclitaxel is found in the bark of the Pacific yew *Taxus brevifolia*, which is native to the Pacific North West of North America (Holmes et al., 1991; Weaver, 2014). Docetaxel is an esterified product of the non-cytotoxic precursor 10-deacetyl baccatin III, which was isolated from the leaves of the European yew *Taxus baccata* (Colin et al., 1989). *T. baccata* and *T. brevifolia* are closely related species, and paclitaxel and docetaxel are related chemicals in the taxane class of secondary metabolites. Despite the similarities, docetaxel is two times more active than paclitaxel in microtubule polymerisation assays *in vitro* (Buey et al., 2005). The differences in properties have a major difference in how the molecules are used as drugs, for example docetaxel is more active than Taxol<sup>®</sup> against metastatic breast cancer (Crown et al., 2004). Therefore, it is rational to pursue the investigation of secondary metabolites from related plant species.

### *1.6 The natural products sesquiterpenes*

Relevant to this thesis is the class of secondary metabolites known as sesquiterpenes, which are produced by plants from different families. During the course of our experiments we have isolated and identified a natural molecule that belongs to the class of sesquiterpenes. This class of secondary metabolites has been widely investigated with over 5000 reported structures (Heinrich et al., 1998; Chadwick et al., 2013). The chemical structure of some sesquiterpenes is typically characterized by unsaturated carbonyl moiety such as in  $\alpha$ -methylene- $\gamma$ -lactones, which contain a methylene group at

the  $\alpha$  position. Sesquiterpenes react by a Michael-type addition with nucleophiles, for example with the sulfhydryl groups of cysteines, which in principle would make these chemicals very reactive to many different proteins (Kupchan et al., 1970; Schmidt, 1997; Scotti et al., 2007). Because of their peculiar chemical structure, these compounds have a variety of activities upon human cells, such as anti-inflammatory and anti-cancer properties (Picman, 1986; Hehner et al., 1998; Zhang et al., 2005). The sesquiterpene lactone that we isolated is presented in this thesis and it has anti-cancer properties, specifically an anti-mitotic activity upon human cancer cells. The anti-mitotic aspect of sesquiterpenes is an emerging property of these plant metabolites that has not been previously reviewed. In chapter 2, we provide a detailed review on sesquiterpenes and their anti-mitotic activities.

### *1.7 Investigating plant species in Canadian ecological zones*

Canada is a large country with a variety of ecological zones, such as the prairies, where plants have been little investigated for their bioactive secondary metabolites. With a low population density, there are regions in Canada where plants grow undisturbed by anthropomorphic activities. Prairie plant species have coevolved with two factors that likely influence secondary metabolite composition: a harsh climate and grazing herbivores. Therefore, abiotic and biotic pressures provide evolutionary pressures upon prairie plant species to be able to reproduce under these conditions. Herbivory, likely grazing animals such as the buffalo, could be repelled by secondary metabolites that enable sessile plants to survive (Stebbins, 1981). For example, purified alkaloid mixtures prepared from *Thermopsis montana* and *Thermopsis rhombifolia*, species growing in the prairie ecological zone, are toxic to grazing domestic animals, and have poisoned human beings when ingested (Keeler et al., 1990; McGrath-Hill et al., 1997). These observations

indicate that plants such as *T. rhombifolia* synthesize chemicals that might affect specific cellular pathways (Kerneis et al., 2015). The abundance of untested plant species in Canada and the diversity of bioactive compounds that they might produce could lead to the discovery of new compounds with different activities upon cells, including anti-cancer properties.

### 1.7.1 Traditional knowledge of Canadian plant species

Canada is among the countries with records of the use of medicinal plants by First Nations' communities (Hellsen et al., 1974; Lafranière et al., 1997; Moerman, 2009). The use of Canadian medicinal plants by First Nations' communities supports the hypothesis that there are plant species in Canada that produce compounds of medical or pharmaceutical interest. Prairie plants were used to treat many diseases including some of those imported to North America from Europe (e.g. smallpox) (Camazine et al., 1980; Kindscher, 1992).

Although cancer is diagnosed among First Nations' communities, there are few secondary records of cancer or remedies used to treat it in a traditional medicine system. However, some prairie plants that were used by traditional healers contain chemicals that scored positive in cancer cell inhibitory assays (Voaden et al., 1972; Piatak et al., 1985; Kindscher, 1992). An example is the plant *Tephrosia virginiana* (commonly known as the goat's rue), which was used by different North American aboriginal tribes to treat coughing, lassitude or worms (Kindscher, 1992). A dose of this plant, one tablespoon of tea made from the roots, was considered to be a powerful medicine. *T. virginiana* contains various flavonoids, including deguelin, a molecule with anti-cancer properties (Suh et al., 2013). In my research, however, we are still building a working relationship with First Nations' communities to explore this avenue, and we did not use traditional

knowledge. In future studies, the information provided by First Nations about the medicinal properties of prairie plants might be useful in selecting plants for investigation.

### *1.7.2 Criteria of selection of plant species for investigation of secondary metabolites*

We decided to investigate plant species with the following characteristics:

1. The plant species flourishes in the prairie ecological zone in southern Alberta;
2. The plant must not be widely distributed in other ecological zones;
3. There should be little scientific information about the secondary metabolites available from peer-reviewed literature;
4. Anecdotal evidence of toxicity to animals.

Albertan prairies extend over undisturbed areas where plants need to defend themselves from insects and mammalian predators. Moreover, the winter season is long and characterized by cold temperatures, snow and ice, so plants have only a short time to reproduce, thus providing biotic and abiotic conditions to produce secondary metabolites.

Alberta hosts more than 1775 vascular plant species (ferns, conifers, and flowering plants) that are either native or were imported and are established in the province (Moss et al., 1983). We have selected the prairie plant species *Gaillardia aristata* for investigation of its anti-cancer properties, because, as will be described later, it meets the criteria listed above.

### *1.8 Gaillardia aristata*

*Gaillardia aristata*, commonly known as “blanket flower”, is an herbaceous plant distributed in the semi-arid ecological zones in North America. It is a member of the taxonomical family of Asteraceae (sunflower) (Kuijt, 1982; Bain et al., 2014). It is native to western Canada, but it was exported to many other locations across Europe, Australia and South America. In southern Alberta *G. aristata* typically grows on the lower part of

the coulees, but can also be found on hills. This perennial plant blooms in June until mid-August allowing for an easier identification of the plant, and exhibits large flower heads with yellow three-lobed petals (see Figure 2.1) and a reddish-brown base, around an orange-brown centre (Bain et al., 2014). It grows to 70 cm in height, *G. aristata* is also characterized by lance-shaped leaves with long petioles situated at the base, long stems, trichomes over the stems and leaves.

This species is known in the Blackfoot and Plateau tribes traditional medical system for its properties against ailments such as fever, skin conditions and wounds (Hellson et al., 1974; Hunn et al., 1990; Moerman, 2009). In particular, the Blackfoot tribe would use a mixture of powdered roots applied directly to a wound or skin disorder, or they would make an infusion that was rubbed on sore skin, or used as eyewash.

In Western modern medicine *Gaillardia aristata* has not been studied. By contrast, the plant has been described to cause contact dermatitis, a skin condition (Burry, 1980; Burry et al., 1982). Other plants belonging to the Asteraceae family, including *Gaillardia* spp., are known to cause contact dermatitis, suggesting these species produce chemicals that cause irritation after direct contact with the plant (Arlette et al., 1981; Paulsen, 1992). The chemicals that cause allergenic irritation are believed to be members of the sesquiterpene family (Mitchell et al., 1971). In fact *G. aristata* appears in the Food and Drug Administration (FDA) database of poisonous plants as a toxic plant (F.D.A., 2016).

From an agricultural perspective *Gaillardia aristata* is considered of low forage value for cattle (Pahl et al., 1999) and there is anecdotal evidence that it is not consumed by grazing herbivores when mature.

Extracts prepared from *G. aristata* have been tested upon MCF-7 breast and HCT116 colon cancer cells (Salama et al., 2012). The authors prepared an alcohol extraction from the leaves, and several fractions were then separated with chloroform, ethyl acetate and n-hexane. The most active fraction was prepared with chloroform extraction and exhibited an IC<sub>50</sub> of 0.43 µg/mL on colon cancer cells. By biology-guided fractionation they isolated two sesquiterpene lactones (neopulchellin and 6α-hydroxyneopulchellin), five flavonoids, a sterol and its glycoside and a triterpene. The two sesquiterpene lactones were the most cytotoxic compounds on the two cell lines. We considered the information about *G. aristata*, and were intrigued that the cause of the cytotoxicity to human cells had not been investigated. Furthermore, with relatively abundant supply of the plant under natural conditions, we decided to pursue the analysis of *G. aristata* extracts by phenotypic assays.

#### *1.9 Type of assay: phenotypic screens applied to the hallmarks of cancer*

One approach to discover biologically active natural products is that of phenotypic assays. These are powerful assays to test compounds in relatively complex biological systems (e.g. representative of a disease) without prior knowledge of mechanisms of action (Moffat et al., 2014). Phenotypic screenings frequently use cell-based assays that measure changes in cell processes such as viability, morphology, proliferation, motility, or production of a product. When compared to a targeted approach, cell-based assays have several advantages: they involve all cellular and biochemical targets and take bioavailability phenomena into account, promptly discriminating between compounds that cross cell membranes and those that do not (Cos et al., 2006). In some cases, this approach can detect synergism between different molecules in a mixture, and biologically inactive compounds that can be metabolized in the cell to produce an active molecule

(Verpoorte et al., 2005). Typically, however, phenotypic assays are best to detect single molecules. Furthermore, these assays require only a limited knowledge of the molecular mechanisms of action of the cell, thus can be used to discover new pathways (Kell, 2013). Historically, phenotypic assays have been more successful than targeted approaches in the discovery of novel molecules with bio-activity (Kell, 2013).

The phenotypic approach has led to the discovery of many drugs that are currently being used, including paclitaxel (Taxol<sup>®</sup>) and camptothecin (Wall, 1998). A study by Swinney et al. in 2011 reported that between 1999 and 2008 out of the 183 new small molecule drugs approved by the F.D.A., 58% were discovered using phenotype-based approach (Swinney et al., 2011; Moffat et al., 2014). The authors also concluded that phenotypic screens are more likely to identify drug leads with therapeutically relevant molecular mechanisms of action than targeted approaches (Moffat et al., 2014). In the research project described in my thesis, we used phenotypic screens based upon the hallmarks of cancer. The screens were designed to identify secondary metabolites that inhibit replicative immortality, and resistance to cell death, with an impact on genome instability and mutation (Hanahan et al., 2011). This approach was successfully used in other studies and led to the isolation of natural products from marine sources (Schumacher et al., 2011).

#### *1.10 Biology-guided fractionation*

Biology-guided fractionation is a powerful method to isolate a pure, active compound from a complex mixture. The pure compound is frequently needed to identify the target and the molecular mechanism of action of its activity, and to determine the chemical structure. Biology-guided fractionation involves sequential cycles of biological testing to identify the bioactivity followed by chemical separation of compounds. These

cycles are repeated until a purified compound is isolated. Also known as bioassay-guided fractionation, this technique is commonly used in natural products and drug discovery (Abel et al., 2002; Koehn et al., 2005), and the application of this approach is also frequent in cancer research (Mehta et al., 2002; Kinghorn et al., 2003). Paclitaxel, for example, was isolated from the bark of the Pacific Yew tree *Taxus brevifolia* using bioassay-guided fractionation (Wani et al., 1971; Wall, 1998). Many variations of biology-guided fractionations are available depending on the degree of purity and convenience (Eldridge et al., 2002).

### *1.11 The cell cycle*

The cell cycle is comprised of a series of tightly regulated events in which the cell duplicates its content and divides into two equal cells. The cell cycle is comprised of four different phases: G<sub>1</sub> (Gap1) is a growth phase during which the cell secures that the conditions are suitable prior to DNA synthesis; S (synthesis) phase during which the DNA is duplicated; G<sub>2</sub> (Gap2) phase during which the cell continues to grow in preparation for mitosis; and M (mitosis) phase, marked by chromosome segregation and cell division (cytokinesis, or cytoplasm division). Collectively, G<sub>1</sub>, S and G<sub>2</sub> phases are called interphase. When extracellular conditions are not favourable for growth, or if cell replication is not needed, cells exit G<sub>1</sub> phase to enter a G<sub>0</sub> phase in which they can remain as terminally differentiated cells.

Human cells in G<sub>1</sub> phase contain 2c (c = haploid DNA content) DNA, or 23 chromosome pairs, which are duplicated during S phase in a semi-conservative process to 4n at the end of S phase and during G<sub>2</sub> phase until the division (M phase). Therefore, the cell content of DNA is indicative of the stage of the cell cycle, and can be analysed using flow cytometry (see sections 3.3.7 and 3.4.6).

S phase is dedicated to the accurate replication of the chromosomes that will be incorporated in the two daughter cells. It begins with the unwinding of the highly condensed double helix by the DNA helicase, leading to the elongation phase when the replication machinery is assembled at the origins and moves along the replication fork. Ahead of the replication fork however, the DNA becomes overwound and for every ten replicated nucleotide pairs, one complete turn of the double helix must be unwound. The unwinding caused by the advancement of the replication fork is relieved by DNA topoisomerase enzymes. S phase also implicates the duplication of chromatin proteins to assemble the DNA properly, as chromatin packing partly controls gene expression. When each chromosome is duplicated, the two sister chromatids are held together by multiple units of cohesin proteins that surround the chromatids.

Mitosis is divided into five phases: prophase, prometaphase, metaphase, anaphase and telophase, followed by the cytoplasm division into two daughter cells (cytokinesis). During prophase the chromosomes become condensed, and the two sister chromatids are resolved into distinct units. DNA condensation is accompanied by chromatin modifications such as the phosphorylation of histone H3 on Ser10 (Hendzel et al., 1997). Meanwhile, the tubulin dimers assemble outside the nucleus to form the mitotic spindle that originates from the centrosomes. In prometaphase the nuclear envelope breakdown is initiated by the disassembly of the nuclear pores and the nuclear lamina, the attachment of the microtubule array to the kinetochores (the centromeric regions of chromatids) and positioning of the spindle apparatus to the opposite poles of the cell. Prometaphase is followed by metaphase, during which the chromatids are aligned at the metaphase plate (situated at the equator of the spindle) by the spindle microtubules. The metaphase-anaphase transition is a crucial step, and it is characterized by the activation of the

anaphase-promoting complex/cyclosome (APC/C, discussed in the following section) that degrades early mitotic proteins and marks the exit from mitosis. Anaphase begins with the degradation of cohesins, which hold the sister chromatids together, by the separase enzyme. Separase is a specific protease that becomes active to degrade cohesins when its natural protein inhibitor, securin, is in turn degraded by APC/C. At this stage, sister chromatids are no longer held together and are free to be directed to the opposite poles by the mitotic spindle. In telophase the two sets of chromosomes at the poles are reassembled into the daughter nuclei. The mitotic spindle disassembles, and the chromosomes decondense. Cytokinesis is the final stage of cell division during which the contractile ring cleaves the mother cell into two new cells.

Mitosis is characterized by numerous regulated complex events, and exhibits characteristics that differentiate it from interphase both at a morphological and biochemical level. The majority of eukaryotic cells without a rigid wall almost always adopt a spherical shape during mitosis (Cadart et al., 2014). Upon entry to mitosis, cells disassemble focal adhesion and actin stress fibers, thereby decreasing adhesion to the substrate (Dao et al., 2009). In addition, water flows in cells, causing them to swell (Stewart et al., 2011). Therefore, a dividing cell will acquire a spherical shape that can easily be distinguished from flattened interphase cells. In experiments, this feature also enables the separation of the mitotic population from the interphase cells because with fewer attachments to the substratus, mitotic cells can be collected by mechanical shake-off (Terasima et al., 1963; Kubara et al., 2012).

Another feature that distinguishes mitotic cells from interphase cells is the presence of the spindle apparatus (Nicklas, 1997). At the onset of mitosis, tubulin dimers assemble to form microtubule fibers from the two replicated centrosomes, creating a

bipolar array of microtubules composed of the interpolar microtubules, which slide across other fibers from the opposite pole, the kinetochore microtubules that are attached to the kinetochores of sister chromatids, and the astral microtubules, which contact the cell cortex and help the apparatus to reposition itself (Jordan et al., 1998). One can detect the presence or absence of the spindle apparatus, thereby determining if a cell is dividing or it is in interphase (Wu et al., 2013). In this thesis, we used the compound nocodazole, a toxin that binds directly to tubulin, to induce mitotic arrest (Vasquez et al., 1997). Nocodazole prevents tubulin polymerization and impedes the formation of mitotic spindles.

Cells that undergo mitosis experience a concomitant phosphorylation on histone H3 that occurs almost exclusively during mitosis, and can therefore be used as a marker to discern mitotic cells from the interphase population (Hendzel et al., 1997).

### *1.12 Cell cycle regulation*

The cell cycle consists of a series of highly regulated biochemical switches that are specific to each stage. The control system ensures cells undergo each phase at the right time and that it is completed successfully. There are three particularly important transitions: a control at the restriction point, between G<sub>1</sub> and S phases (G<sub>1</sub>/S checkpoint), when the cell commits to entry to the cell cycle and starts DNA replication, the G<sub>2</sub>/M checkpoint, when cells are committed in mitosis (in absence of damaged DNA), and the metaphase-anaphase transition, where the control system stimulates completion of mitosis and separation of the daughter cells.

The central components that control cell cycle progression are the cyclin-dependent kinases (Cdks), a family of conserved serine/threonine protein kinases that are activated at each stage by the coupling to their cyclin regulator and phosphorylate many

substrates (Malumbres et al., 2005). There are five Cdks regulating the different stages and four classes of cyclins: Cdk4 and Cdk6 are active when coupled to cyclin D and are essential to entry into G<sub>1</sub>, Cdk2 binds cyclin E and regulates progression from G<sub>1</sub> to S phase, and it also binds cyclin A during S phase, and Cdk1 regulates mitotic progression and is bound to cyclin B in late G<sub>2</sub> until the metaphase-anaphase transition (Vermeulen et al., 2003). Other important regulators of the cell cycle are two ubiquitin ligase complexes, the anaphase-promoting complex, or cyclosome (APC/C), and the Skp1/Cul1/F-box complex (SCF), which mark cyclins for degradation by the proteasome and inhibit Cdk activity (Vodermaier, 2004; Wei et al., 2004).

The cell cycle is regulated by protein synthesis, protein phosphorylation, and protein degradation. While Cdks are always expressed in cells, the levels of cyclins change during the cell cycle. The protein kinase catalytic domain requires binding to cyclin in order to be active. The Cdk1 complex (Cdk1 enzyme and its cyclin partner) is kept in an inactive state by phosphorylation on amino acids Thr14 by Myt1 kinase and Tyr15 by Wee1 kinase (Den Haese et al., 1995). These phosphates alter adenosine triphosphate (ATP) orientation in the ATP binding site preventing its hydrolysis and inhibiting kinase activity (Deibler et al., 2010). To initiate mitosis, the Cdc25 phosphatase removes these inhibitory phosphates groups, and Cdk1 becomes partially active (Karlsson et al., 1999). To be fully active, Cdk1 must be phosphorylated on Thr161 by the Cdk-activating kinase (CAK), inducing conformational changes in the active site and stabilizing the Cdk1-cyclin B complex (Larochelle et al., 2007; Deibler et al., 2010). Cyclin B levels increase during G<sub>2</sub> and are maximal in M phase, leading to a corresponding accumulation of Cdk1-cyclin B dimers. The crucial event that drives a cell into mitosis is the dephosphorylation of the inhibitory phosphates on Thr14 and Tyr15 of

Cdk1 by the Cdc25 phosphatase and the concomitant activating phosphorylation by CAK. At this point Cdk1-cyclin B is fully active and phosphorylates more than 70 different proteins in mammalian cells (Malumbres et al., 2005). The dimer controls several activities and promotes chromosome condensation by phosphorylating condensins (Abe et al., 2011). It is also responsible for nuclear envelope breakdown following phosphorylation of nuclear lamins, which provide structural function to the nucleus (Margalit et al., 2005), and also promotes assembly of microtubules through activation of microtubule motor proteins (Fourest-Lieuvain et al., 2006). Finally, this complex regulates proteins involved in replication, translation, and ubiquitin-dependent proteolysis. An active state of Cdk1 is therefore indicative of mitosis, and can be verified by examining Cdk1 phospho-activity. One Cdk1 substrate is the serine/threonine protein phosphatase 1 (PP1 $\alpha$ ), which is phosphorylated at Thr320 (Kwon et al., 1997). When phosphorylated, PP1 $\alpha$  is inactive, leading to an increased pool of phosphorylated proteins that are also substrates for Cdk1-cyclin B and are required for entry into mitosis (Kwon et al., 1997). On the contrary, when PP1 $\alpha$  is dephosphorylated during anaphase it is activated, contributing to exit from mitosis (Kwon et al., 1997). By examining the presence or absence of phospho-Thr320-PP1 $\alpha$ , it is possible to detect Cdk1 protein kinase activity, and establish whether a cell is undergoing mitosis or not (Lewis et al., 2013).

At the metaphase-anaphase transition, the mitotic spindles have migrated towards opposite poles, the chromosomes are aligned on the metaphase plate and are attached to the spindle fibers, ready to be separated. However, sister chromatids are not able to separate because they are held together by cohesin proteins (Peters et al., 2008). The APC/C ubiquitin ligase attaches ubiquitin units to proteins, which are later recognized by the proteasome and undergo proteolytic degradation (Sudakin et al., 1995). To be fully

active, APC/C must be bound to the regulator protein Cdc20, enabling entry into anaphase (Kramer et al., 2000). From telophase to late G<sub>1</sub> however, APC/C is bound to its activator Cdh1, which was inhibited earlier by Cdk1-cyclin B phosphorylation (Kramer et al., 2000). Therefore, APC/C<sup>Cdc20</sup> is necessary for progression through anaphase, whereas APC/C<sup>Cdh1</sup> regulates late mitosis ensuring the degradation of cyclins. Activation of the APC/C ubiquitin ligase complex promotes securin destruction, separase activation and eventually cleavage of cohesins, and the chromatids are now able to move to the opposite poles (Funabiki et al., 1996; Waizenegger et al., 2002; Thornton et al., 2003). Inactivation of Cdk1-cyclin B leaves a pool of proteins that were activated by phosphorylation to begin mitosis, and are then dephosphorylated by phosphatases, promoting mitotic exit. The APC/C targets cyclin A as well, inhibiting all Cdk activity until G<sub>1</sub> (Peters, 2006).

SCF is a multi-protein E3 ubiquitin ligase complex that attaches ubiquitin to phosphorylated proteins (Pagano et al., 2004). This complex contains three subunits: the S-phase-kinase-associated protein-1 (Skp1), the Cullin 1 protein (Cul1), and the F-box proteins, which determine how the SCF targets specific proteins (Skowyra et al., 1997; Deshaies, 1999; Pagano et al., 2004).

SCF regulates the cell cycle by labelling key proteins with ubiquitin, which is required to direct them to the proteasome. Some of its substrates include small protein inhibitors of cyclin dependent kinases (CKIs) (Pagano et al., 2004). Its activity is constant during the cell cycle, and is controlled by changes in the phosphorylation state of target proteins (Skowyra et al., 1997). SCF complex and APC/C regulate each other (Pagano et al., 2004; Vodermaier, 2004). In particular, during metaphase the SCF complex is conjugated to the F-box protein  $\beta$ -TrCP1 (SCF <sup>$\beta$ -TrCP</sup>), which is responsible for the degradation of early mitotic inhibitor 1 (Emi1), a natural inhibitor of APC<sup>Cdc20</sup> (Reimann

et al., 2001; Margottin-Goguet et al., 2003). The destruction of Emi1 enables APC<sup>Cdc20</sup> to initiate anaphase.

If APC/C is inhibited, mitotic cells will contain high levels of cyclin B and securin and will not be able to enter anaphase. By measuring levels of securin in mitotic cells it is possible to detect inhibition of APC/C pathway.

### *1.13 Ubiquitin-mediated proteasomal degradation*

Cells proteolyse a large number of proteins which, in conjunction with protein synthesis, maintain homeostatic functions as environmental conditions change. Proteolysis also removes damaged proteins. This process is highly specific to ensure that proteins are not degraded inappropriately. The majority of intracellular proteins are degraded by the ubiquitin (Ub)-proteasome pathway (UPP) (Rock et al., 1994; Lecker et al., 2006). The UPP consists of a sequence of enzymatic reactions that ligate ubiquitin chains to target proteins that are then recognized by the 26S proteasome, a large multi-catalytic complex that degrades ubiquitinated proteins to peptides (Baumeister et al., 1998). The task of covalently attaching Ub onto proteins is accomplished by three components, the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2) and the Ub-protein ligase (E3). In an ATP-mediated reaction, E1 activates ubiquitin at the C-terminus by a thiol-ester bond with a cysteine in its active site to generate the high energy intermediate E1-S-Ub (Glickman et al., 2002). The activated ubiquitin bound to E1 is then transferred to a cysteine sulfhydryl group of E2 carrier proteins, which then covalently conjugate Ub to the target protein or to an E3 ligase (Hershko et al., 1992). Finally, the E3 ligase transfers an activated Ub from the E2 onto a lysine amino acid on the selected protein, and to a lysine on Ub molecules, creating a Ub chain (Lecker et al., 2006).

There are two classes of E3 ligases: HECT (homologous to E6-AP carboxyl terminus) class or RING finger class (Jackson et al., 2000). HECT E3 ligases have a C-terminal HECT domain that forms a thiol-ester linkage with a cysteine on the Ub active site from the E2s, generating a third high energy thiol-ester intermediate (Ub-S-E3) and eventually transferring it to the substrate (Scheffner et al., 1995; Ingham et al., 2004). The RING finger class uses RING instead of HECT domains. RING finger domains contain zinc-binding motifs that contain 40 to 60 amino acids rich in cysteine and histidine (Jackson et al., 2000). RING finger E3s are scaffolds that bring the substrate and the E2 into proximity, an optimal condition for Ub conjugation (Deshaies et al., 2005). This class of E3 ligases covalently attaches the Ub moiety to the specific substrate: the Ub is transferred to an amino group of an internal lysine amino acid in the substrate with the formation of a covalent isopeptide bond (Glickman et al., 2002). By using the same mechanism, the E3 enzyme attaches subsequent Ub units to the previously added Ub, creating a polyubiquitin chain that is recognized by the 26S proteasome.

The 26S proteasome is composed of the 20S barrel-shaped core particle complex, which has protease activity, and the 19S subunit at the proximal and distal portion of the 20S complex, which recognizes ubiquitinated proteins (Voges et al., 1999). Inner subunits of the 20S particle contain the proteolytic sites whereas the outer subunits surround a narrow gate through which the substrate enters and the peptides exit (Huber et al., 2000). Substrate entry is catalyzed by the 19S complex, which contains six ATPases that hydrolyse ATP to unfold and translocate the ubiquitinated proteins into the 20S complex (Benaroudj et al., 2003). The distal portion of the 19S particle binds the polyubiquitin chains on the target and two deubiquitinating enzymes that disassemble the Ub chain to re-use Ub units for future protein degradation (Glickman et al., 2002). Once in the 20S

degradative complex, the polypeptide is hydrolysed into small peptides of three to twenty-five amino acids, which are released to the cytosol (Lecker et al., 2006). The 20S particle is composed of outer rings containing seven distinct but homologous  $\alpha$  subunits, and inner rings which contain seven homologous  $\beta$  subunits (Baumeister et al., 1998; Voges et al., 1999). The proteolytic sites are embedded in the inner side of the  $\beta$  subunits that have a chymotrypsin-like, trypsin-like and caspase-like sites (Goldberg, 2012). However, the chymotrypsin-like site seems to be the most important in protein breakdown, and it is the target of proteasome inhibitors (Voges et al., 1999). The active sites inside the  $\beta$  subunits use the hydroxyl group of the N-terminal threonines to react with peptide bonds (Goldberg, 2012).

#### *1.13.1 Inhibitors of the ubiquitin-proteasome pathway*

Aberrations in the UPP cause a number of diseases. Pathologies arising from a dysregulated/mutated UPP are grouped into two categories, pathologies that result from loss of function, eventually leading to stabilization of proteins, and pathologies that result from gain of function caused by abnormal degradation of the protein target (Glickman et al., 2002). Among these pathologies are neurodegenerative diseases, cystic fibrosis, muscle wasting, immune and inflammatory responses, Liddle's and Angelman's syndromes, and cancer.

Some of the targets of the UPP are growth promoting factors that, if not properly degraded, can promote excessive proliferation and cancer. These targets include tumour suppressor proteins such as p53 and p27, for which destabilization is implicated in the pathogenesis of multiple types of cancer. For example, uterine cervical carcinoma caused by infection with human papilloma virus (HPV) has very low levels of p53 protein due to excessive proteolytic degradation by an HPV E3 oncoprotein (Scheffner et al., 1990).

Removal of p53 by the viral E3 protein is considered an important mechanism which the virus uses to transform cells. Similarly, colorectal, prostate and breast cancers have low levels of the p27 cyclin-dependent kinase inhibitor caused by excessive activity of the UPP (Loda et al., 1997).

The SCF complex also plays a part in tumourigenesis. Skp1 is overexpressed in lung cancers and it is associated with poor prognosis. Furthermore, silencing Skp1 expression has an inhibitory effect on cancer cell lines (Liu et al., 2015). Another component of the SCF, the scaffold protein Cullin 1, is involved in tumourigenesis in breast cancer as its expression correlates with aggressive clinico-pathological parameters (Chen et al., 2010). Cull1 overexpression was frequently detected in melanomas, and it was upregulated in several types of lung carcinomas (Salon et al., 2007; Chen et al., 2010; Min et al., 2012). F-box proteins can function as oncogenes, or as tumour suppressors, and their mutation or dysregulation increases the chance of malignant transformations. The F-box protein Skp2 behaves as an oncoprotein in the pathogenesis of different cancers (Wang et al., 2014). Skp2 mediates the ubiquitination and degradation of various cell cycle regulators, such as p27, and activation of a protein kinase, Akt, which has a role in cancer progression (Chan et al., 2012). Skp2 inhibition was shown to inhibit tumourigenesis by inducing apoptosis (Hershko, 2008; Lin et al., 2010; Chan et al., 2012; Wei et al., 2012). On the contrary, the F-box protein FBXO11 is a tumour suppressor protein that targets the Bcl-6 oncoprotein for degradation (Duan et al., 2012; Wang et al., 2014). Overexpression of FBXO11 inhibited cell growth and induced cell death through Bcl-6 degradation in diffuse large B cell lymphoma (DLBCL) cells (Duan et al., 2012). Furthermore, mutation or deletion of FBXO11 was detected in patients diagnosed with B

cell lymphoma, and was associated with the poor survival of patients with pancreatic cancer (Duan et al., 2012; Mann et al., 2012).

Inhibitors of the proteasome and its pathways are of interest for their biological and medical properties. The first proteasome inhibitors were peptide aldehydes that were synthesized based on the knowledge of the substrate specificity of the proteasome (Rock et al., 1994; Lee et al., 1996). To date, there are five classes of proteasome inhibitors: peptide aldehydes, peptide vinyl sulphones, peptide boronates, peptide epoxiketones, and  $\beta$ -lactones (Adams, 2004). The compound Bortezomib was the first anti-cancer drug that inhibits the proteasome and is used to treat multiple myeloma (Goldberg et al., 1976; Raab et al., 2009; Messinger et al., 2012). Bortezomib is active against myeloma cells because they overproduce abnormal, toxic immunoglobulins. Furthermore, myeloma cells rely on the transcription of NF- $\kappa$ B, which promotes expression of growth factors and cytokines that cause tumour pathogenesis by inhibiting apoptosis (Hideshima et al., 2002). The proteasome regulates NF- $\kappa$ B signaling by degrading the protein I- $\kappa$ B, which binds to NF- $\kappa$ B. In the absence of I- $\kappa$ B, NF- $\kappa$ B is active. Therefore, proteasome inhibitors prevent activation of NF- $\kappa$ B and cause accumulation of misfolded proteins, which eventually leads to apoptosis. In non-small-cell lung cancer (NSCLC) H460 cells Bortezomib-mediated proteasome inhibition leads to Bcl-2 phosphorylation and cleavage followed by G<sub>2</sub>/M arrest and apoptosis (Ling et al., 2002).

### *1.13.2 Proteasome inhibitor MG132*

MG132 is a low molecular weight peptide aldehyde N-benzyloxycarbonyl (Z)-Leu-Leu-leucine that inhibits the 26S proteasome (Lee et al., 1998). It inhibits the chymotrypsin-like activity in the  $\beta$  subunit of the 20S proteasome, without impairing the ATPase or isopeptidase activities (Rock et al., 1994; Lee et al., 1996). It is a reversible

inhibitor that has a  $K_i$  in the low nanomolar range for the chymotryptic activity of pure 20S proteasomes, and an  $IC_{50}$  in the low micromolar range for the proteolysis inhibition in cultured cells. This compound, however, did not reach clinical trials because of its clearance by the cytochrome P450 (CYP3A) enzyme in human hepatocytes (Lee et al., 2010). Bortezomib is a derivative of MG132, and has a boronate group that enhances its potency, stability and selectivity (Goldberg, 2012). MG132 inhibits proliferation, glycolysis and mitochondrial oxidation in gastric cancer cells, and it causes S, G<sub>2</sub>/M or non-specific phase arrest of the cell cycle as well as apoptosis and loss of mitochondrial membrane potential in cervix cancer cells (Han et al., 2009; Lu et al., 2016). In this thesis we use MG132 as a positive control of proteasome inhibition in assays to detect the presence of one of its targets.

#### *1.14 DNA damage*

One of the hallmarks of cancer that was investigated in this thesis was genome instability and mutation. When the DNA is exposed to insults, a DNA damage response (DDR) reaction is activated (Zhou et al., 2000). Histone H2AX is a variant of histone H2A and it is phosphorylated on the amino acid Ser139 upon DNA damage, to become histone  $\gamma$ H2AX (Furuta et al., 2003).  $\gamma$ H2AX foci appear as early as 1-3 minutes after the DNA is damaged (Paull et al., 2000). The enzymes responsible for phosphorylation of H2AX are ataxia telangectasia mutated (ATM), and ATM and Rad3 related (ATR), which are activated in response to double-stranded breaks (DSBs) or single-stranded breaks (SSBs), respectively (Kastan et al., 1991; Zhou et al., 2000). It is proposed that all genotoxic compounds, regardless of the pathways they activate, lead to phosphorylation of histone H2AX (Cahuzac et al., 2010). If the damage is repaired, the signal is removed through dephosphorylation of  $\gamma$ H2AX by protein phosphatase 1 (PP1) and protein

phosphatase 2 (PP2) (Nazarov et al., 2003; Chowdhury et al., 2005). The rapidity of action of  $\gamma$ H2AX and the fact that it is dephosphorylated after repair of the lesion, make  $\gamma$ H2AX an excellent marker to detect damaged DNA (Furuta et al., 2003; Mah et al., 2010).

Camptothecin (CPT) is a genotoxic quinoline alkaloid isolated from the Chinese tree *Camptotheca acuminata*, which inhibits DNA topoisomerase I (Top1) (Wall, 1998; Pommier, 2006). DNA topoisomerase enzyme I relieves DNA supercoiling during replication and transcription, by creating a nick on one strand of DNA (Nitiss, 2009). CPT binds to TopI preventing DNA re-ligation and causing DNA damage, which results in S-phase arrest of the cell cycle followed by checkpoint adaptation and cell death (Hsiang et al., 1985; Adams et al., 2006; Pommier et al., 2006; Kubara et al., 2012). CPT was used in this thesis as a positive control treatment for DNA damage and induction of  $\gamma$ H2AX foci.

### *1.15 Cancer cell lines*

In this thesis we used colon carcinoma HT-29 cells, M059K glioblastoma cells and the non-cancerous lung fibroblastic cell line WI-38. HT-29 colorectal carcinoma cells represent one of the types of cancer with the highest mortality rate in Canada and in other countries across the world (C.C.S., 2015; W.H.O., 2015). This polarized epithelial cell line is a good experimental system because it has a dynamic cytoskeleton that responds to changes in cell division or cell death pathways (Cohen et al., 1999). These morphological changes are distinguishable by light microscopy. Furthermore, HT-29 show a pronounced mitotic arrest as compared to other cultured cells (Gascoigne et al., 2008). The tests were also performed on a cell line of fibroblastic origin, the M059K glioblastoma multiforme. Glioblastoma is an aggressive type of cancer characterized by high mortality rates, poor

prognosis, invasiveness and high proliferative rate (Adamson et al., 2009; Alves et al., 2011). In addition, these cells are optically clear, as compared to other cell lines. WI-38 are a diploid non-cancerous cell line of fibroblastic origin, and were used to investigate if *G. aristata* extracts affect a normal cell line or only processes that are specific to cancer cells. WI-38 cells have a number of cell divisions limited to approximately 50 passages (Hayflick, 1965).

#### *1.16 Research aims and objectives*

One of our long-term aim is to understand how human cells divide. To achieve this aim, our laboratory has undertaken a research program to discover chemical inhibitors of mitosis. The source from which we are looking for novel inhibitors are plant species from the Canadian prairie ecological zone. Furthermore, we are investigating novel chemicals for potential use as anti-cancer drugs. Historically, more than 50% of current anti-cancer drugs come from natural products or derivatives thereof (Newman et al., 2016). Plants are an abundant source of natural products, and some very successful anti-cancer drugs were isolated from plants (Paclitaxel, camptothecin, vinka alkaloids etc.) (Johnson et al., 1963; Wani et al., 1971; Wall, 1998). Canadian prairies harbour plants that have coevolved with grazing herbivores and with harsh climatic conditions, thus these plants are likely to produce natural products as defense mechanisms (Clardy et al., 2004).

When I joined the laboratory, it had been observed that extracts prepared from *G. aristata* leaves induced a striking morphology change in treated HT-29 cells, and induced cell cycle arrest. For this reason, and the criteria used to select plants for investigation, we chose to investigate the chemicals from *G. aristata* and their effects upon human cells.

To summarize, our objectives are:

1. To characterize the effects of *G. aristata* extracts upon HT-29, M059K, and WI-38 cells;
2. To determine if the effects of *G. aristata* extracts upon cells are related to the biochemical processes involved in the hallmarks of cancer, especially proliferation and mitosis;
3. To isolate and identify the compound(s) that cause the cellular effects.

As will be described, we discovered a compound from *Gaillardia aristata* that arrests cells in mitosis, possibly by inhibiting a key step in the proteasome pathway.

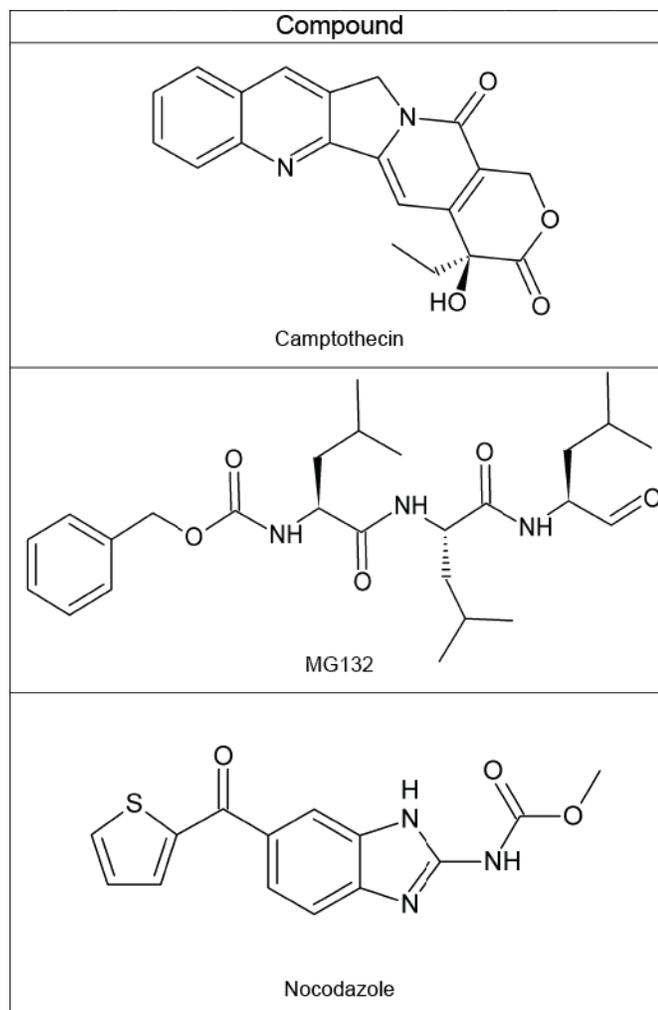


Figure 1.1: Structures of camptothecin, MG132 and nocodazole. These compounds were used as controls in this thesis. The first is the DNA damaging agent camptothecin (CPT), used in genotoxicity and cytotoxicity assays, the second is the proteasome inhibitor MG132, used in biochemical analyses, and the third is the tubulin toxin nocodazole, used in morphology, immunofluorescence and biochemical assays.

## CHAPTER 2

### **Emerging anti-mitotic activities and other bio-activities of sesquiterpene compounds upon human cells**

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## **2.1 Abstract**

We review the bio-activities of natural product sesquiterpenes and present the first description of their effects upon mitosis. This type of biological effect upon cells is unexpected because sesquiterpenes are believed to inactivate proteins through Michael-type additions that cause non-specific cytotoxicity. Yet, certain types of sesquiterpenes can arrest cells in mitosis as measured by cell biology, biochemical and imaging techniques. We have listed the sesquiterpenes that arrest cells in mitosis and analysed the biological data that support those observations. In view of the biochemical complexity of mitosis, we propose that a subset of sesquiterpenes have a unique chemical structure that can target a precise protein(s) required for mitosis. Since the process of mitotic arrest precedes that of cell death, it is possible that some sesquiterpenes that are currently classified as cytotoxic might also induce a mitotic arrest. Our analysis provides a new perspective of sesquiterpene chemical biology.

## **2.2 Introduction**

Sesquiterpene lactones are a class of natural product chemicals that are commonly synthesized by plant species. They are colourless, stable, and lipophilic chemicals that have a 15 carbon core structure (hence the prefix sesqui-), which is derived from the synthesis of three isoprene units and a five member lactone ring (Rodriguez et al., 1976; Yu et al., 2009). Lactone rings are cyclic esters of hydroxy carboxylic acids. Sesquiterpenes are classified into different subgroups based on their chemical structure (Figure 2.1); the largest subgroup is the germacranolides, which contain a 10-membered ring, whereas eudesmanolides are bicyclic 6/6 compounds, and guaianolides and pseudoguaianolides are bicyclic 5/7 compounds (Zhang et al., 2005; Fraga, 2012).

Sesquiterpenes can be acyclic, although the majority are cyclic and contain a lactone ring. The lactone component is characterized by an  $\alpha$ -methylene- $\gamma$ -lactone structure, which is an oxygen-containing ring with a carbonyl moiety ( $O=C-C=CH_2$ ) (Rodriguez et al., 1976; Chadwick et al., 2013) (Figure 2.1). The lactone is thought to be responsible for the majority of biological activities induced by sesquiterpene lactones when administered to organisms or cells. The unsaturated carbonyl structures react by a Michael-type addition with nucleophiles in biological systems, such as the sulfhydryl group in the amino acid cysteine (Kupchan et al., 1970; Schmidt, 1997; Scotti et al., 2007). One might assume that alkylation of thiol groups would have a non-specific effect upon cells because over 90% of the polypeptides encoded by the human genome contain a cysteine amino acid (Miseta et al., 2000). By testing sesquiterpene lactones in cell based assays, however, it has been found that some have very specific effects, and possibly specific targets in cells. We review the literature of the bio-activity of sesquiterpene lactones and describe that a small number of them arrest cells in mitosis.

### **2.3 Sesquiterpene lactones as natural products and their sources**

Over 5000 sesquiterpenes have been identified from plant sources (Heinrich et al., 1998; Chadwick et al., 2013). Sesquiterpenes are commonly found in a number of plant taxonomical families (Table 2.1). The Asteraceae family is particularly rich in sesquiterpene lactones, with over 3000 reported structures (Robles et al., 1995; Heinrich et al., 1998). These chemicals contribute up to 3% of the dry weight of a plant as in the case of tenulin, produced by *Helenium amarum* (Ivie et al., 1975). Some plant species from Asteraceae have the ability to shift the production of classes of terpenoids in response to herbivory, and store compounds in tissues upon which herbivores feed, such

as leaves, trichomes, phyllaries or achenes (Seaman, 1983). In fact, some sesquiterpenes function as a deterrent to grazing by sheep and cattle, and are toxic to various insects (González et al., 1993; Cifuentes et al., 2002; Schnee et al., 2002; War et al., 2012). The large number of structurally distinct sesquiterpene lactones in plants is directed by many sesquiterpene synthases encoded by the genome of a species. Chemical structure diversity across species correlates with the genetic diversity of synthases across species (Bennett et al., 2002; Cheng et al., 2007; Picaud et al., 2007). In addition, abiotic chemical modifications, such as thermolysis, can give rise to new structures (Lange et al., 1987; Little et al., 2002; Schnee et al., 2002; Chadwick et al., 2013). For example, the two recombinant sesquiterpene synthases prepared from *Abies grandis* (grand fir),  $\delta$ -selinene synthase and  $\gamma$ -humulene synthase, can produce more than 30 sesquiterpene olefins each using the acyclic precursor farnesyl diphosphate (Little et al., 2002). From the representative classes of sesquiterpene lactones, germacranolides are derived from the oxidation of the three carbon side chain, which results in the lactone ring, and eudesmanolides, guaianolides and pseudoguaianolides are derived from germacranolides (Seaman, 1983).

#### **2.4 Biological activities of sesquiterpene lactones**

As a consequence of sesquiterpene lactones structural diversity, these chemicals have a range of effects on the physiology of metazoan species, including humans. These compounds were also reported to have antifungal and antibacterial activities, which are described elsewhere (Lee et al., 1977; Picman, 1986; Cowan, 1999; Barrero et al., 2000; Barrero et al., 2005; Portillo et al., 2005).

#### **2.4.1 Effects upon insects and grazing herbivores**

Sesquiterpene lactones play important roles in the defense of the plants against herbivores such as insects or mammals. These compounds repel or poison grazing herbivores and attract parasite predators that attack the herbivore organism, thereby decreasing feeding upon the producer plants. The anti-feedant properties of sesquiterpene lactones were first demonstrated by Burnett et al. in 1974, who conducted larval feeding experiments on three *Vernonia* spp. (Burnett et al., 1974). They reported that two sesquiterpene lactones, glaucolide A and alantolactone, deterred feeding and reduced the survival of several insect species (Burnett et al., 1974). In particular, a concentration of glaucolide A lower than 0.5% in *Vernonia* spp. resulted in increased levels of feeding, whereas concentrations >1.0% of glaucolide A reduced feeding levels. This observation was supported by Rossiter et al. who observed that *Helianthus* spp. plants deterred feeding by sunflower moth larvae by 50% when the plants contained more than 1% dry weight of the sesquiterpene 8 $\beta$ -sarracinoyloxycumambranolide (8 $\beta$ -SC) (Rossiter et al., 1986). Many other studies highlight the importance of sesquiterpenes in defending the plant from insects (Picman, 1986; González et al., 1993; Cifuentes et al., 2002; Cheng et al., 2007; Bleeker et al., 2012; War et al., 2012; Irmisch et al., 2014). Volatile sesquiterpenes can repel or attract insects; citrus leaves release higher amounts of sesquiterpenes when in a juvenile state than a mature state (Killiny et al., 2016). Volatile sesquiterpenes are released to attract parasite predators, which help the plant defend itself against insect herbivores (Holopainen, 2004).

Mammals are also affected by contact with sesquiterpenes, either as a consequence of their toxicity or, in some cases, by taste. The observation that mammals respond adversely to these chemicals suggests that the capacity to produce these

secondary metabolites may have coevolved with grazing mammals (Burnett et al., 1977). For instance, rabbits and deer show avoidance behaviour to the sesquiterpene glaucolide A from *Vernonia* spp. (Burnett et al., 1977). In addition, *Helenium amarum* produces the sesquiterpene tenulin, which is toxic to livestock (Ivie et al., 1975). The sesquiterpene lactone helenalin, isolated from *Helenium microcephalum*, is toxic to cattle, sheep and goats (Witzel et al., 1976). Overall, there is a considerable number of sesquiterpenes, many of which were isolated from Asteraceae species, which are reported to affect the survival of mammals or have mammalian feeding deterrent properties (Picman, 1986).

#### **2.4.2 Effects of sesquiterpene lactones upon humans**

The chemical reactivity of sesquiterpenes and their effects upon grazing mammals make it likely that these molecules would affect human physiology. More than 200 species of Asteraceae have been reported to cause contact dermatitis, with cases documented in Australia, Europe and America (Burry, 1980; Arlette et al., 1981; Paulsen, 1992; Killiny et al., 2016). This condition is due to an inflammation of the skin after direct contact with plants (Arlette et al., 1981). It consists of localized itchy and burning rashes on skin that in some cases develop blisters. The Asteraceae plants in particular cause a more widespread eczema due to contact with airborne particles of the plant, defining the Compositae (synonym of Asteraceae) dermatitis (Paulsen, 1992), and sesquiterpene lactones have been identified as the causative agent (Mitchell et al., 1971; Arlette et al., 1981; Spettoli et al., 1998; Jack et al., 2013). The methylene group attached to the lactone ring is necessary but not sufficient to induce contact dermatitis (Mitchell et al., 1971). Sesquiterpene lactones that are structurally different can cause cross-reactions,

whereas identical sesquiterpenes from different plant species can cause false reactions. As a result of the great number of sesquiterpenes, the cross-reactions among them and the different proportions in plant species, the clinical description of contact dermatitis is complex (Mitchell et al., 1971; Rozas-Munoz et al., 2012).

## **2.5 Medicinal properties of sesquiterpene lactones**

Several medically important sesquiterpenes have been identified. They have been used for treatments of cardiovascular diseases (Rodriguez et al., 1976; Wong et al., 1999), ulcers (Giordano et al., 1990), or minor illnesses and symptoms such as diarrhoea, flu, neurodegeneration, migraines, burns, and pain (Heinrich et al., 1998; Ahlemeyer et al., 1999; Canales et al., 2005; Wesółowska et al., 2006). *Ambrosia tenuifolia* is an Asteraceae plant that harbours psilostachyins, which are sesquiterpenes with anti-parasitic activity (Picman, 1986; Sülsen et al., 2016). These compounds are active against *Leishmania* spp., which are responsible for severe forms of leishmaniasis, with an IC<sub>50</sub> value of 0.12 µg/mL (Sülsen et al., 2008).

Artemisinin is one of the most significant medicines at a global level. It is a sesquiterpene discovered and isolated from the Chinese herb *Artemisia annua* by Tu YouYou (Klayman, 1985; Tu, 2011). For this discovery Tu YouYou was awarded the 2015 Nobel Prize in Medicine (Tu, 2016). Artemisinin is active against *Plasmodium falciparum*, the causative organism of malaria. A derivative of artemisinin is now a standard worldwide treatment for malaria (W.H.O., 2010; Tu, 2011; W.H.O., 2012; W.H.O., 2015; Corey et al., 2016). Although the mechanism of action of artemisinin is not well understood, studies suggest that its endoperoxide bridge generates free radicals

that damage vital proteins in the parasite system, resulting in its death (Meshnick, 2002; Winzeler et al., 2014; Cravo et al., 2015). It was shown that artemisinin generates toxic free radicals, which interact with the intraparasitic heme of the parasite (Cui et al., 2009).

## **2.6 Anti-inflammatory effects**

Sesquiterpene lactones modulate several inflammatory processes, such as oxidative phosphorylation, platelet aggregation, histamine and serotonin release. However, the main inflammatory response inhibited by sesquiterpenes involves Nuclear Factor - kappa B (NF- $\kappa$ B) (Rüngeler et al., 1999; Siedle et al., 2004). A comprehensive study by Bork et al. showed that 54 Mexican Indian medicinal plants, all rich in sesquiterpene lactones, had potent inhibitory effects on the NF- $\kappa$ B pathway (Bork et al., 1997). NF- $\kappa$ B is a family of proteins that control DNA transcription, cytokine production, and cell survival. The proteins form either a hetero- or homo-dimer cytoplasmic complex comprised of the subunits, p50 and p65. Its activity is tightly regulated by interaction with the natural inhibitor I- $\kappa$ B, which sequesters the NF- $\kappa$ B dimer in the cytosol (Gilmore, 2006). Pathogenic or inflammatory stimuli lead to the production of reactive oxygen species (ROS) and phosphorylation and ubiquitination of I- $\kappa$ B. Once I- $\kappa$ B is ubiquitinated, it is recognized by the proteasome and degraded (Driscoll et al., 1990; Kretz-Remy et al., 1996). The absence of I- $\kappa$ B leaves the NF- $\kappa$ B dimer free to translocate to the nucleus and induce transcription of target genes (Napetschnig et al., 2013). NF- $\kappa$ B regulates over 150 genes in pathways that mediate inflammatory or immune processes in response to injury, or bacterial and viral infections (Rüngeler et al., 1999). As elucidated by Rüngeler et al., a possible mechanism of inhibition of NF- $\kappa$ B by sesquiterpenes is

through alkylation of the amino acids Cys38 and Cys120 in the DNA-binding domain of the p65 subunit (Rüngeler et al., 1999). Cys38 forms a hydrogen bond with the backbone of the  $\kappa$ B-DNA motif, participating in DNA binding. The sulfur atom of Cys120 is in proximity to Cys38, and the space between the two amino acids normally positions the phenol ring of Tyr36, which is essential for DNA binding. A sesquiterpene lactone adduct in which both sulfur atoms are alkylated creates a cross link between Cys38 and Cys120 in the p65 subunit, and impairs DNA binding.

A comprehensive study conducted by Siedle et al. characterized 103 sesquiterpene lactones from six subclasses in their capacity to inhibit NF- $\kappa$ B DNA binding (Siedle et al., 2004). They found that the majority of active sesquiterpene lactones belonged to the guaianolides subclass and that the presence of the  $\alpha,\beta$ -unsaturated carbonyl group in the  $\alpha$ -methylene- $\gamma$ -lactones played a major role in cytotoxicity (Siedle et al., 2004). Zerumbone and parthenolide were two of the sesquiterpenes with anti-inflammatory activities (Guzman et al., 2005; Takada et al., 2005). Zerumbone treatment at 50  $\mu$ M for 12 h inhibited the activation of NF- $\kappa$ B and NF- $\kappa$ B-regulated gene expression induced by carcinogens and various inflammatory agents (such as okadaic acid, tumour necrosis factor -TNF-, cigarette smoke and hydrogen peroxide) on H1299 lung adenocarcinoma, KBM-5 chronic myelogenous leukemia, A293 embryonic kidney, and FaDu squamous cell carcinoma cell lines. Zerumbone treatment at 25  $\mu$ M for 12 h also reduced expression of NF- $\kappa$ B-dependent gene products involved in cell proliferation, anti-apoptosis and invasion (Takada et al., 2005). Parthenolide, a sesquiterpene lactone that is structurally different from zerumbone, induced apoptosis in human acute myelogenous leukemia stem and progenitor cells through inhibition of NF- $\kappa$ B, proapoptotic activation of p53, and increasing amounts of reactive oxygen species (ROS) (Guzman et al., 2005). Parthenolide

was also described as an inhibitor of NF- $\kappa$ B activation in HeLa cells by binding directly to I- $\kappa$ B-kinase (IKK), which prevents it from phosphorylating the I- $\kappa$ B (Kwok et al., 2001) and maintaining I- $\kappa$ B association with NF- $\kappa$ B.

## 2.7 Anti-tumour activities

There are numerous reports describing the activities of sesquiterpenes upon different pathways in human cancer cells (Zhang et al., 2005; Ghantous et al., 2010) of which the predominant effect is cytotoxicity. Lee et al. investigated the cytotoxicity of sesquiterpenes on normal lung fibroblastic cells WI-38, HEP2 epidermoid carcinoma of larynx cells and the W18-Va2 buccal mucosa (fibroblast-like) cells and reported that 16 out of 18 sesquiterpenoids showed cytotoxicity to three cell lines tested, the most active being helenalin with IC<sub>50</sub> ranging from 0.03 to 0.18  $\mu$ g/mL (Lee et al., 1971). It was later postulated that helenalin reacts with the cysteines of telomerase proteins and inactivates enzyme activity in T-cell leukemia (Jurkat cells) and HL60 promyelocytic leukemia (Huang et al., 2005). Telomerase maintains telomeres length ensuring immortality of cancer cells (Huang et al., 2005). Lee et al. concluded that the unsaturated carbonyl (O=C-C=CH<sub>2</sub>), independently of whether it was included in a lactone or cyclopentanone, was required for cytotoxicity (Lee et al., 1971; Rodriguez et al., 1976). However, the presence of additional alkylating groups such as cyclopentanone, or  $\alpha$ -methylene- $\gamma$ -lactone appeared to enhance cytotoxicity, as the latter plays an important role in alkylating enzymes (Lee et al., 1972; Lee et al., 1973; Hall et al., 1977). Other structure-cytotoxicity studies on sesquiterpenes showed that  $\alpha$ -methylene- $\gamma$ -lactones react rapidly with cysteines to form stable adducts, whereas endocyclic  $\alpha,\beta$ -unsaturated- $\gamma$ -lactones

react slowly with cysteine, to form unstable adducts (Kupchan et al., 1970). Furthermore, Lee and Hall established that the  $\alpha$ -methylene- $\gamma$ -lactone moiety, a  $\beta$ -unsaturated cyclopentanone ring or an  $\alpha$ -epoxycyclopentanone system are the essential structures for anti-tumour activity *in vivo* (Lee et al., 1977; Hall et al., 1978). Overall, sesquiterpene lactones selectively alkylate nucleophilic groups in many enzymes, including those involved in the control of cell division (Rodriguez et al., 1976; Picman, 1986). Kupchan suggested that the tumour inhibitory activity is selective for thiols, for sulfhydryl enzymes and for sulfhydryl groups within enzymes (Kupchan, 1974). For example, phosphofructokinase and other sulfhydryl enzymes from rabbit skeletal muscle lose their activity *in vitro* after reaction with sesquiterpenes (Hanson et al., 1970; Smith et al., 1972). It was found that sesquiterpene lactones inhibited DNA polymerase and thymidylate synthase enzymatic activity in tumour cells, thereby inhibiting nuclear DNA synthesis (Hall et al., 1977; Lee et al., 1977; Hall et al., 1988).

Increases in NF- $\kappa$ B activity can contribute to cancer development and progression, and it provides a mechanism by which tumour cells escape immune surveillance and resist chemotherapy and radiotherapy (Baud et al., 2009). This nuclear factor plays an important role in prevention of carcinogenesis, and many human tumours have a constitutively active NF- $\kappa$ B (Ravi et al., 2004). When NF- $\kappa$ B is active it promotes cell cycle entry and inhibits apoptosis (Ravi et al., 2004). Therefore, NF- $\kappa$ B inhibitors can sensitize tumour cells to apoptosis signaling pathways activated by multiple stimuli, or to the effects of other anti-tumour agent, or they can prevent cell proliferation.

### 2.7.1 Clinical trials of sesquiterpene lactones

Three sesquiterpene lactones entered clinical trials by virtue of the chemical properties such as alkylating center reactivity, lipophilicity, molecular geometry and electronic features (Ghantous et al., 2010). These compounds are: L12ADT, a peptide prodrug derived from thapsigargin, isolated from *Thapsia*; artesunate, a derivative of artemisinin from *Artemisia annua L.*; and dimethylamino-parthenolide (or LC-1), an analogue of parthenolide from *Tanacetum parthenum* (Figure 2.2). These compounds have selective activities toward tumour and cancer stem cells by targeting specific signaling pathways involved in cell differentiation, cell proliferation, and apoptosis through mitochondrial and caspase signaling pathways and through an increase of the cytosolic concentration of calcium (Ghantous et al., 2010). Artesunate has shown promising results in the treatment of laryngeal carcinomas, uveal melanomas and pituitary macroadenomas, and is currently undergoing phase I clinical trials against cervical intraepithelial neoplasia, colorectal cancer and other solid tumours (Krishna et al., 2015; N.I.H., 2016; N.I.H., 2016; Ren et al., 2016). Artesunate targets the iron group content by catalyzing the generation of free radicals from the bridged endoperoxide group (Efferth et al., 2001). Furthermore, artesunate reverses multi-drug resistance by reducing the ATP-binding cassette subfamily G member 2 (ABCG2), a multidrug transporter, expressed in esophageal cancer (Liu et al., 2013; Ren et al., 2016). L12ADT (8-*O*-(12-{L-leucinoylamino}dodecanoyl)-8-*O*-debutanoyl-thapsigargin) underwent phase I clinical trials for treatment of refractory, advanced or metastatic solid tumours, and is currently undergoing phase II clinical trials for the treatment of glioblastoma (Mahalingam et al., 2016; N.C.I., 2016). By targeting the sarco/endoplasmic reticulum (ER) calcium ATPase (SERCA) pump, thapsigargin causes apoptosis (Doan et al., 2015). Dimethylamino-

parthenolide (LC-1), an oral bioavailable parthenolide analogue (Guzman et al., 2007), was investigated in a phase I clinical trial against acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and other blood and lymph node cancers in 2012 (Ghantous et al., 2010; Ren et al., 2016). However, the phase I clinical trial was suspended after one year (AdisInsight, 2012).

### **2.7.2 Anti-mitotic activities of sesquiterpenes**

Research from our laboratory led us to identify a novel sesquiterpene lactone from the Asteraceae family member *Gaillardia aristata*, using phenotypic assays to detect anti-mitotic compounds. The discovery of an anti-mitotic sesquiterpene lactone was surprising to us because very few have been described to have this activity although more than 1500 publications have reported anti-cancer and anti-inflammatory properties (Lyss et al., 1998; Wen et al., 2002; Ghantous et al., 2010). Furthermore, the predicted mechanism of action of sesquiterpene lactones is counterintuitive to a protein target that would have a specific mitotic phenotype.

#### *6-O-Angeloylplenolin (6-OAP)*

6-O-Angeloylplenolin (Figure 2.3) was isolated from the Chinese medicinal herb, *Centipeda minima*, from the Asteraceae family (Liu et al., 2011). 6-OAP has anti-proliferative properties on MM.1R, MM.1S, U266 and RPMI 8226 multiple myeloma cells and induces a G<sub>2</sub>/M-phase arrest. The arrest is characterized by an increase of cyclin B levels and a decrease in phospho-Tyr15 Cdk1 (Liu et al., 2011). Treatment of cells with 7.5 μM 6-OAP for 24 h induced a mitotic arrest, which in turn activated the spindle assembly checkpoint (SAC) proteins BubR1 and Mad2. The authors showed that 6-OAP

facilitated binding between the proteins Mad2 and Cdc20, which prevented activation of the anaphase-promoting complex/cyclosome (APC/C). As a consequence, levels of ubiquitinated cyclin B decreased, which is believed to prevent cyclin B degradation by the proteasome. The mitotic arrest induced by 6-OAP treatment was confirmed by observations of an increase of phosphorylated levels of histone H3 (PH3), and the formation of a mitotic spindle (Liu et al., 2011). In a subsequent study, Liu et al. reported that 6-OAP inhibited the Skp1 protein in A549 lung adenocarcinoma and NCI-H1975 non-small cell lung cancer cell lines (Killiny et al., 2016). Skp1 is a component of the Skp1-Cullin-F-box containing (SCF) complex, an E3 ubiquitin ligase. This complex promotes the ubiquitination of regulatory proteins that target them for degradation by the proteasome (Jia et al., 2011; Liu et al., 2015). Computational docking analysis and co-immunoprecipitation analysis suggested that 6-OAP treatment at 7.5  $\mu$ M binds Skp1 at the Skp1-Skp2 interface, and attenuates Skp1-Skp2 interaction. This causes dissociation and proteolysis of E3 ligase complexes NIPA, Skp2, and  $\beta$ -TrCP, and accumulation of their substrates cyclin B, p27 and E-cadherin. In a murine model *in vivo*, 20 mg/kg of 6-OAP for 30 days reduced tumour mass and prolonged mice survival. 6-OAP treatment *in vivo* did not affect body weight or serum concentration of control proteins. Another target of 6-OAP is the transcription factor STAT3, which promotes STAT3-dependent Skp2 transcription (Cheng et al., 2017). Therefore, 6-OAP may repress Skp2 activity in a dual action by inhibition of STAT3 which impedes Skp2 transcription, and by binding to Skp1, causing dissociation and proteolysis of Skp2 (Liu et al., 2015; Cheng et al., 2017).

#### *9 $\beta$ -Acetoxycostunolide and Santamarine*

9 $\beta$ -acetoxycostunolide is a derivative of the sesquiterpene lactone costunolide, and it is structurally related to santamarine (Figure 2.3). Both compounds were isolated from

the Asteraceae Chinese herb *Cyathocline purpurea*. Santamarine and 9 $\beta$ -acetoxycostunolide block L1210 murine leukemia cells in the G<sub>2</sub>/M-phase of the cell cycle in a concentration- and time-dependent fashion with a corresponding decrease of cells in the G<sub>1</sub> phase, and subsequent cell death. The duration of the treatments were 2 and 48 h, with concentrations of either compound ranging between 1 and 10  $\mu$ M. However, the target causing the arrest had not been identified and it was not determined if cells were in mitosis (Ma et al., 2009).

#### *Artemisinin and its derivatives artesunate and dihydroartemisinin*

Artemisinin is a sesquiterpene lactone isolated from the plant species *Artemisia annua* (Asteraceae). Its derivative, artesunate, is an effective therapy against malaria (Tu, 2011; W.H.O., 2015; Tu, 2016) (Figure 2.3). Artesunate can induce mitotic arrest in human cells in culture (Steinbruck et al., 2010; Luo et al., 2013). Artesunate induced a G<sub>2</sub>/M-phase arrest on four cell lines when applied at 26  $\mu$ M for 48 h (J-Jhan and H69) or at 78  $\mu$ M for 24 h (HCT116 and U251) (Steinbruck et al., 2010). Although specific biochemical measures of mitosis were not described, further analysis showed that treated cells exhibited remote centrosomes and two nuclei per cell, indicating that the cells had duplicated the DNA but could not divide properly (Steinbruck et al., 2010). The multiple centrosomes, multiple spindles and multinucleated cells suggested that artesunate also caused a defect in cytokinesis. Dihydroartemisinin is another semi-synthetic derivative of artemisinin and was shown to have anti-cancer properties (Luo et al., 2013). When HeLa cells were radio-sensitized with 6 Gy x-irradiation for 24 h and treated with 20  $\mu$ M dihydroartemisinin, they exited the G<sub>2</sub> block and exhibited downregulated Wee1 and upregulated cyclin B levels. Combined treatment with radiation and dihydroartemisinin

led HeLa cells to abrogate the G<sub>2</sub>/M checkpoint and enter mitosis with a highly rearranged genome, eventually leading to mitotic cell death.

### *Coronopilin*

Coronopilin (Figure 2.3) is a sesquiterpene lactone isolated from *Ambrosia arborescens*, an aromatic plant that belongs to the Asteraceae family, and grows in western South America. Cotugno et al. found that treatment of the U937 leukemia cell line by 20  $\mu$ M coronopilin for 24 h induced 52% of the cells to arrest in the G<sub>2</sub>/M-phase, as compared to 12% of control samples (Cotugno et al., 2012). The fraction of mitotic cells increased from 6.6% to 18.0% after treatment. The mitotic arrest was characterized by Cdk1-cyclin B activity and sustained levels of cyclin B, and high levels of PH3. The authors found that coronopilin could bind covalently with tubulin and cause an increase of hyperpolymerized tubulin. The  $\alpha,\beta$ -unsaturated carbonyl group of coronopilin is likely responsible for the activities of coronopilin because the structural analogue, dihydrocoronopilin, which lacked an  $\alpha,\beta$ -unsaturated carbonyl group, did not inhibit leukemia cell population growth (Cotugno et al., 2012).

### *Costunolide*

Costunolide (Figure 2.3) is a sesquiterpene lactone isolated from *Michelia compressa*, a small tree that belongs to the Magnoliaceae family. Costunolide inhibits tubulin carboxypeptidase activity in Bt-549, MDA-MB-436 and MDA-MB-157 breast cancer cells in a manner similar to that of parthenolide (see parthenolide section), at concentrations ranging from 5 to 25  $\mu$ M and at 6 h post treatment (Whipple et al., 2013). In HA22T/VGH hepatocellular carcinoma cells, treatment by 5  $\mu$ M costunolide caused a mitotic arrest as measured by an increase in PH3 positive cells from 3.6% in the control to 25.8% in treated cells (Liu et al., 2011). Cells were arrested in metaphase as shown by

the mitotic spindle and DNA configuration accompanied by upregulation of phosphorylated Cdc25c (Ser216) and cyclin B (Liu et al., 2011). In a separate study, 100 nM costunolide at 24 h post treatment had anti-proliferative activity upon MCF-7 breast cancer cells with low cytotoxicity, correlated with interference with microtubule dynamics, forming short and dense microtubule fibers (Bocca et al., 2004). It was suggested that interaction of costunolide with tubulin involves the nucleophilic reaction with sulfhydryl groups of cysteines (Legault et al., 2000; Bocca et al., 2004). However, the precise mechanism of the interaction between costunolide and tubulin was not shown experimentally. Rasul et al. also reported a G<sub>2</sub>/M-phase arrest by costunolide in T24 bladder cancer cells in which the percentage of cells in G<sub>2</sub>/M increased from 13.8% in the control group to 25.6% or 41.3% in cells treated with 25 or 50 μM costunolide for 24 h, respectively (Rasul et al., 2013). The precise mechanism of costunolide activity has not been characterized, although it seems to affect microtubule dynamics.

#### *Dehydroleucodine*

The sesquiterpene lactone dehydroleucodine (Figure 2.3) was isolated from the aromatic herb *Artemisia douglasiana* (Asteraceae family). When applied to HeLa S3 cervix cancer cells for 24 h, 20 μM dehydroleucodine increased duration of the mitotic phase, which included an increase in the number of cells positive for PH3 (Costantino et al., 2013). The G<sub>2</sub>/M delay was also accompanied by decreased levels of cyclin B, in contrast to other results reviewed here. When treated cells exited mitosis, they did not undergo cell death but arrested in the G<sub>1</sub> phase. However, Costantino et al. found that dehydroleucodine induced DNA lesions after 8 h of treatment, characterized by the presence of phosphorylated ATM, histone γH2AX, and increased levels of the DNA double-stranded break (DSB) marker 53BP1. These findings suggest that DNA damage

causes slower transitions through S and G<sub>2</sub>/M phases followed by activation of apoptosis or senescence responses (Costantino et al., 2013).

### *NP136*

NP136 (Figure 2.3) and the related compounds NP339 and NP176 are sesquiterpene lactones that were identified by a screen for natural products that modulates centriole number (Graciotti et al., 2016). It was reported that these compounds do not affect the cell cycle when tested at 7.5  $\mu$ M for 48 h. However, when the compounds were tested at 7.5  $\mu$ M, they reduced the number of centrosomal components of the mitotic spindle in mitotic HeLa cells. The effects of one of the compounds, NP339, were suggested to be linked to cysteine modification and the NF- $\kappa$ B pathway.

### *Parthenolide*

The sesquiterpene lactone parthenolide was isolated from the feverfew, *Tanacetum parthenium* (Asteraceae family) (Figure 2.3). Fonrose et al. showed that 10  $\mu$ M parthenolide inhibited tubulin carboxypeptidase (TCP) in HeLa cells and impaired tumour progression (Fonrose et al., 2007). Detyrosinated tubulin is a form of  $\alpha$ -tubulin monomer that lacks the tyrosine at the C-terminus of the protein, which exposes a glutamic acid (Lafanechère et al., 2000). This form of detyrosinated tubulin destabilizes microtubules and impairs mitotic spindles, increasing the likelihood of tumour invasiveness and progression (Lafanechère et al., 2000). The removal of the C-terminal tyrosine amino acid is catalyzed by an ill-defined TCP, whereas the re-addition of tyrosine is mediated by the tubulin tyrosine ligase enzyme (TTL), which is frequently suppressed in tumour cells, leading to the accumulation of Glu-tubulin in these cells (Barra et al., 1988; Lafanechère et al., 1998). It is postulated that inhibition of TCP might reverse Glu-tubulin accumulation in tumour cells and restore normal tyr-tubulin levels.

The  $\alpha$ -methylene- $\gamma$ -lactone moiety was indispensable for the inhibitory effect of parthenolide on TCP, which was not correlated to parthenolide inhibition of NF- $\kappa$ B pathway (Fonrose et al., 2007). Parthenolide inhibited TCP in MDA-MB-157, MDA-MB-436, and Bt-549 breast carcinoma cells; it decreased the pool of deetyrosinated tubulin without impairing the microtubule network (Whipple et al., 2013). As a consequence, parthenolide reduced microtentacle formation and tumour cell attachment (Whipple et al., 2013). Tang et al. showed that 16  $\mu$ M parthenolide inhibited U373 glioblastoma cells proliferation by causing a G<sub>2</sub>/M-phase arrest followed by apoptosis (Tang et al., 2015), but the mechanisms of its action were not elucidated and it was not reported if cells were in mitosis.

#### *Psilostachyin A and psilostachyin C*

These psilostachyins are sesquiterpene lactones isolated from the Asteraceae plant *Ambrosia artemisifolia* (Figure 2.3). They decreased the percentage of MCF-7 breast cancer cells arrested in the G<sub>2</sub> phase with a concomitant increase of mitotic cells, in an assay to detect compounds that relieve a DNA damage checkpoint (Sturgeon et al., 2005). Psilostachyin A and C (50  $\mu$ M) induced a 40% and 50% increase in the number of cells to override G<sub>2</sub> phase arrest, respectively. Cells that were not pretreated with a genotoxic agent (i.e. single treatments similar to those to detect other mitotic sesquiterpene lactones) also arrested in mitosis. Treated cells exhibited condensed chromosomes that failed to align, with mitotic spindles that were not properly organized. Microtubules in treated cells formed long and thick fibers, and no metaphase, anaphase or telophase arrangements were observed. Microtubule polymerization was not stimulated *in vitro* by psilostachyins A and C, suggesting that these sesquiterpene lactones do not target microtubules or tubulin. Furthermore, to determine which reactive group was responsible for the activity,

a mercapto-psilostachyin A derivative of the  $\alpha$ -lactone was synthesized. This new compound no longer inhibited mitosis, demonstrating a requirement for the  $\alpha,\beta$ -unsaturated carbonyl group for inhibitory activity. This is the first demonstration of a structure-activity relationship between the  $\alpha,\beta$ -unsaturated carbonyl group and mitotic arrest.

#### *$\alpha$ - and $\beta$ - Santalols*

$\alpha$ - and  $\beta$ - santalols are sesquiterpenes extracted from sandalwood oil, produced by the distillation of the heartwood of the *Santalum album* tree, of the Santalaceae family (Lee et al., 2015). These compounds lack the lactone moiety (Figure 2.3). Lee et al. found that these sesquiterpenes are toxic to seven different human head and neck squamous carcinomas cell lines (SCC-4, CAL 27, HSC-3, SCC-9, SCC-25, HN5, HSC-2 and HNSCC), and cause a G<sub>2</sub>/M-phase arrest at concentrations ranging from 7.7 to 45  $\mu$ M, which corresponded to a maximal G<sub>2</sub>/M arrest (Lee et al., 2015). Treated cells became rounded, arrested in mitosis and exhibited aberrant mitotic spindles (punctate, multipolar or monopolar spindles). These observations correlated with the ability of santalols to inhibit the polymerization of purified tubulin. In a turbidimetric assay, 50  $\mu$ M  $\alpha$ -santalol decreased polymerization of purified tubulin by 30% compared to 50  $\mu$ M  $\beta$ -santalol. Rhodium protein docking simulation program lead to the prediction that both chemicals bind tubulin at the colchicine site, with a low affinity (K<sub>i</sub> of 5.5 and 6.6  $\mu$ M, respectively). The authors concluded that  $\alpha$ - and  $\beta$ -santalols inhibit proliferation of several human head and neck squamous carcinomas with low potency, targeting tubulin polymerization and disrupting mitotic spindle formation (Lee et al., 2015). Another study by Zhang et al. reported that 24 h treatment of human A431 epidermoid carcinoma and UACC-62 melanoma cells with  $\alpha$ -santalol at concentrations above 50  $\mu$ M reduced cell

viability by 20-30% and caused progressive accumulation of cells in G<sub>2</sub>/M-phase (Zhang et al., 2010). An increase of expression of cyclin B occurred in the epidermoid carcinoma, whereas a downregulation of cyclin B and A in the melanoma cell line suggested the arrest was at the G<sub>2</sub> phase. Furthermore,  $\alpha$ -santalol inhibited microtubule polymerization in melanoma cells, indicating that the anti-mitotic activities of this sesquiterpene rely on its interaction with the microtubule network (Zhang et al., 2010). A third study by Santha et al. elucidated the effects of  $\alpha$ -santalol on MCF-7 and MDA-MB-231 breast cancer cell lines and reported that it caused a G<sub>2</sub>/M-phase arrest in both cell lines, which was associated with low cyclin A levels (Santha et al., 2013). However, the authors did not investigate further the anti-mitotic mechanisms (Santha et al., 2013).

#### *Xanthatin*

Xanthatin is a xanthanolide sesquiterpene lactone isolated from plants of the *Xanthanum* spp. (Asteraceae family). This compound does not have a 5-membered ring but contains an  $\alpha$ -methylene- $\gamma$ -lactone (Figure 2.3). Treatment of A549 non-small-cell lung cancer cells with 40  $\mu$ M xanthatin for 24 h induced accumulation of cells at the G<sub>2</sub>/M-phase. The arrest correlated with a dose-dependent reduction of Chk1, Chk2, and an increase in phosphorylation of Cdk1, suggesting a G<sub>2</sub> arrest, followed by apoptosis (Zhang et al., 2012). The authors did not provide a description of the target or the molecular pathway that was affected by xanthatin.

#### *Zerumbone*

Zerumbone (Figure 2.3) is a sesquiterpene isolated from the essential volatile oil of rhizomes from the edible wild ginger *Zingiber zerumbet* (Zingiberaceae family) (Rahman et al., 2014). It was reported that zerumbone did not affect normal human

peripheral blood mononuclear cells, but was cytotoxic to Jurkat leukemia cells. It arrested treated cells in the G<sub>2</sub>/M-phase of the cell cycle in a time (24, 48, and 72 h) and concentration (26, 42, and 57 μM) dependent manner, followed by apoptosis (Rahman et al., 2014). Chan et al. found that 30 μM zerumbone had anti-proliferative activity to PC-3 and DU-145 prostate cancer cell lines inducing a G<sub>2</sub>/M-phase arrest at 8 h, and increased the levels of PH3, cyclin B, and MPM2 expression, a mitotic marker (Chan et al., 2015). It was shown that zerumbone caused mitotic arrest by targeting tubulin/microtubules and disrupting microtubule dynamics, which led to the formation of aberrant monopolar and multipolar spindles (Chan et al., 2015). The authors described other effects upon cells treated with zerumbone, which included endoplasmic reticulum stress and mitochondria-mediated apoptosis. In addition to caspase-dependent apoptosis, zerumbone also induced autophagic cell death mediated by a decrease of LC3, a marker for autophagy (Tanida et al., 2008; Chan et al., 2015). Xian et al. reported that a 24 h treatment of NB4 leukemia cells with 10 μM zerumbone arrested them in the G<sub>2</sub> phase of the cell cycle, as characterized by phosphorylation of Cdk1 on Tyr15. This arrest was followed by apoptosis, due to loss of the mitochondrial membrane potential (Xian et al., 2007). When tested on Caov-3 ovarian cancer cells, zerumbone induced apoptosis and cycle arrest at G<sub>2</sub>/M-phase in a concentration-dependent manner (from 4 to 45 μM) (Abdelwahab et al., 2012).

## **2.8 Conclusions**

In the review of sesquiterpene molecules that affect human cells in assays *in vitro*, we highlighted those for which the data clearly demonstrated an arrest in the M-phase of the cell cycle. These data include evidence of molecular or cellular events such as

phosphorylation of histone H3 (PH3), condensed chromosomes, or a mitotic spindle (microtubules). These data could be supplemented with other data such as elevated levels of cyclin B, and non-Tyr15 phosphorylated Cdk1, which would be present in mitotic cells, but singly would be insufficient to determine if a cell is in mitosis or in G<sub>2</sub> phase. In some publications, measurements of 4N DNA amounts by flow cytometry that indicated a cell cycle arrest; however, without additional data, it was not possible to know if the arrest was in M-phase. The sesquiterpenes that arrest cells in mitosis includes 6-OAP, artesunate, coronopilin, costunolide, NP136, NP176, NP339, parthenolide, psilostachyins A and C,  $\alpha$ - and  $\beta$ - santalols, and zerumbone. A second group of sesquiterpenes caused G<sub>1</sub> or G<sub>2</sub>-phase arrest. This group includes 9 $\beta$ -acetoxycostunolide, dihydroartemisinin, dehydroleucodine, santamarine, and xanthatin.

The bio-molecules that were targeted by the sesquiterpenes include components that regulate the mitotic spindle or interact with tubulin directly, which caused changes in tubulin stability, or microtubule hyperpolymerization or cytokinesis defects (Figure 2.4). A second activity that caused mitotic arrest was linked to inhibition of the ubiquitin-proteasome pathway. In the majority of cases, the pathway leading to a deformed spindle was not identified. The precise mechanism of how sesquiterpenes cause mitotic arrest is not known, although there is a consensus that it might be linked to the metaphase-anaphase transition (Rundle et al., 2001; Sturgeon et al., 2005; Liu et al., 2011; Cotugno et al., 2012; Liu et al., 2015). As described by Liu et al. in their analysis of 6-OAP, these phenotypes could be caused by inhibition of regulatory steps required for proteolytic pathways (Liu et al., 2015).

A structural comparison of the compounds that induce mitotic arrest produces a complex picture because both sesquiterpene lactones and non-lactone sesquiterpenes are

present. The group comprised of sesquiterpene lactones includes: 6-OAP, coronopilin, costunolide, 9 $\beta$ -acetoxycostunolide, dehydroleucodine, NP136, NP176, NP339, parthenolide, psilostachyins A and C, santamarine, and xanthatin. The second group includes artesunate and dihydroartemisinin, the  $\alpha$ - and  $\beta$ - santalols, and zerumbone. It is hypothesized that the  $\alpha,\beta$ -unsaturated carbonyl group is responsible for the activity of sesquiterpenes, including the anti-mitotic activity and checkpoint abrogation activity (Rundle et al., 2001; Bocca et al., 2004; Sturgeon et al., 2005; Fonrose et al., 2007; Cotugno et al., 2012). The hypothesis is supported by the demonstration that modification of the  $\alpha,\beta$ -unsaturated carbonyl group by reaction with  $\beta$ -mercaptoethanol renders sesquiterpenes inactive in mitotic and checkpoint assays (Sturgeon et al., 2005). These data are further supported by mechanism of action studies in non-mitotic processes in which cysteines are covalently modified by a Michael addition reaction, rendering the polypeptide inactive.

Another compound, 13-hydroxy-15-oxozoapatlin (OZ), with a very similar structure to sesquiterpene lactones, exhibits similar anti-mitotic activities (Figure 2.5) (Rundle et al., 2001). This natural product is not a member of the sesquiterpene lactone class because it is an ent-kaurane diterpenoid. OZ was isolated from the bark of the South African tree *Parinari curatellifolia* from the Chrysobalanaceae family. Although OZ has a core structure different from the sesquiterpene lactones reviewed here, it has an  $\alpha,\beta$ -unsaturated carbonyl group that makes it reactive to nucleophiles and it has anti-mitotic properties upon treated cells. OZ treated cells exhibited atypical, disorganized mitotic spindles, although microtubule polymerization or depolymerization were not affected when tested in a purified system *in vitro*. Furthermore, OZ was rendered inactive after reaction with  $\beta$ -mercaptoethanol in a DNA damage checkpoint assay (Rundle et al.,

2001). These data further support the notion that the  $\alpha,\beta$ -unsaturated carbonyl group plays a key role in the mitotic arrest as well as other biological activities of these natural products.

Knowing that 90% of polypeptides in human cells have cysteines, one could assume that sesquiterpenes could react with nearly any protein. The presumed mechanism of action of generic cysteine modification is at odds with the specificity required to arrest a human cell in mitosis. A cell that enters mitosis requires the exquisite coordination of thousands of active proteins, including those involved in the most fundamental processes such as ribosome function to synthesize the cyclin required to initiate Cdk1 activity, and ATP synthesis for metabolism and phosphorylation events that characterize mitosis (such as phospho-histone H3). Yet the mitotic arrest phenotype described in the literature and observed by our laboratory indicates that a non-specific cysteine modification of proteins is insufficient to explain the action of sesquiterpenes. We predict that specific protein targets are inhibited by a subset of sesquiterpene molecules and this protein(s) is required for a mid to late event in mitosis, such as the metaphase-to-anaphase transition. Among the presumed targets of sesquiterpenes, tubulin and protein degradation pathways have been considered. Although these targets may not be exclusive, they would need to account for one observation common to all mitotic arrest phenotypes observed by sesquiterpene inhibition, the percentage of cells arrested in mitosis never arrives to near 100% as is the case with tubulin poisons such as nocodazole (Kubara et al., 2012).

We propose that some of the chemical biology of sesquiterpenes be reinvestigated. In our analysis of the literature, the most common biological activity was that of cytotoxicity, which would be expected from non-specific Michael-type additions. Tests in our laboratory led us to the observation that a subset of sesquiterpene molecules inhibit

mitosis, and this is a step that occurs before cell death (Swift et al., 2016; Swift et al., 2016). It is possible that the number of compounds with anti-mitotic activities amongst the 5000 described sesquiterpene molecules is underestimated. We predict that as natural compounds, some sesquiterpene molecules may have invaluable medicinal properties through their cell cycle arrest activities. Furthermore, investigation of the chemical biology of sesquiterpene lactones opens the possibility of gaining further insight into the process of mitosis.

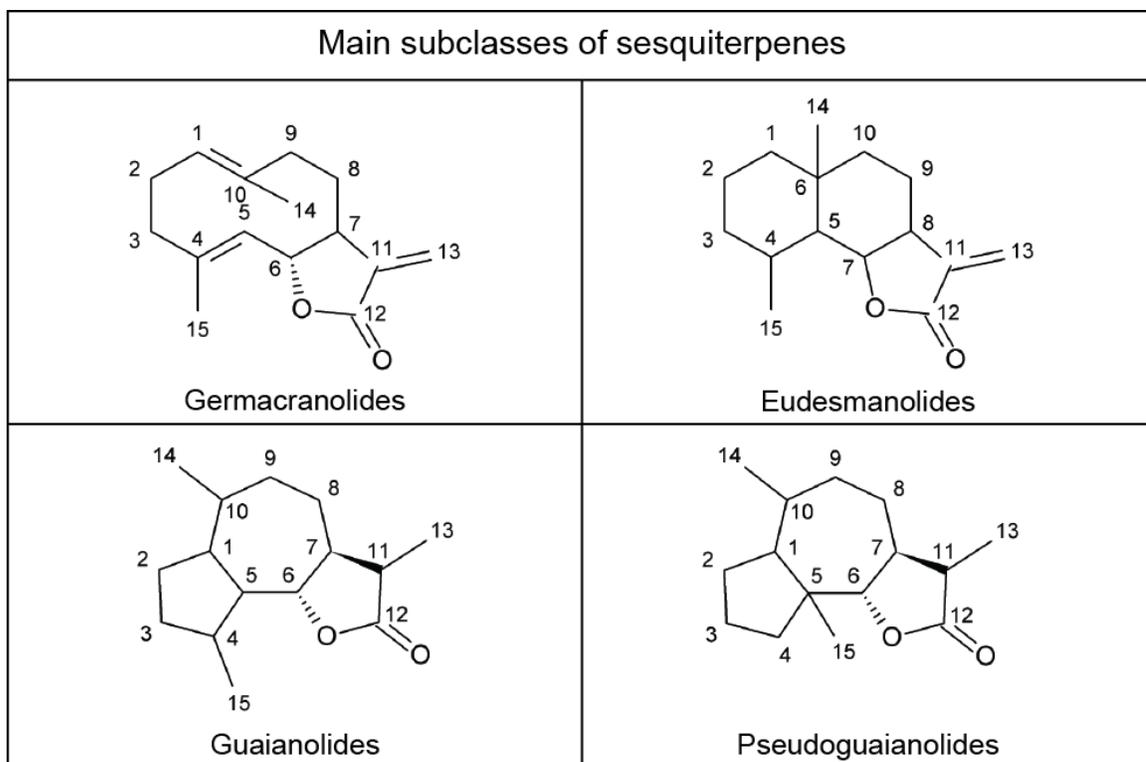


Figure 2.1: The chemical structures representing the main subclasses of sesquiterpenes. The positions of carbons are numbered.

Table 2.1: The names of plant taxonomical families that are known producers of sesquiterpene molecules. The common names and references are also provided.

Plant family	Common name	Reference
Acantaceae	Acanthus family	Picman, 1986
Anacardiaceae	Cashew family	Robles et al., 1995
Apiaceae	Celery family	Picman, 1986
Araceae	Aroids family	Chadwick et al., 2013 Canales et al., 2005
Asteraceae	Sunflower family	Chadwick et al., 2013 Heinrich et al., 1998
Cactaceae	Cactus family	Chadwick et al., 2013 Canales et al., 2005
Euphorbiaceae	Spurge family	Chadwick et al., 2013 Canales et al., 2005
Lauraceae	Laurel family	Picman, 1986
Magnoliaceae	Magnolia family	Picman, 1986
Menispermaceae	Moonseed family	Picman, 1986
Rutaceae	Citrus family	Robles et al., 1995
Solanaceae	Nightshades family	Chadwick et al., 2013 Canales et al., 2005
Winteraceae	Winter's Bark family	Picman, 1986

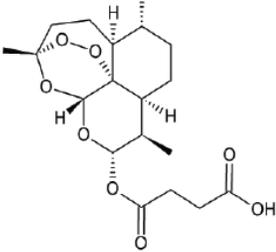
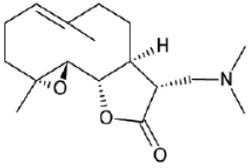
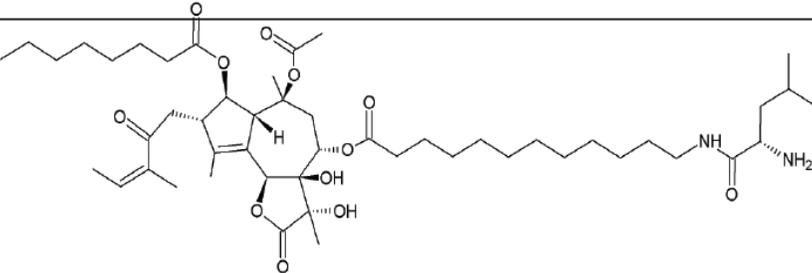
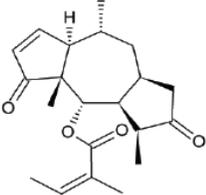
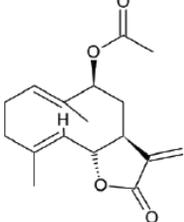
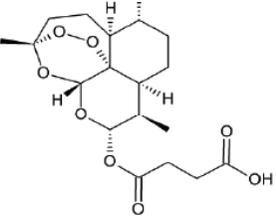
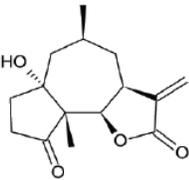
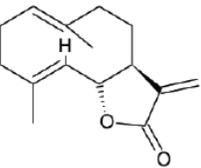
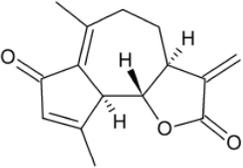
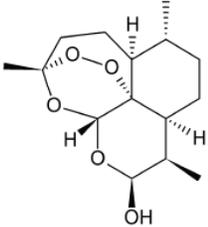
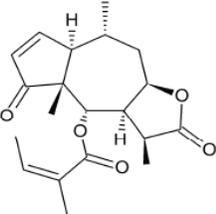
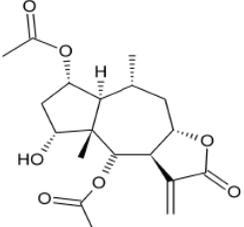
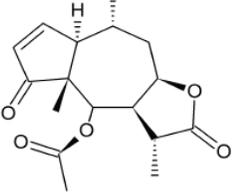
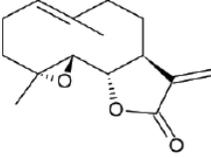
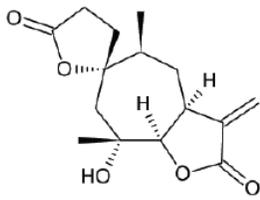
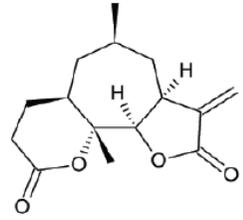
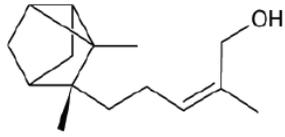
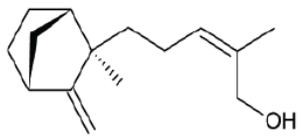
	<p><b>Artesunate</b></p> <ul style="list-style-type: none"> <li>• Semi-synthetic derivative of artemisinin.</li> <li>• Targets the iron group content by catalyzing the generation of free radicals from the bridged endoperoxide group.</li> <li>• Phase II clinical trials for cervical intraepithelial neoplasia, colorectal cancer, non-small cell lung cancer, metastatic uveal melanoma, and laryngeal squamous cell carcinoma.</li> <li>• References: Efferth et al., 2001; Krishna et al., 2016; Liu et al., 2013; NIH 2016; NIH 2016; Ren et al., 2016.</li> </ul>
	<p><b>Dimethylaminoparthenolide (LC-1)</b></p> <ul style="list-style-type: none"> <li>• Oral bioavailable analogue of parthenolide.</li> <li>• Inhibition of NF-<math>\kappa</math>B DNA binding and activation of p53 protein.</li> <li>• Phase I clinical trials against acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and other blood and lymph node cancers.</li> <li>• References: AdisInsight 2012; Ghantous et al., 2010; Guzman et al., 2007; Ren et al., 2016.</li> </ul>
	<p><b>L12ADT (8-O-(12-{L-leucinoylamino}dodecanoyl)-8-O-debutanoyl-thapsigargin)</b></p> <ul style="list-style-type: none"> <li>• Semi-synthetic derivative of thapsigargin.</li> <li>• Causes apoptosis via depletion of androgen receptor (AR) protein by inhibition of protein synthesis, and via inhibition of sarco/endoplasmic reticulum (ER) calcium ATPase (SERCA) pump.</li> <li>• Phase I clinical trials for refractory, advanced or metastatic solid tumours, and phase II clinical trials for glioblastoma.</li> <li>• References: Doan et al., 2015; Mahalingam et al., 2016; NCI 2016; Vander et al., 2009.</li> </ul>

Figure 2.2: Sesquiterpene lactones that are in clinical trials as anti-cancer drugs. The figure shows the structures of the three compounds (artesianate, LC-1, and L12ADT), the sources, the mechanisms of action and references.

Compound	Anti-mitotic activity	Reference(s)
 <p><b>6-O-angeloylplenolin</b> Isolated from <i>Centipeda minima</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• Prometaphase arrest, activation of the Cdk1 dimer and increase of PH3 levels.</li> <li>• Activation of the spindle assembly checkpoint, failed activation of APC/C and decrease of ubiquitinated cyclin B levels.</li> <li>• Binding at the Skp1-Skp2 interface and inhibition of the SCF-NIPA complex.</li> </ul>	<p>Cheng et al., 2017 Liu et al., 2011 Liu et al., 2015</p>
 <p><b>9β-acetoxycostunolide</b> Isolated from <i>Cyathocline purpurea</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• G2/M phase arrest (not specified if it is a G2 or an M arrest).</li> </ul>	<p>Ma et al., 2009</p>
 <p><b>Artesunate</b> Derivative of artemisinin, isolated from <i>Artemisia annua</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• G2/M phase arrest.</li> <li>• Presence of multiple centrosomes, multiple spindle poles and multinucleated cells.</li> <li>• Cell cycle arrest caused by a defect in cytokinesis.</li> </ul>	<p>Luo et al., 2013 Steinbruck et al., 2010</p>
 <p><b>Coronopilin</b> Isolated from <i>Ambrosia arborescens</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• Mitotic arrest.</li> <li>• Sustained levels of cyclin B and PH3, suggesting metaphase arrest.</li> <li>• Covalent interaction with tubulin nucleophilic groups, causing hyperpolymerization of tubulin.</li> </ul>	<p>Cotugno et al., 2012</p>
 <p><b>Costunolide</b> Isolated from <i>Michelia compressa</i> (Magnoliaceae)</p>	<ul style="list-style-type: none"> <li>• Inhibition of tubulin carboxypeptidase activity and restoration of normal levels of tyr-tubulin.</li> <li>• Mitotic arrest followed by increased phosphorylation of PH3.</li> <li>• Metaphase arrest and formation of short and dense microtubule fibers.</li> </ul>	<p>Bocca et al., 2004 Liu et al., 2011 Rasul et al., 2013 Whipple et al., 2013</p>

Compound	Anti-mitotic activity	Reference(s)
 <p><b>Dehydroleucodine</b> Isolated from <i>Artemisia douglasiana</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• Delay in mitotic entry and an increased duration of the mitotic phase.</li> <li>• Upregulation of PH3.</li> <li>• Temporary mitotic arrest and final accumulation of cells in a G1 phase senescence.</li> </ul>	<p>Costantino et al., 2013</p>
 <p><b>Dihydroartemisinin</b> Derivative of artemisinin, isolated from <i>Artemisia annua</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• G2/M phase arrest (not specified if it is a G2 or an M phase arrest).</li> <li>• Increased levels of cyclin B and decreased levels of Wee1.</li> </ul>	<p>Luo et al., 2013</p>
 <p><b>NP136</b> Natural product isolated from an unknown source</p>	<ul style="list-style-type: none"> <li>• Underduplication of centrioles.</li> <li>• Formation of monopolar spindles.</li> <li>• Aberrant chromosome segregation.</li> </ul>	<p>Graciotti et al., 2016</p>
 <p><b>NP176</b> Natural product isolated from an unknown source</p>	<ul style="list-style-type: none"> <li>• Underduplication of centrioles.</li> <li>• Formation of monopolar spindles.</li> <li>• Aberrant chromosome segregation.</li> </ul>	<p>Graciotti et al., 2016</p>
 <p><b>NP339</b> Natural product isolated from an unknown source</p>	<ul style="list-style-type: none"> <li>• Underduplication of centrioles, formation of monopolar spindles, aberrant chromosome segregation.</li> <li>• Does not affect a non-transformed cell line.</li> <li>• Impairs centriole formation by modulating NF-κB signaling.</li> </ul>	<p>Graciotti et al., 2016</p>

Compound	Anti-mitotic activity	Reference(s)
 <p><b>Parthenolide</b> Isolated from <i>Tanacetum parthenium</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• M phase arrest.</li> <li>• Inhibition of tubulin carboxypeptidase.</li> <li>• Decrease of the pool of deetyrosinated tubulin and stabilization of microtubules.</li> <li>• Reduction of microtentacle formation and tumour cell attachment.</li> </ul>	<p>Fonrose et al., 2007</p> <p>Tang et al., 2015</p> <p>Whipple et al., 2013</p>
 <p><b>Psilostachyin A</b> Isolated from <i>Ambrosia artemisiifolia</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• Prometaphase-like arrest.</li> <li>• Condensed chromosomes not properly aligned.</li> <li>• Polymerization <i>in vitro</i> of purified tubulin was not affected.</li> <li>• Disorganized mitotic spindles.</li> <li>• Mercaptoethanol-psilostachyin A does not cause a prometaphase-like arrest.</li> </ul>	<p>Sturgeon et al., 2005</p>
 <p><b>Psilostachyin C</b> Isolated from <i>Ambrosia artemisiifolia</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• Mitotic arrest at a prometaphase-like stage.</li> <li>• Condensed chromosomes not properly aligned.</li> <li>• <i>In vitro</i> polymerization of purified tubulin was not affected.</li> <li>• Disorganized mitotic spindles.</li> </ul>	<p>Sturgeon et al., 2005</p>
 <p><b><math>\alpha</math>-santalol</b> Isolated from <i>Santalum album</i> (Santalaceae)</p>	<ul style="list-style-type: none"> <li>• G2/M phase arrest.</li> <li>• Formation of aberrant mitotic spindles (punctate, multipolar or monopolar).</li> <li>• Decreased polymerization of purified tubulin <i>in vitro</i>.</li> <li>• Binding to the colchicine site on tubulin.</li> </ul>	<p>Lee et al., 2015</p> <p>Santha et al., 2013</p> <p>Zhang et al., 2010</p>
 <p><b><math>\beta</math>-santalol</b> Isolated from <i>Santalum album</i> (Santalaceae)</p>	<ul style="list-style-type: none"> <li>• G2/M phase arrest.</li> <li>• Formation of aberrant mitotic spindles (punctate, multipolar or monopolar).</li> <li>• Decreased polymerization of purified tubulin <i>in vitro</i>.</li> <li>• Binding to the colchicine site on tubulin.</li> </ul>	<p>Lee et al., 2015</p>

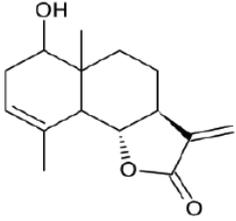
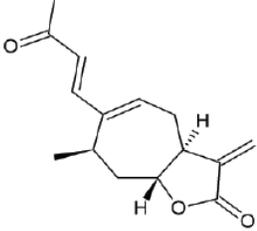
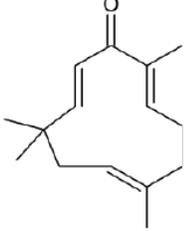
Compound	Anti-mitotic activity	Reference(s)
 <p><b>Santamarine</b> Isolated from <i>Cyathocline purpurea</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• G2/M phase arrest (not specified if it is a G2 or an M phase arrest).</li> </ul>	<p>Ma et al., 2009</p>
 <p><b>Xanthatin</b> Isolated from <i>Xanthium strumarium</i> (Asteraceae)</p>	<ul style="list-style-type: none"> <li>• Accumulation of cells in G2 phase.</li> <li>• Phosphorylation of Cdk1.</li> </ul>	<p>Zhang et al., 2012</p>
 <p><b>Zerumbone</b> Isolated from <i>Zingiber zerumbet</i> (Zingiberaceae)</p>	<ul style="list-style-type: none"> <li>• Mitotic arrest characterized by increased levels of PH3, cyclin B1, MPM2 expression.</li> <li>• Disruption of microtubule dynamics and formation of aberrant monopolar and multipolar spindles.</li> </ul>	<p>Abdelwahab et al., 2012 Chan et al., 2015 Rahman et al., 2014 Xian et al., 2007</p>

Figure 2.3: The names and structures of sesquiterpenes that have cell cycle arrest or mitotic activity.

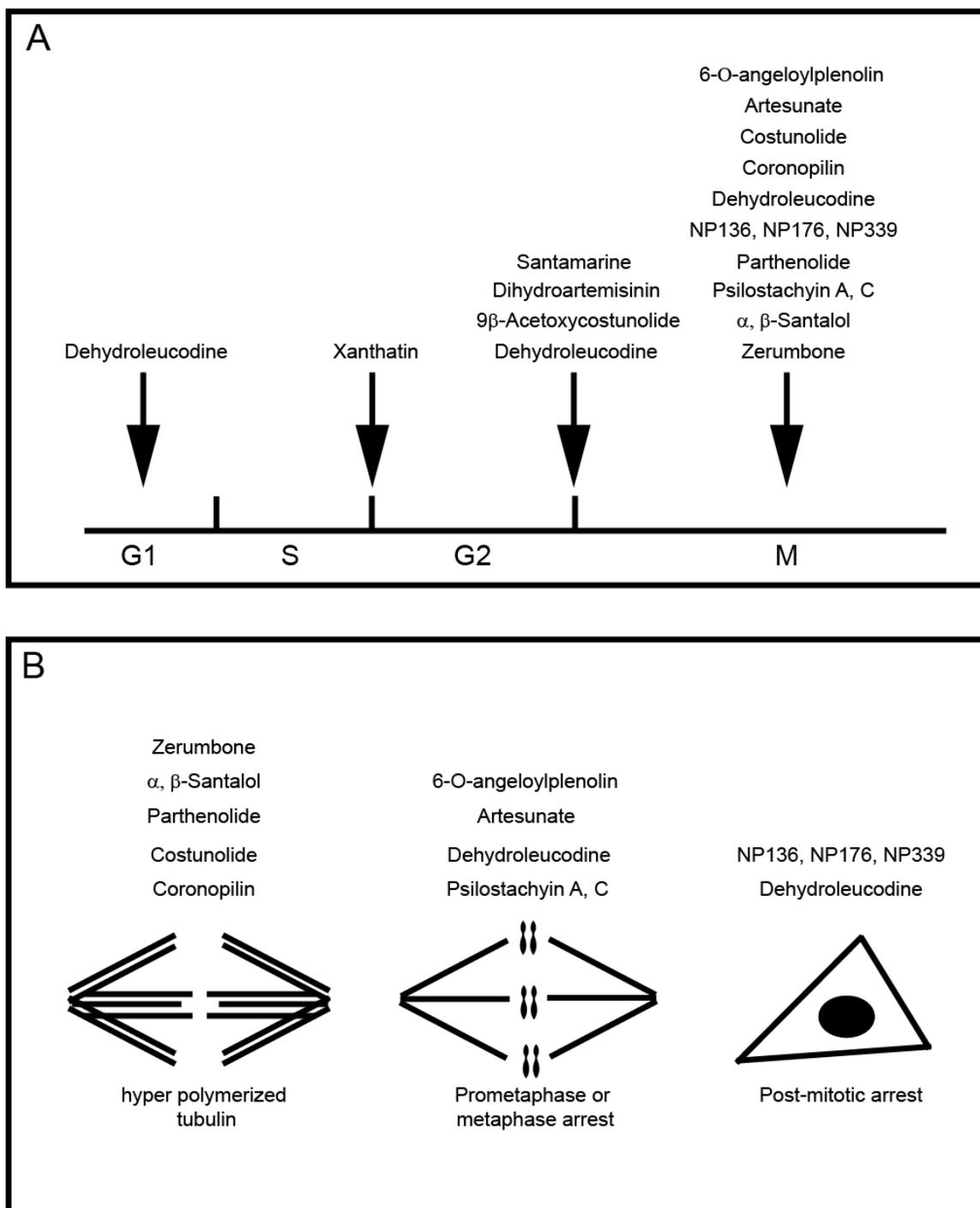


Figure 2.4: Cell cycle arrests and mechanism of the anti-mitotic activity of sesquiterpenes. A) The position of the cell cycle arrest relative to the phases of the cell cycle are given for the compounds. B) The types of mitotic and post mitotic arrests are given for the compounds.

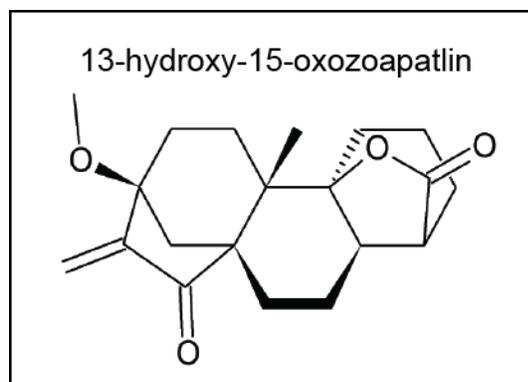


Figure 2.5: Structure of the ent-kaurane 13-hydroxy-15-oxozoapatlin. This natural product has an  $\alpha,\beta$ -unsaturated carbonyl group, and has an anti-mitotic activity similar to some of the sesquiterpene lactones reviewed here.

## CHAPTER 3

### **Investigation of the properties of *Gaillardia aristata* extracts on two cancerous cell lines and one non-cancerous cell line.**

#### **3.1 Abstract**

The prairie plant species *Gaillardia aristata* was selected for investigation of its effects upon human cells. It is resistant to herbivory by grazing mammals, reported to be cytotoxic, recognized as a medicinal plant in traditional knowledge, and present in sustainable quantities in the prairie ecological zone. The precise effects of the chemicals from this plant upon human (or any) cells had not been described, nor was it likely that all secondary molecules had been identified. In this chapter, we describe a new anti-mitotic activity from *Gaillardia aristata* extracts.

#### **3.2 Introduction**

Cancer is the number one cause of death in Canada (C.C.S., 2015) and it is among the leading causes of morbidity and mortality worldwide (W.H.O., 2015). Moreover, cancer incidence is expected to increase by 70% in the next two decades (W.H.O., 2015). Although therapies are available, conventional therapeutic and surgical approaches have not been able to control effectively the incidence or cure many types of cancer. Despite the efforts made to prevent the disease and to improve the outcome, cancer still is a major disease in Western nations. It is clear that cancer is an unmet medical need, therefore there is a high demand for new and effective therapies.

One strategy to address this challenge is to discover new chemotherapeutic drugs. Among the chemotherapeutics that are currently available, 67% of anticancer drugs are natural products or natural product derivatives (Balunas et al., 2005; Newman et al., 2016). Historically, plants with ethnopharmacological uses have been the primary sources of medicines for early drug discovery (Chin et al., 2006). In 1960 the National Cancer Institute launched a large-scale screening program for anti-tumour agents, which evaluated 35000 plant samples. A very successful example that emerged from this evaluation was Paclitaxel (Taxol<sup>®</sup>), the most effective anti-cancer drug (Wani et al., 1971; Weaver, 2014).

Despite the extensive studies on plants, of the estimated 250,000 to 500,000 existing plant species only a small percentage have been tested for therapeutic effects (Hostettmann et al., 2002; Miller, 2011; Ngo et al., 2013). One reason could be that the majority of the world's plant species are not common, and they have restricted geographical distribution (Miller, 2011). Thus, rare plants with medical potential are less likely to be discovered if they grow in a remote area, and it is also less likely that they are used if the access is restricted. This suggests that many new plant species and their compounds are yet to be discovered.

Few plants from northern ecological zones, such as Canadian prairies, have been analysed, although there are examples in which some produce secondary metabolites with interesting biological properties (Uprety et al., 2012). In particular, it was reported that some metabolites found in prairie plants cause intoxication in grazing domestic animals, and poisoning in human beings following ingestion (Keeler et al., 1990; McGrath-Hill et al., 1997).

An ethnomedical approach is beneficial in selecting plants for investigation of their biological properties. The selection of plants is partly based upon oral or written information on their medicinal use from traditional medical systems (Cos et al., 2006). Interestingly, 70-80% of people worldwide rely on traditional herbal medicine to meet their primary health care needs (Farnsworth et al., 1991; W.H.O., 2002). Canada in particular has some secondary records of the use of medicinal plants by aboriginal people (Hellson et al., 1974; Lafranière et al., 1997). These records are scattered and there is not any systematic study available, since most of the First Nations' knowledge is poorly documented and it is based mostly on oral tradition. It is likely, however, that plants have served as a source of medicines for aboriginal people in Canada for millennia. The prairie ecological zones offer a wide range of plants that have been little investigated by scientific methods, especially for anti-cancer compounds.

We therefore decided to investigate native plants of the prairie ecological zone. The selection of plants was based on several criteria: the plant is not widely distributed in other ecological zones and flourishes in the prairie ecological zone in southern Alberta; there is little scientific information available from peer-reviewed literature on its secondary metabolites; there is possible anecdotal evidence of toxicity.

There are 1775 known vascular plant species (among which are ferns, conifers, and flowering plants) that are native to Alberta, or that were imported and now have become established and grow wild in the province (Moss et al., 1983). We chose to investigate the plant *Gaillardia aristata* for anti-cancer compounds for the following reasons: there is previous knowledge of its toxic effects upon cancer cells, but the biological mechanisms behind its action remain unknown (Salama et al., 2012); it is a medicinal plant in the Blackfoot and Plateau tribes culture for its beneficial effects against

skin conditions, wounds and fever (Hellsen et al., 1974; Hunn et al., 1990; Moerman, 2009). Furthermore, evidence was reported that direct contact with *G. aristata* leaves can cause contact dermatitis (Burry, 1980), and there is anecdotal evidence that *G. aristata* is not consumed by grazing herbivores (Pahl et al., 1999). These features suggest that this species likely produces chemicals with interesting biological properties.

*G. aristata* is one of the 23 species that belong to the Asteraceae family (sunflower family) and it is a perennial herbaceous plant native to most of the prairie ecological zone in North America. It grows to a height of 70 cm, and blooms from mid-summer to early fall. This plant is characterized by lance-shaped leaves situated at the base, long stems and large flower heads with a brown center and yellow or red petals (Kuijt, 1982).

In this chapter we investigate the effects of *Gaillardia aristata* upon two human cancer cell lines, HT-29 (colorectal adenocarcinoma) and M059K (glioblastoma), and on a human non-cancerous cell line, WI-38 (lung fibroblast). It is useful to test the extracts on a non-cancerous cell line to determine whether their effects, if any, are selective on cancer cells or affect common pathways. The HT-29 cell line is colorectal cancer, which is one of the most common types of cancer in Canada, and its incidence is expected to increase in the next 15 years; it is also associated with one of the highest cancer mortality rates (C.C.S., 2015). Additionally, HT-29 cells undergo striking morphology changes in response to different conditions (Cohen et al., 1999). These cells in particular exhibit a pronounced rounded morphology when in mitosis (Gascoigne et al., 2008; Kubara et al., 2012). M059K cells represent glioblastoma, which is extremely difficult to treat. Gliomas account for the majority of malignant brain tumours in adults (Goodenberger et al., 2012). A successful way to investigate the bio-activities of plant extracts is by phenotypic

assays. These are unbiased assays and are more likely to discover novel compounds or novel mechanisms of action than a targeted approach, because they do not require prior knowledge of the molecular mechanism of action. Phenotypic screens also involve nearly all targets, take bioavailability phenomena into account and are successful in detecting synergism between different molecules (Verpoorte et al., 2005; Cos et al., 2006).

Of the multitude of phenotypic assays available, we focus on those that represent the hallmarks of cancer (Hanahan et al., 2011). The hallmarks of cancer described by Hanahan and Weinberg are ten alterations in cell physiology that cause and promote malignant cell growth. Specifically, we focus on the three processes: enabling replicative immortality, resisting cell death, and genome instability and mutation. The application of this approach has had positive results in other studies, as demonstrated by Diederich et al. (Schumacher et al., 2011), who investigated compounds from marine organisms.

### **3.3 Materials and Methods**

#### *3.3.1 Collection of plants*

*Gaillardia aristata* aerial plant parts were collected at three different undisturbed sites, with the following GPS coordinates: 49°58'32.4"/ -114°05'21.5" (Porcupine Hills, at an altitude of 1683 meters, 01/08/2014), 49°40'49.2"/ -112°51'37.4" (University of Lethbridge campus, at 886 meters, 02/07/2015), and 49°57'14.3"/ -114°04'21.4" (Porcupine Hills, at 1683 meters, 08/07/2015). During each harvest the amount of wet plant collected was, respectively, 1.4 kg, 7.1 kg and 16 kg. The harvest of plants was undertaken by sustainable practice; one every two plants was collected when they were abundant, whereas only one in five was collected when they were scarce. Plant taxonomy was confirmed to species based on documents of the Flora of southern Alberta (Kuijt,

1982; Moss et al., 1983; G.P.F.A. et al., 1986), and verified by Dr. John Bain, the director of the University of Lethbridge Herbarium. Information including collection date, site description, associated species and location were stored with a voucher specimen in the Herbarium database. A “molecular” voucher consisting of frozen plant tissue was collected for DNA extractions to obtain the genomic sequence of *Gaillardia aristata*. Additional information on the genomic sequence of this species could be obtained using the “bar coding” method, which involves short orthologous DNA sequences, known as “DNA barcodes”.

Following the harvest, plants were dried (at room temperature for the first day, then in an oven at 40-50°C for three or four days) and the different parts were separated (flower heads, stems and leaves), catalogued and stored in paper bags in a dry environment at room temperature until extraction.

### 3.3.2 Preparation of plant extracts

Plant extracts were prepared by grinding dried material with a mortar and pestle or a blender to a fine powder. The powder was suspended to 10% (w/v), with either dichloromethane (DCM), 75% ethanol (EtOH) or water (H<sub>2</sub>O), and stirred. After overnight incubation at room temperature the suspension was filtered to collect the soluble fraction, which was dried with a roto evaporator, then placed in an oven at 40°C for eight hours. The dried material was collected, weighed, labeled, given a code number and stored in darkness at room temperature. To test the extract in biological assays, samples of dried material were dissolved in dimethyl sulfoxide (DMSO) to 100 mg/mL. Extracts suspended in DMSO were stored at -20°C in small aliquots to avoid freeze-thaw cycles.

### 3.3.3 Cell culture

The human cell lines HT-29 (ATCC HTB-38), M059K (ATCC CRL-2365) and WI-38 (ATCC CCL-75) were obtained from the American Type Culture Collection (ATCC). The colorectal carcinoma cell line HT-29 was maintained in RPMI 1640 medium (Gibco; 21870-092) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco; 12484028) and 1.6 mM GlutaMAX (Gibco; 35050-061). M059K glioblastoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Gibco; 11320-082) supplemented with 10% (v/v) heat inactivated FBS (Gibco; 12484028), 2 mM Modified Eagle Medium non-essential amino acids (Gibco; 11140050) and 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4. The human lung fibroblast cells WI-38 were maintained in DMEM/High glucose (Sigma; D6546) supplemented with 10% heat inactivated FBS (Gibco; 12484028), 2 mM Modified Eagle Medium non-essential amino acids (Gibco; 11140050), and 1.6 mM GlutaMAX (Gibco; 35050-061). Cells were grown at 37°C in 5% CO<sub>2</sub> and the media were changed every two-three days. HT-29 cells were plated at a density of 1.0 x 10<sup>6</sup> cells/75 cm<sup>2</sup> flask and cultured for 48 h prior to treatment. M059K and WI-38 cells were plated at 5.0 x 10<sup>5</sup> cells/75 cm<sup>2</sup> flask and cultured for 48 h prior to treatment. The compounds camptothecin (CPT) (Sigma; 7689-03-4) and nocodazole (Sigma; M1404-10MG) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D2438) to a concentration of 10 mM and 200 µg/mL respectively. The extracts were suspended in DMSO at 100 mg/mL and all extracts and other compounds listed were stored at -20°C. In not-treated (NT) cells DMSO was added to a final concentration of 0.1% (v/v) as a solvent vehicle control.

### 3.3.4 Cytotoxicity assay

The cytotoxicities of camptothecin and of *G. aristata* extracts on HT-29, M059K and WI-38 cells were measured by the MTT (3-((4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Sigma-Aldrich; M2128-1G). HT-29 cells were plated at  $5 \times 10^5$  cells/96 well culture plate and cultured at 37°C for 72 h prior to treatment. M059K and WI-38 cells were plated at  $2.5 \times 10^5$  cells/96 well culture plate and cultured at 37°C for 72 h prior to treatment. All treatments were run in triplicate at 96 h and experiments were performed three times. At the end of the 96 h, 20  $\mu$ L of MTT solution (5 mg/mL MTT in phosphate buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 18 mM KH<sub>2</sub>PO<sub>4</sub>) was added to the media in each well and the plates were incubated at 37°C for 3.5 h. The media were then aspirated and 100  $\mu$ L MTT solvent (4 mM HCl, 0.1% (v/v) IPEGAL (octylphenoxy polyethoxy ethanol), in isopropanol) was added to each well. Plates were placed on a shaker for 30 minutes in the dark, and absorbance was measured at 590 nm using a BioTek microplate spectrophotometer operated by Eon software. Results were expressed as IC<sub>50</sub> concentrations; the concentration of the compound that reduced the absorbance of MTT by 50%, by comparison to 0.1% (v/v) DMSO treated cells. The normalized percent absorbance was calculated as shown:

$$\text{Normalized percent absorbance} = (\text{absorbance/DMSO absorbance}) \times 100$$

The log concentrations of the compounds were plotted against the normalized percent absorbances using Microsoft Excel software. Analysis was performed with GraphPad Prism 5 software, using non-linear regression (log(inhibitor) versus normalized response), to estimate the IC<sub>50</sub> concentrations. Standard curves were plotted using the equation:

$$Y = \text{maximum} + (\text{maximum} - \text{minimum}) / (1 + 10^{(x - \text{LogIC}_{50})})$$

where maximum is the percentage of viable cells after treatment with 0.1% DMSO, minimum is the percentage of viable cells after treatment with the highest concentration of the genotoxic molecule and x is the log<sub>10</sub> value of the treatment concentration.

### *3.3.5 Light microscopy*

HT-29 cells were seeded at  $1.0 \times 10^5$ /well in a 6 well culture plate and incubated at 37°C for 48 h prior to treatment. M059K and WI-38 cells were seeded at  $5 \times 10^4$ /well in a 6 well culture plate and incubated at 37°C for 48 h prior to treatment. Images were captured at room temperature with an Infinity 1 camera operated by Infinity Capture imaging software (Lumenera Corporation) on an Olympus CKX41 inverted microscope using an Olympus LUCPlanFLN 20x objective with 0.45 numerical aperture. Images were processed using Adobe Photoshop (CC 2014.1.0). Rounded cells were counted using Image J software (IJ 1.46r). All experiments were performed three times, unless specified otherwise.

### *3.3.6 Time-lapse video microscopy*

HT-29 cells were plated at  $3.0 \times 10^5$ /25 cm<sup>2</sup> flask and incubated at 37°C for 48 h prior to treatment. Time-lapse video microscopy images were collected from the start of treatment, using a Lumascope 500 microscope (etaluma) powered by LumaView software (etaluma; V.13.4.25.99). Images were captured at 37°C every ten minutes for 96 h using a Meiji Techno Japan UPlan 20x objective with 0.40 numerical aperture. Cells were manually scored for a rounded morphology, indicative of mitosis, between 0 and 96 h. At least 200 HT-29 cells were counted for each treatment. Experiments were performed three times.

### 3.3.7 Flow cytometry

HT-29 cells were plated at  $1 \times 10^6$  cells/75 cm<sup>2</sup> flask and incubated at 37°C for 48 h prior to treatment. Total cell cultures were collected by trypsinisation. Cells were washed in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 18 mM KH<sub>2</sub>PO<sub>4</sub>) and fixed in 70% ethanol (2208C) for at least 24 h. Samples were incubated for 20 minutes in wash buffer with 0.02 mg/mL propidium iodide (Invitrogen) and analysed by a FACS Canto II flow cytometer (BD Biosciences) using BD FACSDiva (BD Biosciences) software. Gating was set using control samples of cells either not-treated or treated with 50 nM CPT (Kubara et al., 2012). Experiments were repeated twice and the average values are provided.

### 3.3.8 Statistics

Data and statistical analysis were performed using Microsoft Excel 2010 software. Data were plotted as means from three separate experiments  $\pm$  standard errors of the means. Statistical significance was calculated using the student's t-test for two paired sample means and values were considered significantly different when  $p < 0.05$ . GraphPad Prism 5 software was used to estimate IC<sub>50</sub> concentrations.

## 3.4 Results

### 3.4.1 Collection of *Gaillardia aristata*

Prior to my joining the laboratory, a first test collection of *G. aristata* had been made in summer 2014, in an area located on the Porcupine hills with the following geographic coordinates: 49°58'32.4"/ -114°05'21.5". Extracts from this collection had been prepared and I confirmed that they were cytotoxic in morphology assays. Although I

had sufficient material for the initial experiments described here, I helped organize the second and third major collections in summer 2015. *G. aristata* aerial parts (flower heads, stems and leaves) were collected at two different undisturbed sites (not subjected to cultivation, pesticide or herbicide treatment), with the following GPS coordinates: 49°40'49.2"/ -112°51'37.4" (University of Lethbridge campus on 02/07/2015), and 49°57'14.3"/ -114°04'21.4" (Porcupine Hills on 08/07/2015). All experiments included in this thesis were performed using extracts prepared from aerial plant parts of *Gaillardia aristata* collected during summer 2014. We have tested extracts of *G. aristata* harvested during summer 2015 and determined that the effects of all extracts were similar when tested on the HT-29 cell line, indicating that the plant likely produces similar groups of compounds, regardless of the different locations where it grows. Plants were confirmed to species as described in section 3.3.1, processed and stored until extraction.

#### 3.4.2 Preparation of extracts

Extracts were then prepared as described in section 3.3.2 from the aerial parts of *G. aristata* harvested at the three different sites (Figure 3.2). To do so, we first prepared 10% (w/v) extracts from the leaves (Figure 3.1) using one of three solvents, dichloromethane, 75% ethanol, or water. Dichloromethane favours the extraction of non-polar compounds, whereas ethanol favours the extraction of polar molecules (Verpoorte et al., 2005; Cos et al., 2006). We also used water as a solvent because it dissolves a wide range of polar molecules and could be more effective in extracting substances that would otherwise be lost during extractions with the other selected solvents. These three extracts, PP-050 (prepared with DCM), PP-051AB (prepared with 75% ethanol) and PP-053AB (prepared with water) were made from the leaves of *G. aristata* that were collected during the first harvest (Table 3.1). By a first morphology test for cell rounding we observed that

the dichloromethane extract was more potent than the ethanol extract (see section 3.4.4), and that the water extract did not show any activity. These findings were later confirmed when the cytotoxicity test was performed (see section 3.4.3). Therefore, we proceeded in preparing all the extracts using dichloromethane as solvent. Later, two other extracts, named PP-180 and PP-190, were prepared from *G. aristata* leaves collected at, respectively, the second and third harvest. A stems and a flower heads extracts were also prepared from *G. aristata* collected on the first harvest, and were named PP-182A and PP-191A.

#### *3.4.3 Extracts from G. aristata aerial parts are cytotoxic to HT-29 cell line*

With adequate supplies of *G. aristata* plant parts and extracts, we then set to investigate the bioactivity of extracts upon human cells. We tested the cytotoxicity of leaf extracts PP-050 (DCM) and PP-051AB (EtOH) upon HT-29 cells. Cells were treated with 0.3 to 1000  $\mu\text{g}/\text{mL}$  concentrations of either extract, and cell viability was measured by MTT assay after 96 h.  $\text{IC}_{50}$  values are shown in Table 3.2A. The  $\text{IC}_{50}$  value for the dichloromethane extract was  $44.4 \pm 1.3 \mu\text{g}/\text{mL}$ , and for the ethanolic extract it was  $139.6 \pm 13.9 \mu\text{g}/\text{mL}$ . Camptothecin was used as a positive control for cytotoxicity and the  $\text{IC}_{50}$  value on HT-29 cells was, as expected,  $26.0 \pm 1.4 \text{ nM}$ . Although both leaves extracts were cytotoxic, the extraction prepared using dichloromethane was approximately three fold more toxic.

We then compared the cytotoxic effects of the aerial plant parts (leaves, stems and flower heads). Cell viability at 96 h after treatment by the three extracts was measured by MTT assay on HT-29 cells. The  $\text{IC}_{50}$  for the stems and flower heads extracts were  $312.4 \pm 23.9$  and  $48.2 \pm 3.6$  (Table 3.2B), respectively, suggesting that the most cytotoxic extract

was the one prepared from the leaves. The IC<sub>50</sub> value for the positive control CPT was 24.0 ± 0.8 nM.

#### 3.4.4 Characterization of morphological changes induced after treatment with *G. aristata* extracts

Before I joined this project, the laboratory had observed that test samples of extracts of *G. aristata* induced a change in cell morphology. In a first set of experiments, we sought to characterize this change in morphology and compare the morphology of leaves extracts (L.E.) prepared using three different solvents such as dichloromethane, 75% ethanol or water. HT-29 cells were treated with either DMSO, used as a negative control, or with 200 ng/mL of nocodazole, a tubulin toxin that induces cell rounding by a mitotic arrest (Vasquez et al., 1997). In addition, cells were treated with 50 µg/mL of L.E. prepared with one of the three solvents. Images were taken at 24 h (Figure 3.3A). Cells treated with DMSO had a normal morphology with most cells in a flattened shape and a small number of rounded cells (as pointed by the arrow). By contrast, nearly all cells treated by nocodazole were rounded (arrow). Interestingly, leaf extracts prepared with either dichloromethane or 75% ethanol induced rounded cells, whereas the water extraction did not. The frequency of rounded cells in cultures treated with DMSO was 3.0 ± 0.3%, and it was 98.3 ± 1.9% for nocodazole, as expected. The percentage of rounded cells accumulated after treatment with the dichloromethane, ethanolic and water extracts at 50 µg/mL each were 34.0 ± 0.4%, 12.8 ± 2.3% and 3.5 ± 0.3%, respectively (Figure 3.3B). This result showed that the most pronounced effect was caused by treatment with the dichloromethane extract. Therefore, we pursued the investigation of *Gaillardia aristata* extracts using dichloromethane as solvent.

We then investigated which plant part harbours the chemical(s) that induced cell rounding. HT-29 cells were treated with DMSO only, 50  $\mu\text{g}/\text{mL}$  dichloromethane extracts from leaves (L.E.), stems (S.E.) and flower heads (F.E.), and 200  $\text{ng}/\text{mL}$  nocodazole as a positive control. Images were taken at 24 h post treatment (Figure 3.4A), and the percentage of rounded cells was counted (Figure 3.4B). Consistent with the previous experiment, DMSO and nocodazole induced  $2.8 \pm 0.7\%$  and  $98.0 \pm 1.7\%$ , respectively, of rounded cells. Of the plant parts, the highest percentage of rounded cells accumulated after treatment with the L.E. ( $35.3 \pm 1.5\%$ ), whereas the S.E. and F.E. were less potent ( $7.0 \pm 1.3\%$  and  $16.0 \pm 1.9\%$  respectively) (Figure 3.4B). We noted that although leaf and flower head extracts had similar cytotoxicities by the MTT assay (Table 3.2B), the leaf extract (L.E.) was more active in the morphology assay, and that dichloromethane extracted a greater amount of this activity, when compared to the 75% ethanol extract.

Both the L.E. extract and nocodazole induced a rounded morphology when added to HT-29 cells, however, the percentage of rounded cells by 24 h was different between the two treatments. We investigated the two rounding activities, and that of DMSO treatment by time-lapse video microscopy every hour for 24 h (Figure 3.5). While the percentage of rounded cells in the DMSO population changed little during the experiment (ranging from  $1.2 \pm 0.4\%$  to  $3.7 \pm 0.4\%$ ), L.E. and nocodazole treated cells (Figure 3.5A) accumulated rounded cells, albeit different numbers, reaching a maximum at 24 h of, respectively,  $35.3 \pm 0.3\%$  and  $99.0 \pm 0.3\%$  (Figure 3.5B). Although the L.E. caused a morphological change similar to that induced by nocodazole, it did so at a different rate, suggesting that cells might be arresting in mitosis, but by different mechanisms, as suggested by the significantly different percentages of rounded cells at 24 h post treatment. The rounded cell phenomenon is of great interest as it could be indicative of

mitosis (Cadart et al., 2014). We decided to investigate it further by first confirming that the rounded phenotype occurred in other cell lines.

#### 3.4.5 Effect of *G. aristata* extracts upon WI-38 and M059K cell lines

We tested the *G. aristata* extracts on a human non-cancerous cell line, WI-38 (lung fibroblasts), and on a second cancer cell line, M059K (glioblastoma). WI-38 and M059K cells were treated with different concentrations of L.E., S.E., and F.E., and CPT was used as a positive control, and cell viability was measured by MTT assay at 96 h. The IC<sub>50</sub> values for L.E., S.E., F.E., and CPT were, respectively,  $44.4 \pm 5.5$   $\mu\text{g/mL}$ ,  $258.8 \pm 12.2$   $\mu\text{g/mL}$ ,  $28.6 \pm 3.6$   $\mu\text{g/mL}$  and  $368.7 \pm 56.3$  nM upon WI-38 and  $13.3 \pm 1.3$   $\mu\text{g/mL}$ ,  $81.2 \pm 8.3$   $\mu\text{g/mL}$ ,  $19.5 \pm 0.4$   $\mu\text{g/mL}$  and  $4.4 \pm 0.4$  nM upon M059K cells (Table 3.2B). This indicated that all extracts, with different potencies, were cytotoxic upon both cell lines, but M059K cells were more sensitive to all three extracts when compared to WI-38 and to HT-29 (see section 3.4.3).

Overall, the L.E. was the most cytotoxic extract and we further tested it for morphological changes on the selected cell lines. WI-38 and M059K cells were incubated with DMSO, with 50  $\mu\text{g/mL}$  L.E., and 200 ng/mL nocodazole and photos were taken after 24 h (Figure 3.6A and 3.6B). As shown in Figure 3.6B, not treated WI-38 cells had few rounded cells, and the number did not increase after treatment with L.E., whereas nocodazole treatment caused a higher number of cells to become rounded. On the contrary, as shown in Figure 3.6A, L.E. treated M059K cells acquired a rounded morphology, and the effect was not as pronounced as that of nocodazole treatment. These data revealed two important observations: first, that L.E. cell rounding activity upon M059K cells is similar to that seen with HT-29 cells, and that the normal cell line did not undergo this morphology change.

### 3.4.6 HT-29 cells treated with a leaf extract (L.E.) arrest at the S- and G<sub>2</sub>/M phases of the cell cycle

It was previously reported that *Gaillardia aristata* is cytotoxic upon different cancer cell lines (Salama et al., 2012), and we confirmed it in our experiments (see section 3.4.3). However, the mechanisms behind its cytotoxicity were unknown. Because the phenomenon of the morphology change had not been previously described and it might be caused by a mitotic arrest (dividing cells normally acquire a spherical shape) (Cadart et al., 2014), we decided to investigate it.

We started by using the technique of flow cytometry to measure DNA content in cells. Cells were treated for 24 h with DMSO only, 50 µg/mL L.E. and 50 nM CPT as a positive control and their DNA content was analysed (Figure 3.7). As shown in Figure 3.7A and 3.7D, most of the DMSO treated cells (62.3%) were in G<sub>1</sub> phase of the cell cycle. Cells treated with CPT, as expected, were predominantly in S phase (56.2%), whereas 12.8% and 29.1% were in G<sub>1</sub> and G<sub>2</sub>/M phases, respectively (Figure 3.7C and 3.7D). By contrast, the L.E. treated cell population displayed a distinct DNA profile, with only 14.7% of the cells in G<sub>1</sub> phase, whereas 50.6% were in S phase and 33.5% were in G<sub>2</sub>/M-phase (Figure 3.7B and 3.7D). Therefore, L.E. treatment caused S- and G<sub>2</sub>/M-phase arrests. Furthermore, the percentage of cells in G<sub>2</sub>/M-phase was consistent with the light microscopy data, supporting the notion that a fraction of the population that were rounded might be mitotic. These data were the foundation for subsequent studies on whether the L.E. extract induces mitosis, and by which mechanism it might do this.

### 3.5 Discussion

We are investigating prairie plant species to determine if they contain natural products that have bioactivities upon human cells. We approached this investigation in two ways: we studied how treated cells respond to the chemicals present in a plant extraction and then we isolated the active compound from the plant extract that is responsible for this bioactivity. This approach is similar to that which has been used in the 19<sup>th</sup> century to discover many of the drugs that make our modern pharmacopeia. The innovative components of our investigation are that we use plant species that have not been previously studied, and that we identify the targeted cellular pathways beyond that of cytotoxicity. In this chapter, we characterized the first critical steps from *Gaillardia aristata* by cytotoxicity and induction of morphology change. With this information, we were able to undertake a large scale, collaborative project to isolate a novel natural product with an anti-mitotic activity, as described in the following chapters.

*Gaillardia aristata* is a prominent plant in the prairie ecological zone of North America. Although this plant is native to North America, it was also imported to various locations across Europe, Australia and South America. *G. aristata*, also known as the blanket flower, is a perennial herbaceous plant with basal leaves growing at ground level and one or more flowering stalks (Bain et al., 2014). Flower heads are large and have yellow three-lobed petals with a brown centre. This plant was known to North American First Nations communities such as the Blackfoot and the Plateau First Nation tribes who rely on indigenous plant species for their primary health care needs. *G. aristata* is a medicinal plant that has beneficial properties against gastroenteritis, skin conditions and fever (Hellson et al., 1974; Hunn et al., 1990; Moerman, 2009). The Blackfoot tribe would use a mixture of powdered roots applied directly to a wound or skin disorder, or

they would make an infusion that was also rubbed on sore skin, or used as eyewash. In Western medicine, *G. aristata* has not been studied; however, it has been described as material that causes contact dermatitis (Burry, 1980; Burry et al., 1982). The dermatitis manifested itself with visible rashes on the face and hands as well as swelling of the upper eyelids, indicating irritation of the body parts that had been in direct contact with the plant. In fact, there are various studies reporting the allergenic properties of chemicals produced by many plants belonging to the Asteraceae family, including *Gaillardia* spp., and their role in contact dermatitis (Arlette et al., 1981; Paulsen, 1992). The chemical source of the irritation is believed to be members of the sesquiterpene family of chemicals, which have an  $\alpha$ -methylene group attached to the  $\gamma$ -lactone ring, and is characteristic of the types of chemicals produced by Asteraceae species (Mitchell et al., 1971). For these reasons *G. aristata* is listed as a toxic plant in the Food and Drug Administration (FDA) database of poisonous plants (F.D.A., 2016). Finally, *G. aristata* is also known in agricultural studies. The plant is considered as poor forage value for cattle (Pahl et al., 1999) and there is anecdotal evidence that it is not consumed by grazing herbivores when mature. Overall, these observations support the notion that *G. aristata* contains chemicals of biological interest.

The first investigation of the effects of *G. aristata* upon human cells was described in 2012. Salama et al. extracted the leaves of *G. aristata* with alcohol and reported its cytotoxic effects upon MCF-7 breast cancer and HTC116 colon cancer cells, as well as a chemical profile of the plant (Salama et al., 2012). The initial alcohol extract was fractionated with chloroform, ethyl acetate and n-hexane, and the extract and the fractions were tested upon both cell lines. It resulted that the chloroform fraction was the most active with  $IC_{50}$  ranging from 0.43 to 0.54  $\mu\text{g/mL}$ . The authors then used bioassay-

guided fractionation to separate the cytotoxic activity from the chloroform fraction and isolate the active compounds, and found two sesquiterpene lactones from the chloroform initial fraction (neopulchellin and 6 $\alpha$ -hydroxyneopulchellin), five flavonoids, a sterol and its glycoside and a triterpene, with the two sesquiterpenes being the most cytotoxic (IC<sub>50</sub> from 0.32 to 0.46  $\mu$ g/mL). The cellular pathways that were affected by the extract or chemicals purified from it were not described (Salama et al., 2012). This first study on human cells is consistent with previous reports of herbivory avoidance. One of our goals was to investigate plants from a natural site and to determine if extracts contained chemicals that inhibit vital cellular pathways that may account for cytotoxicity.

We prepared a first extraction of *G. aristata* leaves in dichloromethane and found that it was cytotoxic to HT-29 cells with an IC<sub>50</sub> of 44.4  $\mu$ g/mL. However, Salama et al. found that extracts prepared from *G. aristata* were 100-fold more toxic on cancer cells compared to our data. By using phenotypic screens to investigate other properties of the extract, we observed that the *G. aristata* extracts caused a striking morphology change on HT-29 cells, in which approximately a third of HT-29 cells became rounded by 24 h treatment. Rounded morphology may arise from two processes, mitosis or apoptosis (Elmore, 2007; Cadart et al., 2014). This phenomenon had not been previously reported for *Gaillardia* spp.

After having identified the cytotoxic and morphologic activities of *G. aristata* upon HT-29 cells, we tested the extraction solvents and plant parts to discern the best methods to isolate a great amount of bioactivity for future exploitation. The three solvents tested differ by polarity value, therefore each solvent extracts different compounds from the plant material. For example, a characteristic of solvents is the polarizability value, which is expressed as  $\pi^*$  and indicates the likeliness of a solvent to interact through

dispersion interactions and dipole interactions (Hansen et al., 2015). DCM has a  $\pi^*$  value of 0.82, ethanol has a  $\pi^*$  value of 0.54, and water of 1.09, demonstrating that these solvents have different properties and extract different chemicals (Hansen et al., 2015). The dichloromethane extract was the most cytotoxic extract of the three solvents with an  $IC_{50}$  of 44.4  $\mu\text{g/mL}$ , followed by the 75% EtOH extract. Similarly, the cell rounding morphology activity was most prominent when cells were treated by the dichloromethane extract. In the morphology assay, the water extract showed no activity. We note that the solvent with an intermediate  $\pi^*$  was the most effective at extracting chemicals that caused a rounding cell activity. Since the most active and potent extract was prepared with dichloromethane, it is likely that the active molecule(s) is a non-polar molecule(s), which is a property of the majority of natural products of interest. Dichloromethane was used to prepare all extracts for future studies, which answered one of the aims of the investigation for this chapter.

We then proceeded to test aerial plant parts (flower heads, stems and leaves) to elucidate where the majority of cytotoxic or rounded morphology inducing chemicals were stored. The leaf extract (L.E.) was the most cytotoxic extract ( $IC_{50}$  of 44.4  $\mu\text{g/mL}$ ), followed closely by the flower head extract (F.E.) ( $IC_{50}$  of 48.2  $\mu\text{g/mL}$ ), whereas the stem extract (S.E.) was far less toxic ( $IC_{50}$  of 312.4  $\mu\text{g/mL}$ ). Interestingly, the L.E. was the most active in morphology assays as well. These results showed that although all plant parts exert their activities, the L.E. was the most toxic and potent. Thus the leaves appear to be the best organ to produce the active chemical(s), consistent with other studies showing that a large portion of secondary metabolites are produced in the leaves (War et al., 2012). Finally, of the total dried aerial plant parts, the leaves make up to 20% of the total dried aerial plant mass, whereas the flowers and stems contribute 30% and 50%

respectively. For future studies, the leaves contained the greatest amount of activity, although all plant parts might be useful.

We found that *G. aristata* leaf extract had similar biological activity on a second cancer cell line, the glioblastoma M059K cells. In addition, these cells were approximately three-fold more sensitive to cytotoxicity. Interestingly, although *G. aristata* extracts were toxic to the non-cancerous lung fibroblastic WI-38 cells, they did not induce the cell rounding morphology on these cells. This suggests that the extract might affect mechanisms that are selective to or differently regulated in cancer cells. As described above, the cytotoxicity and the rounding activity did not seem to be correlated as the extracts were cytotoxic, with different potencies, on all cell lines, but they were not always active for morphology changes. This difference will allow us to study the two activities separately.

The cell rounding activity could be indicative of mitosis (Cadart et al., 2014) or apoptosis (Elmore, 2007). There are many assays available to investigate both phenomena and to discern between the two events. We therefore compared the L.E. activity to nocodazole, which inhibits microtubule polymerization that causes cells to arrest in mitosis with a rounded morphology (Vasquez et al., 1997). Although the nocodazole and L.E. treatments induced similar morphologies, almost all cells were rounded by nocodazole treatment whereas approximately 30% of the cells were rounded by L.E. treatment. This observation was supported by the flow cytometry data, which showed that the L.E. caused a G<sub>2</sub>/M-phase arrest and a concomitant S-phase arrest. This result suggests that while nocodazole arrests the majority of cells in mitosis (Blajeski et al., 2002; Rosner et al., 2013), the L.E. only causes a smaller fraction to accumulate in the G<sub>2</sub>/M-phases, and the two agents are likely to act on different targets. By contrast,

apoptosis inducing agents have a DNA profile in which cells are in sub-G<sub>1</sub> phase and do not undergo cell cycle phase arrest. These data led us to continue to investigate a possible anti-mitotic activity of the L.E.

We report that *Gaillardia aristata* extracts contain cytotoxic and anti-proliferative activity. We supported the initial observation by Salama et al. that *G. aristata* was toxic upon cancer cells, and we expanded it by characterizing dichloromethane as the solvent that extracts chemicals with the highest activity and by identifying the leaves as the plant part that stored the most potent chemicals, or the highest amount of chemicals. We also provided a first indication that this plant had an interesting anti-proliferative activity on cancer cells without affecting normal cells. We later confirmed that we could detect the cell rounding activity in extract prepared from plants collected at two different geographical sites, and prepared from two different years, demonstrating that the activities were sufficiently stable for future investigation. With this first characterization of a striking cellular phenomenon, an analysis of source material and adequate supply, we decided to investigate the cell rounding phenomenon in detail.

A



B



Bar in A = 10 cm

Bar in B = 1 cm

Figure 3.1: *Gaillardia aristata* growing in its natural habitat. A) The photo was taken on the University of Lethbridge coulees, and it shows *G. aristata* aerial parts. B) Image of the seeds of *G. aristata*. The scale bars are shown.

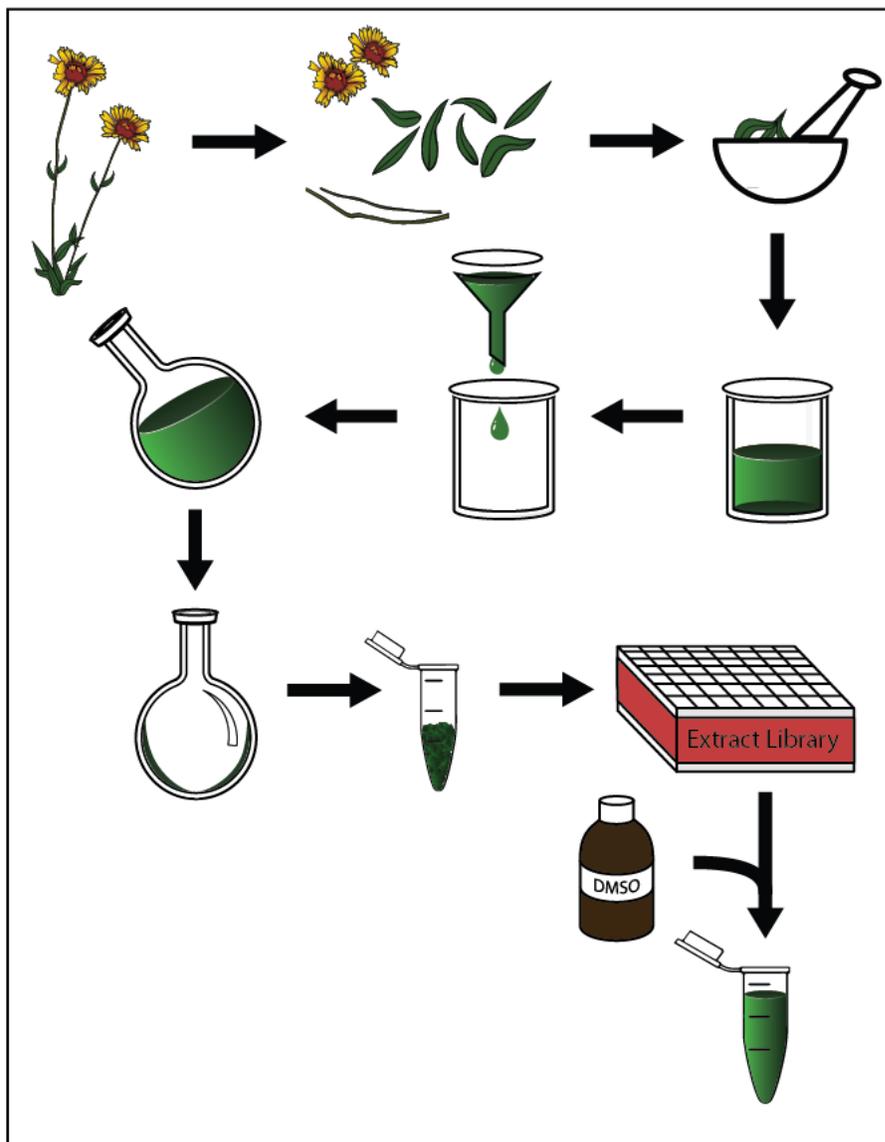


Figure 3.2: A representation of the plant extraction process, from the separation of the plant parts to the storage of the crude extract.

Table 3.1: Plant extracts made from different *G. aristata* plant parts and with different solvents. The names of the plant extracts are shown, including the plant parts and solvents used to prepare them.

Extracts			
Name	Plant species	Plant part	Solvent
PP-050	<i>G. aristata</i>	Leaves	DCM
PP-051AB	<i>G. aristata</i>	Leaves	75% EtOH
PP-053AB	<i>G. aristata</i>	Leaves	Water
PP-180	<i>G. aristata</i>	Leaves	DCM
PP-190	<i>G. aristata</i>	Leaves	DCM
PP-182A	<i>G. aristata</i>	Stems	DCM
PP-191A	<i>G. aristata</i>	Flower heads	DCM

Table 3.2: Mean IC<sub>50</sub> concentrations of different *G. aristata* plant extracts used to treat HT-29, M059K and WI-38 cells for 96 h. A) Mean IC<sub>50</sub> concentrations of HT-29 cells treated for 96 h with a dichloromethane, 75% ethanol or water extractions prepared from the leaf of *G. aristata*. B) Mean IC<sub>50</sub> concentrations of HT-29, M059K and WI-38 cells treated for 96 h with a leaf, stem or flower extract from *G. aristata*.

**A**

	Extract or compound		
Cell line	DCM (µg/mL)	75% EtOH (µg/mL)	CPT (nM)
HT-29	44.4 ± 1.3	139.6 ± 13.9	26.0 ± 1.4

**B**

	Extract or compound			
Cell line	Leaves (µg/mL)	Stems (µg/mL)	Flower heads (µg/mL)	CPT (nM)
HT-29	44.4 ± 1.3	312.4 ± 23.9	48.2 ± 3.6	24.0 ± 0.8
M059K	13.3 ± 1.3	81.2 ± 8.3	19.5 ± 0.4	4.4 ± 0.4
WI-38	44.4 ± 5.5	258.8 ± 12.2	28.6 ± 3.6	368.7 ± 56.3

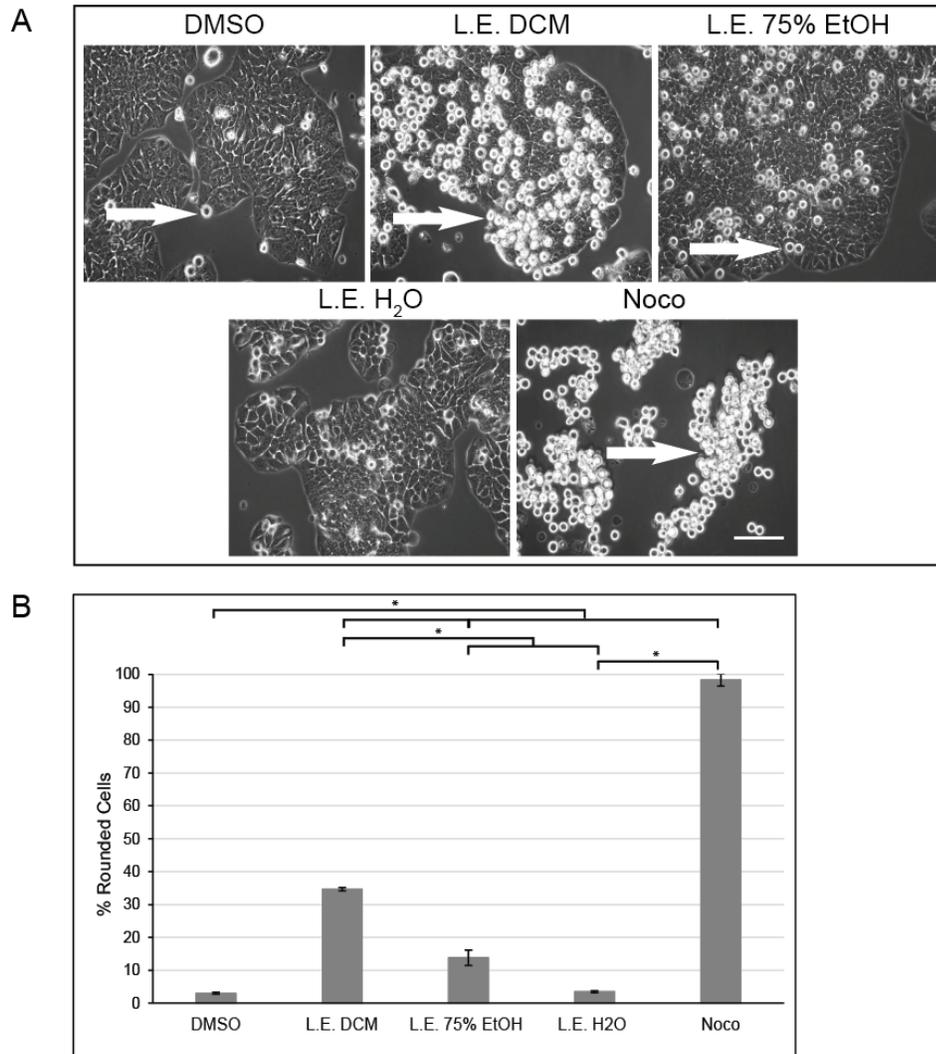


Figure 3.3: HT-29 cells treated with L.E. acquire a rounded morphology. A) Morphologic assay of HT-29 cells treated for 24 h with 50  $\mu\text{g}/\text{mL}$  L.E. prepared with dichloromethane, 75% ethanol or water. DMSO and 200 ng/mL nocodazole treated cells were used as controls. Cells were observed by phase-contrast light microscopy. Arrows indicate rounded cells. Scale bar equals 100  $\mu\text{m}$ . B) HT-29 cells were treated with 50  $\mu\text{g}/\text{mL}$  L.E. prepared with dichloromethane, 75% ethanol or water. DMSO and 200 ng/mL nocodazole treated cells were used as controls. Cells were observed by phase-contrast light microscopy and percentages of rounded cells at 24 h were manually determined using Image J software. At least 200 cells were counted for each treatment per experiment. Mean percentages from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .

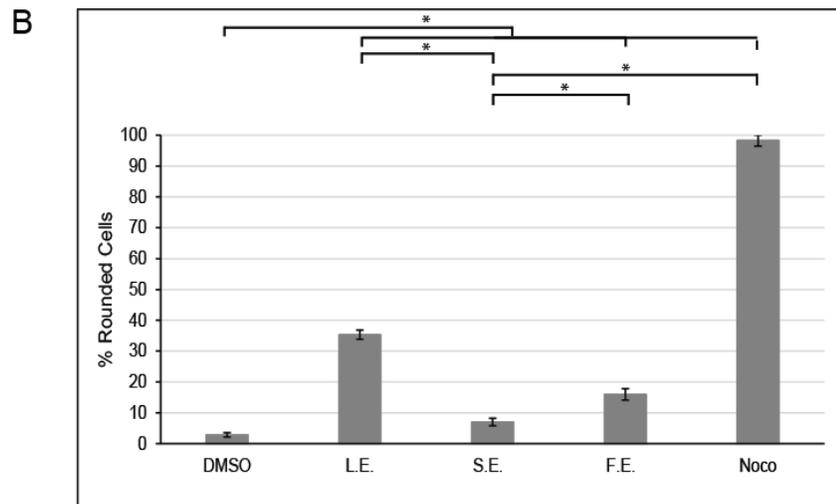
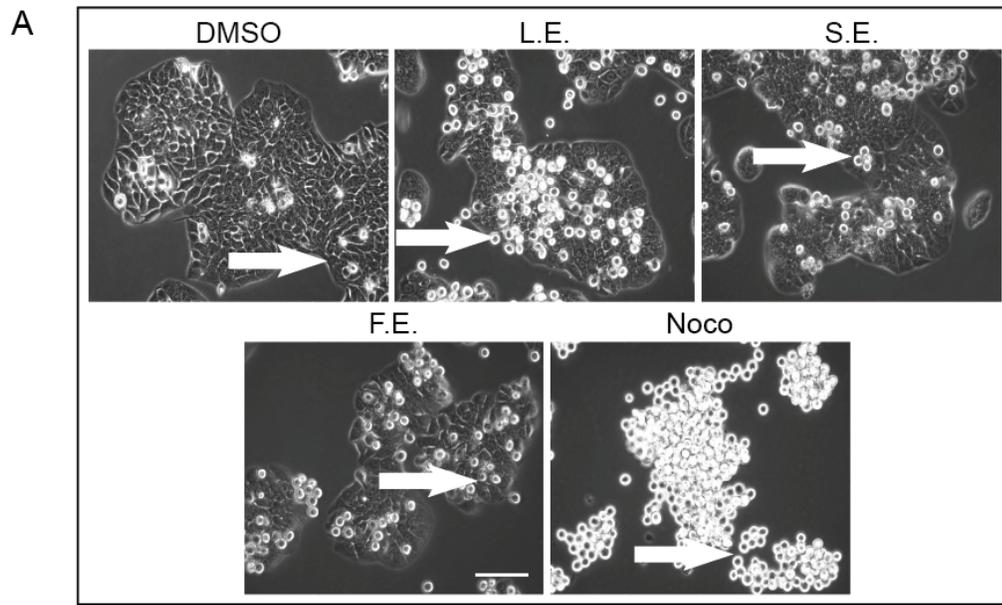


Figure 3.4: HT-29 cells treated with L.E. or F.E. acquire a rounded morphology. A) Morphologic assay of HT29 cells treated for 24 h with 50  $\mu\text{g}/\text{mL}$  L.E., S.E. or F.E. DMSO and 200 ng/mL nocodazole treated cells were used as controls. Cells were observed by phase-contrast light microscopy. Arrows indicate rounded cells. Scale bar equals 100  $\mu\text{m}$ . B) HT-29 cells were treated with 50  $\mu\text{g}/\text{mL}$  L.E., S.E. or F.E. DMSO and 200 ng/mL nocodazole treated cells were used as controls. Cells were observed by phase-contrast light microscopy and percentages of rounded cells at 24 h were manually determined using Image J software. At least 200 cells were counted for each treatment per experiment. Mean percentages from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .

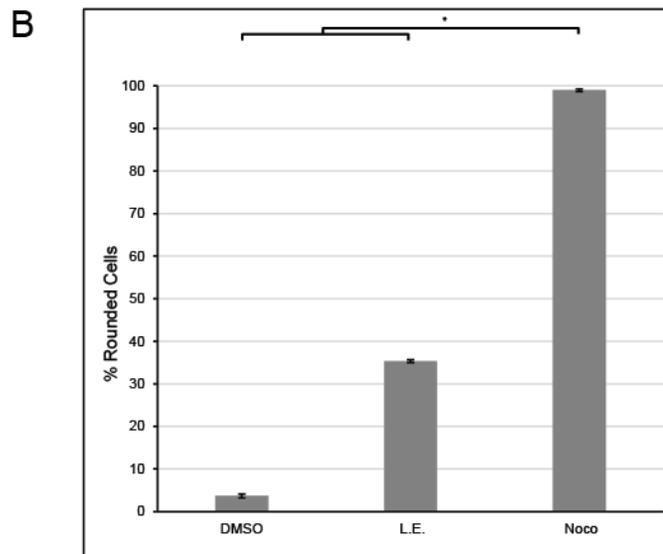
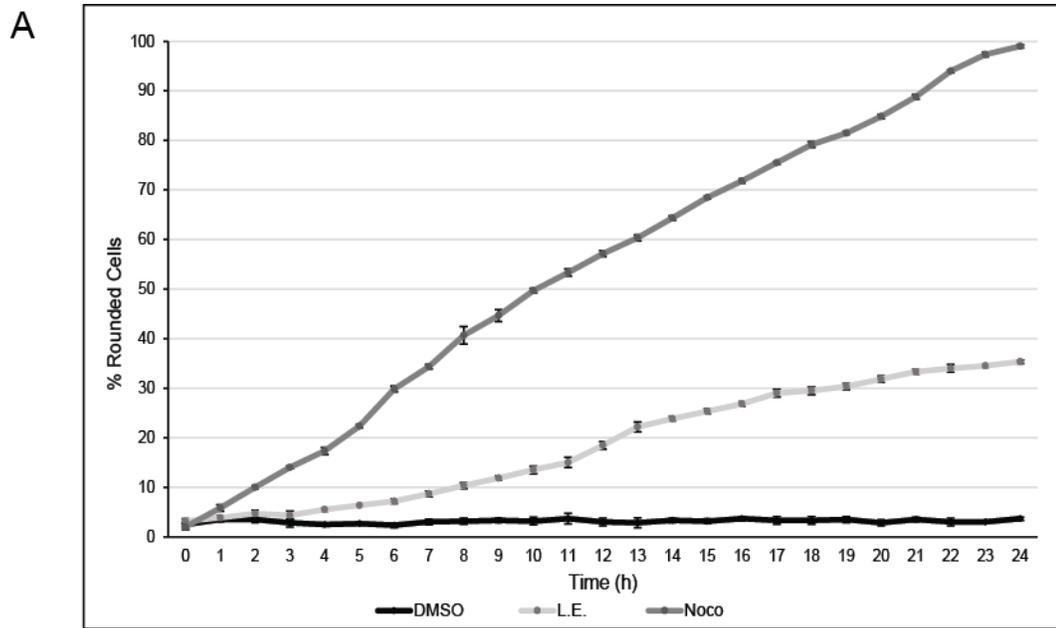


Figure 3.5: HT-29 cells treated with L.E. accumulate rounded cells at a different rate than cells treated by the mitotic inhibitor, nocodazole. A) HT-29 cells were treated with 50  $\mu\text{g}/\text{mL}$  L.E., or DMSO and 200  $\text{ng}/\text{mL}$  nocodazole were used as controls. Treated cells were observed by time-lapse video microscopy, and images were captured every 60 minutes for 24 h. Percentages of rounded cells were manually determined every 60 minutes using Image J software. At least 200 cells were counted for each treatment per experiment. Mean percentages from three separate experiments and standard errors of the means are shown. B) The mean percentages of rounded cells at 24 h post-treatment were calculated from three separate experiments. Standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .

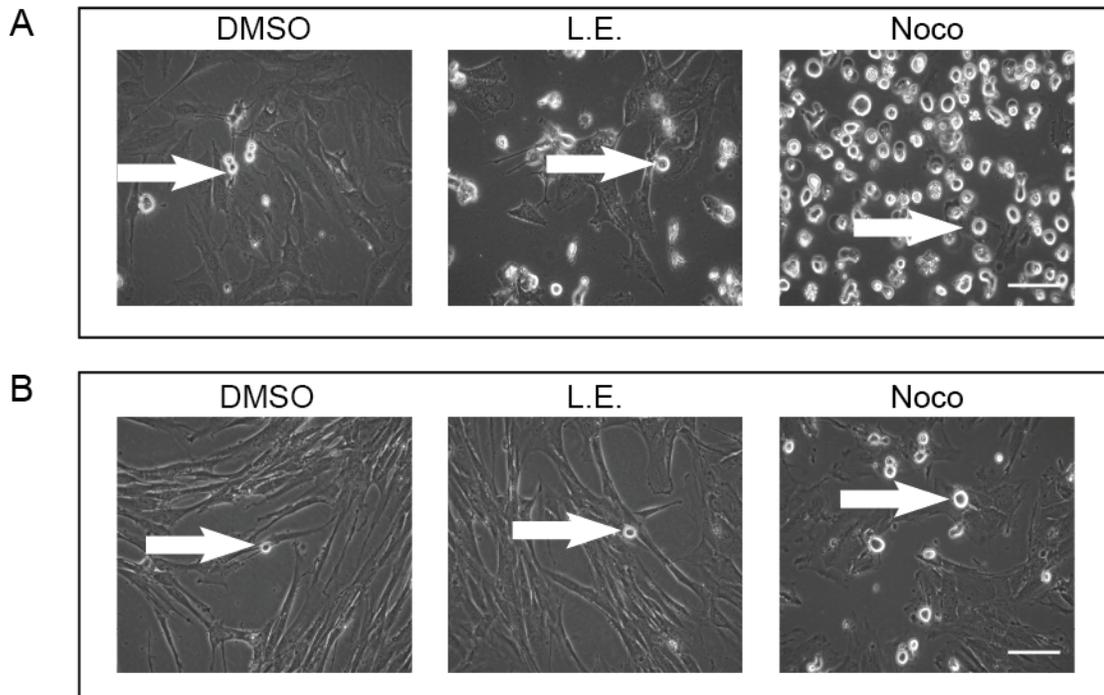


Figure 3.6: The L.E. is active upon another human cancer cell line (the M059K glioblastoma cell line), but not on a non-cancerous human cell line (WI-38 lung fibroblast cell line). A) M059K cells were treated with DMSO, 50  $\mu\text{g}/\text{mL}$  L.E. or 200  $\text{ng}/\text{mL}$  nocodazole, and observed by phase-contrast light microscopy at 24 h. Arrows indicate rounded cells. Scale bar equals 100  $\mu\text{m}$ . The experiment was repeated twice. B) WI-38 cells were treated with DMSO, 50  $\mu\text{g}/\text{mL}$  L.E. or 200  $\text{ng}/\text{mL}$  nocodazole, and observed by phase-contrast light microscopy at 24 h. Arrows indicate rounded cells. Scale bar equals 100  $\mu\text{m}$ . The experiment was repeated twice.

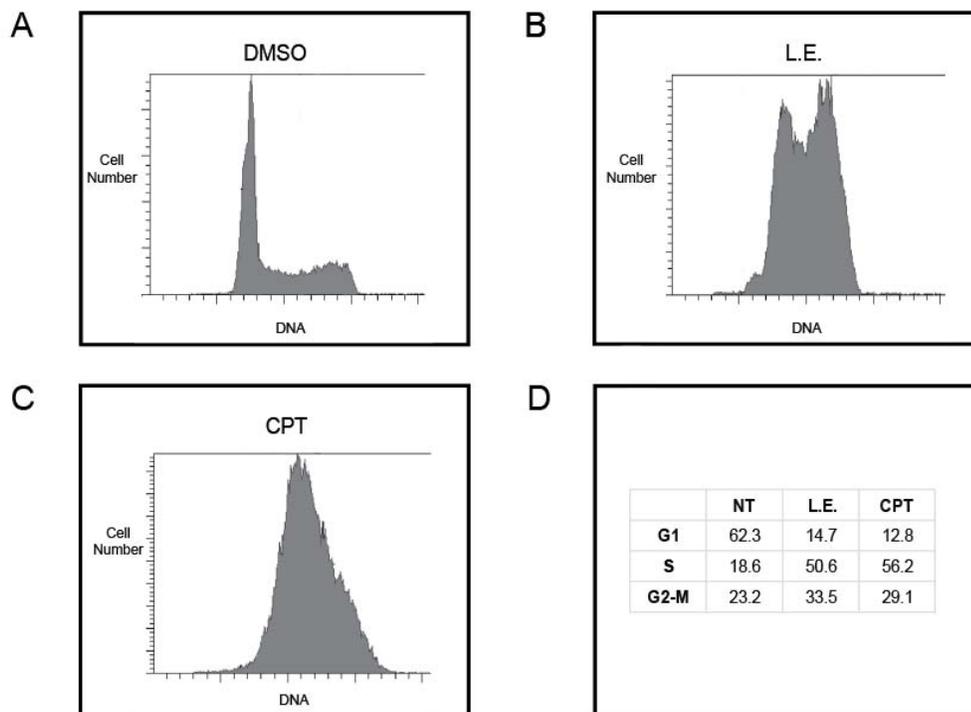


Figure 3.7: HT-29 cells treated with 50  $\mu\text{g}/\text{mL}$  L.E. accumulate in S- and G<sub>2</sub>/M-phases. HT-29 cells were treated for 24 h with DMSO (A), 50  $\mu\text{g}/\text{mL}$  L.E. (B) or 50 nM CPT (C), and analysed by flow cytometry. DNA content was analysed by propidium iodide staining, and the mean percentages of cells in either G1, S or G<sub>2</sub>/M phases of the cell cycle are shown (D). The experiment was repeated twice.

## CHAPTER 4

### **Characterization of the mitotic arrest in human cancer cells induced by a leaf extract prepared from *Gaillardia aristata*.**

#### **4.1 Abstract**

We have shown in chapter 3 that a *Gaillardia aristata* L.E. is cytotoxic to human cancer cell lines and it induces them to become rounded. A first test also indicated that the L.E. causes some cells to arrest in the G<sub>2</sub>/M-phase of the cell cycle, suggesting that the observed rounded cells could be in fact mitotic. Knowing that many anti-mitotic agents are also valuable anti-cancer drugs, we investigated further the rounded morphology phenomenon.

In this chapter, I report that we identified a new mitotic arrest activity from extracts prepared from *Gaillardia aristata* and have determined which cellular pathways might be affected by this chemical. We confirmed that the *G. aristata* L.E. arrests HT-29 cells in mitosis, and that during this prolonged arrest HT-29 cells undergo a drastic change in shape of mitotic spindle, and acquire damaged DNA.

#### **4.2 Introduction**

Enabling replicative immortality is a key hallmark of cancer (Hanahan et al., 2011). At the onset of mitosis major changes are orchestrated in many cellular components (Chan et al., 2012). Among these events are chromosome condensation, nuclear envelope breakdown, cytoskeleton rearrangements and assembly of the mitotic

spindle (Sullivan et al., 2007). In particular, mitotic chromosome condensation is associated with phosphorylation of histone H3 on Ser10. This phosphorylation event can be used as a marker to detect mitotic cells (Hendzel et al., 1997).

During mitosis microtubules assemble to bind to the chromosomes and form a spindle. This structure forms from two Microtubule Organizing Centers (MTOCs), which separate and migrate to opposite poles of the cell at the onset of mitosis (Jordan et al., 1998). Microtubules, therefore, are intimately involved with the replication of cells, and they are required to separate the genome of two daughter cells. By targeting the components of the microtubules ( $\alpha$ - or  $\beta$ -tubulin) it is possible to discern their rearrangement and determine whether a cell is undergoing mitosis or not.

Cell division is arguably one of the most complex and demanding processes undertaken by a cell, and it is finely regulated and coordinated by a control system (Sullivan et al., 2007). The key regulatory components of this system are the cyclin-dependent kinases (Cdks), which are serine/threonine protein kinases whose activity requires their association with the cyclin subunits (Malumbres, 2014). Another key component is the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin-protein ligase that targets several cell cycle proteins for degradation, and promotes unidirectional metaphase-anaphase transition (Vermeulen et al., 2003). The Cdk-cyclin complexes are responsible for phosphorylating a multitude of substrates, tightly regulating mitotic events. A cell is driven into mitosis when the Cdk1 enzyme is active, which promotes chromosome condensation, nuclear envelope breakdown and formation of the mitotic spindle. Cdk1 can become fully active only when coupled to its cyclin, cyclin B, forming the dimer Cdk1-cyclin B (Bloom et al., 2007; Gavet et al., 2010). Cdk1 is then phosphorylated on Thr161 by cyclin-dependent kinase activating kinase (CAK) (Deibler

et al., 2010), stabilizing the interaction between cyclin B and Cdk1 and inducing the conformational rearrangements needed for kinase activity (Laroche et al., 2007). Cdk1, however, is held in its inactive form during G<sub>2</sub> phase when it is phosphorylated on the amino acids Thr14 and Tyr15, which prevent ATP binding (Den Haese et al., 1995). Therefore, to be fully activated Cdk1 must be dephosphorylated on these two amino acids: this is achieved by the Cdc25 phosphatase (Timofeev et al., 2010). Thus, to enter mitosis, cells must have low levels of phosphorylated Cdk1 and high levels of cyclin B.

When the Cdk1-cyclin B complex is active, it can phosphorylate up to 70 different proteins in mammalian cells (Malumbres et al., 2005). Measuring Cdk1 activity can be used as a method for detecting mitosis. For example, one of the Cdk1 substrates is the serine/threonine protein phosphatase 1 (PP1 $\alpha$ ), which is phosphorylated at Thr320 (Kwon et al., 1997). Phosphorylation of PP1 $\alpha$  leads to its inhibition, increasing the phosphorylation of substrates for Cdk1-cyclin B that are required for entry into mitosis, whereas its dephosphorylation (and activation) contributes to completion and exit from mitosis (Kwon et al., 1997). It is possible to measure Cdk1 protein kinase activity by examining the presence (or absence) of phosphorylated PP1 $\alpha$  (Lewis et al., 2013): the presence of phospho-Thr320 PP1 $\alpha$  is indicative of mitosis (Lewis et al., 2016).

Initiation of anaphase requires another major regulatory system: the anaphase-promoting complex (APC/C). The APC/C complex is a ubiquitin-protein ligase that covalently adds ubiquitin chains to regulatory cell cycle proteins, which leads to their degradation. Polyubiquitin chains on proteins cause them to be recognized and degraded by the 26S proteasome complex (Peters, 2006). The APC/C complex is responsible for the degradation of the mitotic cyclins, thereby inactivating Cdk1 enzyme activity enabling the cell to exit mitosis (Cohen-Fix et al., 1996; Wasch et al., 2002). Securin protein is

another target of the APC/C complex. It inhibits the protease separase, which cleaves the cohesin proteins that hold the sister chromatids together (Funabiki et al., 1996; Yanagida, 2000). Cleavage of the cohesins results in release of tension between paired kinetochores to allow sister chromatids to migrate towards opposite poles of the mitotic spindle (Yamamoto et al., 1996; Shirayama et al., 1998). Failure to activate separase, which prevents cleavage of the cohesins, results in a delay in progression through mitosis. In addition, loss of the hSecurin gene in cells causes chromosome loss due to various unsuccessful attempts to segregate chromosomes (Jallepalli et al., 2001). Therefore, securin is essential in ensuring proper progression through mitosis and in preventing aneuploidy.

The APC/C complex is regulated in conjunction with another component: the Skp1/Cullin/F-box (SCF) complex, which is an E3 ubiquitin ligase that ubiquitinates proteins involved in diverse roles (Vodermaier, 2004; Skaar et al., 2013). Substrate specificity of the SCF complex is dictated by interaction with one of the 69 F-box proteins, each targeting multiple substrates (Jin et al., 2004; Skaar et al., 2009). One SCF complex is SCF <sup>$\beta$ -TrCP</sup>, which associates specifically with phosphorylated destruction motifs in I- $\kappa$ B $\alpha$  and  $\beta$ -catenin and stimulates their degradation (Winston et al., 1999). SCF <sup>$\beta$ -TrCP</sup> also plays an important role in regulating mitosis progression through the APC/C complex (Vodermaier, 2004). From early S phase until prometaphase, the APC/C<sup>Cdc20</sup> is also kept inactive by the protein early mitotic inhibitor 1 (Emi1), and its degradation in early mitosis is necessary for the activation of APC/C<sup>Cdc20</sup> in late mitosis (Reimann et al., 2001). Emi1 is phosphorylated on a specific DSGxxS amino acid sequence (Guardavaccaro et al., 2003), which is necessary for the recognition by the SCF ubiquitin ligase complex that contains the F-box protein  $\beta$ -TrCP (Laney et al., 1999;

Maniatis, 1999). Plk1 and Cdk1-cyclin B stimulate the ligation of ubiquitin to Emi1 by SCF <sup>$\beta$ -TrCP</sup>, thus marking the protein for destruction and allowing cells to progress to anaphase (Margottin-Goguet et al., 2003; Moshe et al., 2004). Therefore, Emi1 is also crucial for progression of the cell cycle by regulating APC/C, and when it is not degraded, the cells undergo mitotic arrest.

Increasing evidence suggests that the SCF complex could play a crucial role in the process of tumourigenesis (Wang et al., 2014), and many of the F-box proteins, such as Skp2, NIPA, and  $\beta$ -TrCP, can function as oncoproteins (Skaar et al., 2013; Wang et al., 2014). In particular, it is shown that inhibition of  $\beta$ -TrCP suppresses growth of breast cancer cells (Tang et al., 2005), whereas inhibition of Skp2 suppresses tumourigenesis by inducing apoptosis (Wei et al., 2004; Lin et al., 2010). It is therefore useful to consider the SCF complex as a possible target to inhibit cancer cells and their proliferation.

The majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway (Rock et al., 1994; Lecker et al., 2006). The ubiquitin-proteasome pathway consists of three enzymatic components that are required to link chains of ubiquitin onto proteins that are destined for degradation (Glickman et al., 2002; Pickart, 2004), and the 26S proteasome, which recognizes the tagged proteins and degrades them to small peptides (Baumeister et al., 1998). The proteasome system has been extensively studied as a possible target in cancer cells (Adams et al., 1999) because cancer cells accumulate more misfolded/mutated/damaged proteins than do normal cells (Adams, 2004). Therefore, cancer cells are much more susceptible to proteasome inhibition than normal cells. MG132 is a peptide aldehyde that inhibits the chymotryptic-like activity of the 26S proteasome complex (Lee et al., 1998). MG132 was tested against a human cervix cancer HeLa cell line and was demonstrated to induce apoptosis in a dose-dependent manner,

cell cycle arrest and formation of reactive oxygen species (ROS) (Han et al., 2009). Also, MG132 induces G<sub>2</sub>/M-phase arrest in HT-29 colon cancer cells (Wu et al., 2008). MG132 was used in this thesis as a control compound for proteasome inhibition to investigate the activity of the *G. aristata* extract.

Finally, we addressed the third hallmark of cancer, genomic instability and mutation, by investigating which effects the *G. aristata* extract had upon DNA damage (Hanahan et al., 2011). When DNA is damaged, ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) protein kinases become activated and start a signaling cascade that controls DNA repair pathways (Zhou et al., 2000). The cascade culminates in different events such as cell cycle arrest, activation of cell death or checkpoint adaptation, induced transcription and damage-induced DNA repair (Zhou et al., 2000; Matsuoka et al., 2007). Specifically, ATR is activated when single-stranded breaks occur (Zhou et al., 2000; Zou et al., 2003), whereas ATM is activated in response to double-stranded breaks (Kastan et al., 2004; Keogh et al., 2006). Among the many substrates is histone H2AX, which is phosphorylated by ATR and ATM on amino acid Ser139, leading to the formation of phospho-H2AX (or  $\gamma$ H2AX) foci at sites of damaged DNA (Rogakou et al., 1998; Ward et al., 2001).  $\gamma$ H2AX recruits repair factors on the site containing damaged DNA, and it also activates checkpoint proteins promoting arrest of the cell cycle progression (Podhorecka et al., 2010). If the DNA damage is repaired,  $\gamma$ H2AX is dephosphorylated by PP1 and PP2 (Nazarov et al., 2003; Chowdhury et al., 2005). Therefore,  $\gamma$ H2AX can be used as a marker to detect damaged DNA (Furuta et al., 2003).

Mitosis is a complex and highly regulated phase of the cell cycle, which represents a good point of intervention to block the proliferation of cancer cells. Many chemotherapeutic agents interfere with mitotic processes causing cells to die (Chan et al.,

2012). In this chapter, we will investigate which mitotic mechanisms *Gaillardia aristata* acts upon.

## **4.3 Materials and Methods**

### *4.3.1 Cell culture*

The human cell line HT-29 (ATCC HTB-38) was obtained from the American Type Culture Collection (ATCC). The colorectal carcinoma cell line HT-29 was maintained in RPMI 1640 medium (Gibco; 21870-092) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco; 12484028) and 1.6 mM GlutaMAX (Gibco; 35050-061). Cells were grown at 37°C in 5% CO<sub>2</sub> and the media were changed every two or three days. HT-29 cells were plated at a density of 3.0 x 10<sup>5</sup> cells/25 cm<sup>2</sup> flask and cultured for 48 h prior to treatment. The compounds CPT (Sigma; 7689-03-4) and nocodazole (Sigma; M1404-10MG) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D2438) to a concentration of 10 mM and 200 µg/mL respectively. The compound MG132 was dissolved in DMSO to a concentration of 300 nM. Various extracts prepared from *Gaillardia aristata* were resuspended in DMSO to a concentration of 100 mg/mL. Extracts and other compounds listed were stored at -20°C. In not-treated (NT) cells DMSO was added to a final concentration of 0.1% (v/v) as a solvent vehicle control.

### *4.3.2 Immunofluorescence microscopy*

HT-29 cells were plated on glass coverslips at 1.0 x 10<sup>5</sup>/well in a 6 well culture plate and incubated at 37°C for 48 h prior to treatment. After treatment, cells were fixed at room temperature for 20 minutes in 3% (v/v) formaldehyde (Ted Pella Inc; 18505),

diluted in PBS. Fixation was quenched with 50 mM NH<sub>4</sub>Cl in PBS and cells were permeabilised for five minutes using 0.2% (v/v) Triton X-100 in PBS and blocked for 30 minutes with 3% (w/v) BSA in PBS-T (0.1% (v/v) Tween-20 diluted in PBS). Cells were then incubated with primary antibodies as described: anti-histone  $\gamma$ H2AX (Millipore; 05-636; 1:400) for 1 h at room temperature; anti-phospho-Ser10 histone H3 (Millipore; 06-570(CH); 1:1000) for 18 h at 4°C, and anti- $\alpha$ -tubulin (Santa Cruz Biotechnology; sc-53030; 1:200) for 2 h at room temperature. After washing with PBS-T, cells were incubated with secondary antibodies for 1 h at room temperature as follows: Alexa Fluor 488-conjugated anti-mouse (Life Technologies; A11059; 1:400) for anti-histone  $\gamma$ H2AX, Texas Red-conjugated anti-rabbit (Jackson ImmunoResearch; 111-075-003; 1:400) for anti-phospho-Ser10 histone H3 and Alexa Fluor 488-conjugated anti-rat (Life Technologies; A11006; 1:400) for anti- $\alpha$ -tubulin. Nuclei were stained with 300 nM DAPI (4',6-diamidino-2-phenylindole) in PBS for 15 minutes and coverslips were mounted onto microscope slides using ProLong Gold Antifade reagent (Molecular probes; P36934). Cells were observed at room temperature on an Olympus BX41 microscope using either an Olympus UPlanFL N 20x objective with 0.50 numerical aperture or an Olympus UPlanFL N 60x objective with 1.25 numerical aperture. Images were captured using an Infinity 3 camera operated by Infinity Capture imaging software (Lumenera Corporation), within the linear range of detectors. Images were prepared using Adobe Photoshop (CC 2014.1.0) software. Cells positive for histone  $\gamma$ H2AX, phospho-Ser10 histone H3 and  $\alpha$ -tubulin were counted using Image J (IJ 1.46r) software. At least 20 cells were counted for each treatment, unless otherwise stated, and experiments were performed three times.

#### *4.3.3 Confocal microscopy*

Cell fixation and staining were performed as described for immunofluorescence microscopy. Cells were observed at room temperature on an inverted Nikon C1 Plus Digital Eclipse Modular Confocal Microscope using a Nikon PlanApo VC 60x objective with 1.20 numerical aperture. Images were captured using the DAPI and FITC filters by the photodetector integrated in the microscope, which was operated by the Nikon EZ-C1 3.80 software. Images were prepared using Adobe Photoshop (CC 2014.1.0) software.

#### *4.3.4 Mechanical shake-off*

HT-29 cells were plated at  $1 \times 10^6$  cells/75 cm<sup>2</sup> flask and incubated at 37°C for 48 h prior to treatment. After treatment, medium was aspirated and cells were gently washed with PBS. Fresh medium was added at 3 mL/75 cm<sup>2</sup> and the flask was tapped with moderate force on all edges to displace rounded cells from flattened cells (Kubara et al., 2012). Medium containing the rounded cells was collected and stored in a separate tube until use.

#### *4.3.5 Cell extract preparation*

HT-29 cells were plated at  $1 \times 10^6$  cells/75 cm<sup>2</sup> flask and incubated at 37°C for 48 h prior to treatment. After treatment, cells were either trypsinised or collected by mechanical shake-off and washed with ice cold PBS. Cells were re-suspended in ice cold lysis buffer (50 mM HEPES, pH 7.4, 50 mM NaF, 10 mM EGTA (ethylene glycol tetraacetic acid), 50 mM  $\beta$ -glycerophosphate, 1 mM ATP, 1 mM DTT (dithiothreitol), 1% Triton X-100 (v/v), 10  $\mu$ g/mL RNase A (Sigma-Aldrich; R6513-250MG), 0.4 U/mL DNase I (Invitrogen; I354Ba) and protease inhibitor cocktail (Roche; 11836170001)) at a

concentration of 20,000 cells/ $\mu\text{L}$ , passed through a 26-gauge needle five times and incubated on ice for 30 minutes. The suspension was centrifuged at 10,000 x g for ten minutes at 4°C, aliquoted into 1.5 mL microfuge tubes and stored at -80°C. Extracts were either used for electrophoresis after being boiled for five minutes in the presence of 2x SDS (sodium dodecyl sulphate) sample buffer (20% (v/v) glycerol, 10% (v/v) DTT, 6% (w/v) SDS, 500 mM Tris, pH 6.8) or used to assay Cdk1 activity. The concentration of proteins in each extract was quantified using the Agilent 2100 Bioanalyser and samples were prepared using the Agilent Protein 230 kit: 4  $\mu\text{L}$  of protein sample and 2  $\mu\text{L}$  denaturing solution were mixed in a 0.5 mL microfuge tube and incubated at 95°C for five minutes. The samples were then briefly centrifuged and 84  $\mu\text{L}$  deionised water was added. Samples were mixed by vortexing and loaded onto the Agilent Protein 230 chip which was loaded into the Agilent 2100 Bioanalyser. Samples were analysed using Agilent Expert 2100 software to quantify the amount of protein.

#### *4.3.6 Electrophoresis and western blotting*

Cell extracts were separated on 10% or 12% (v/v) SDS-PAGE (poly-acrylamide gel electrophoresis) gels with 4% (v/v) stacking gels. Precision Plus Dual Colour marker (BioRad; 161-0394) was used to estimate molecular weight in kilodaltons (kDa). Proteins were transferred onto nitrocellulose membranes by semi-dry transfer (BioRad) for 45 minutes at 25 volts. Membranes were blocked with either 5% (w/v) low fat milk or 3% (w/v) BSA in Tris buffered saline with Tween-20 (TBS-T) (50 mM Tris base, 150 mM NaCl and 0.1% (v/v) Tween-20, pH 7.6) for 2 h. Membranes were then incubated with the following primary antibodies at 4°C overnight: anti-Cdk1/Cdc2 (Signalway Antibodies; 21236-2; 1:500); anti-phospho-Tyr15 Cdk1/Cdc2 (Signalway Antibodies; 11244-2; 1:500); anti-cyclin B (Santa Cruz Biotechnology; sc-245; 1:200); anti-actin

(Santa Cruz Biotechnology; sc-58673; 1:200); anti-securin (Thermo Fisher Scientific; 700791; 1:150). The membranes were then washed with TBS-T and incubated with the following secondary antibodies for 1 h at room temperature: alkaline phosphatase coupled anti-mouse IgG (Promega; PRS3721; 1:2500) or alkaline phosphatase coupled anti-rabbit IgG (Promega; PRS3731; 1:2500). The membranes were washed with TBS-T and developed using an alkaline phosphatase conjugate substrate kit (BioRad; 172-1063). Development was stopped with Tris-EDTA (ethylenediaminetetraacetic acid) buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). Western blot analyses were performed three times.

#### *4.3.7 Cdk1 Kinase Assay*

Cdk1 phosphorylation reactions (20  $\mu$ L total volume) were prepared as follows: 10  $\mu$ L 2x Cdk1 phosphorylation buffer (50 mM  $\beta$ -glycerophosphate pH 7.4, 10 mM  $MgCl_2$ , 10 mM NaF, 1 mM DTT) with 200  $\mu$ M ATP and 5  $\mu$ L of either 80 ng/ $\mu$ L glutathione S-transferase (GST) or GST-PP1C-S artificial substrates (Lewis et al., 2013). Reactions were initiated by adding 5  $\mu$ L of whole cell extracts diluted in cold lysis buffer (50 mM HEPES, pH 7.4, 50 mM NaF, 10 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 1 mM ATP, 1 mM DTT, 1% Triton X-100 (v/v), 10  $\mu$ g/mL RNase A (Sigma-Aldrich; R6513-250MG), 0.4 U/mL DNase I (Invitrogen; I354Ba) and protease inhibitor cocktail (Roche; 11836170001) to 100 lysed cells/ $\mu$ L. Reactions were incubated for five minutes at 30°C and were stopped by adding an equal volume of 2x SDS sample buffer and heating at 95°C for five minutes. Reaction mixtures were separated on 12% (v/v) SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes with a semi-dry electroblotter system (BioRad) for 45 minutes at 25 volts. The membranes were blocked with either 5% (w/v) low fat milk or 3% (w/v) BSA in TBS-T and incubated overnight at room temperature with either anti-phospho-Thr320 PP1C $\alpha$  (Abcam; Ab62334; 1:300,000) or

anti-GST (Sigma-Aldrich; G7781; 1:20,000) primary antibodies. After washing with TBS-T, the membranes were incubated with alkaline phosphatase coupled to anti-rabbit IgG (Promega; PRS3731; 1:2500). The membranes were washed with TBS-T and developed using an alkaline phosphatase conjugate substrate kit (BioRad; 172-1063). Development was stopped using Tris-EDTA buffer. Cdk1 assays were performed three times (Lewis et al., 2016).

#### *4.3.8 Statistics*

Data and statistical analysis were performed using Microsoft Excel 2010 software. Data were plotted as means from three separate experiments  $\pm$  standard errors of the means. Statistical significance was calculated using the student's t-test for two paired sample means and values were considered significantly different when  $p < 0.05$ .

## **4.4 Results**

### *4.4.1 HT-29 cells that become rounded after treatment with L.E. are in mitosis*

We reasoned that because L.E. treated cells displayed a rounded phenotype and arrested at the G<sub>2</sub>/M-phases (chapter 3), they might be undergoing mitosis. HT-29 cells were treated with 50  $\mu$ g/mL L.E. for 2 and 24 h and then stained with phospho-Ser10 histone H3 antibodies, and analysed by immunofluorescence microscopy (Figure 4.1A). DMSO and 200 ng/mL nocodazole treatments were used as controls. At 2 h post treatment with L.E.,  $4.3 \pm 0.4\%$  of cells were positive for phospho-Ser10 histone H3 staining, which was similar to that of the DMSO treated cells ( $3.2 \pm 0.4\%$ ). After 24 h treatment with L.E. the number of PH3 positive cells increased to  $27.3 \pm 2.1\%$  whereas, as expected,  $87.8 \pm 12.2\%$  of nocodazole treated cells stained positive for PH3.

#### *4.4.2 HT-29 cells treated with L.E. for 24 h have distorted mitotic spindles*

If L.E. treated cells are in mitosis, then they should have a mitotic spindle. Treated cells were analysed for  $\alpha$ -tubulin staining by immunofluorescence microscopy.  $\alpha$ -tubulin is a protein that polymerizes into microtubules, and microtubules rearrange to form the mitotic spindle. Cells were treated with 50  $\mu\text{g}/\text{mL}$  L.E. for 2 and 24 h, and DMSO and 200 ng/mL nocodazole were used as controls (Figure 4.2A). Nocodazole is a tubulin depolymerizing agent (Jordan et al., 1998) and it prevents the formation of mitotic spindles, arresting cells in mitosis (Vasquez et al., 1997). The percentage of cells with mitotic spindles in DMSO, 2 h L.E., 24 h L.E. and nocodazole treated cells were, respectively,  $3.2 \pm 0.2\%$ ,  $7.2 \pm 0.8\%$ ,  $32.9 \pm 1.4\%$ , and  $0.0\%$  (Figure 4.2B). These data show that cells treated with L.E. contained a mitotic spindle. Upon closer observation, however, the spindles in 2 h L.E. treated cells displayed a regular, bipolar shape, whereas cells treated with L.E. for 24 h had distorted spindles which, in some cases, appeared to be either monopolar or multipolar. For each treatment the number of cells with regular or distorted spindles was counted and normalized to the number of cells that presented a spindle. Cells from the DMSO treatment had only  $4.8 \pm 4.8\%$  distorted spindles and those from the 2 h L.E. treatment had  $7.9 \pm 4.5\%$  distorted spindles. By contrast, cells treated for 24 h with L.E. had  $78.5 \pm 3.9\%$  distorted spindles (Figure 4.2C). Nocodazole treatment was not included as it prevented the formation of a mitotic spindle. These results suggest that when HT-29 cells are treated with L.E. they form a spindle that becomes distorted in shape.

#### *4.4.3 Confirmation of mitotic spindle morphology by confocal microscopy*

We analysed the same populations of treated cells described in section 4.4.2 by confocal microscopy (Figure 4.3). DMSO treated cells that were in mitosis displayed a

regular shape of the mitotic spindle, whereas L.E. treated cells that arrested in mitosis showed a distorted spindle. In addition, the DNA (stained with DAPI) in the mitotic cells that were treated with L.E. for 24 h was not properly aligned on the metaphase plate, as it was at 2 h post treatment (see Figure 4.2).

#### *4.4.4 Cdk1 is dephosphorylated on Tyr15 and cyclin B is overexpressed in rounded HT-29 cells treated with L.E.*

Cdk1 is a protein kinase whose activity is essential for cells to enter mitosis, and it is responsible for the regulation of all the key mitotic events (Malumbres, 2014). To characterize the status of Cdk1 in treated cells, mechanical shake-off was performed on DMSO, 2 h and 24 h 50  $\mu\text{g}/\text{mL}$  L.E. and 200  $\text{ng}/\text{mL}$  nocodazole treated cells to separate the rounded population from the interphase adherent population. The protein composition of the different populations was investigated by western blot analysis using Cdk1, phospho-Tyr15 Cdk1, cyclin B and actin (used as a loading control) antibodies, as shown in Figure 4.4. While Cdk1 was equally expressed in all populations, phospho-Tyr15 Cdk1 levels were low only in the rounded cells from both the L.E. and the nocodazole treatments. These cells also had high amounts of cyclin B. DMSO treated cells and adherent cells from both the 2 h and 24 h L.E. treatment, on the other hand, were positive for phospho-Tyr15 Cdk1 and contained low levels of cyclin B. Finally, the total population of cells treated with L.E. for 24 h contained Cdk1 that was phosphorylated on Tyr15 and had intermediate levels of cyclin B, and the total population of the 2 h L.E. treated cells was positive for phospho-Tyr15 Cdk1, but was not positive for cyclin B. We concluded that the rounded cells had Cdk1 that was not phosphorylated on Tyr15 and had high levels of cyclin B, indicating that Cdk1 might be active in these cells, and that these cells are in mitosis.

#### *4.4.5 Cdk1 is active in rounded cells that accumulate after treatment with L.E.*

To verify whether the Cdk1-cyclin B complex was active in rounded HT-29 cells that were treated with L.E., we measured the activity of the complex. GST-PP1C $\alpha$  is an artificial Cdk1 substrate that contains the Cdk1 consensus sequence found in PP1C $\alpha$  (Lewis et al., 2013). GST alone is used as a negative control substrate because it does not contain the Cdk1 phosphorylation site. We treated cells with 50  $\mu$ g/mL for 2 h and 24 h with L.E., and prepared interphase and mitotic cell extracts. DMSO treated cell extracts, 200 ng/mL nocodazole treated cell extracts, purified Cdk1 and extraction buffer were used as controls. We analysed the presence of phospho-Thr320 by western blotting (Figure 4.5). Mitotic cells from L.E. and nocodazole treatments, and the purified Cdk1 sample contained GST-PP1C $\alpha$  that was phosphorylated on Thr320. The total population of L.E. treated cells showed an intermediate signal of phospho-Thr320 PP1C $\alpha$  whereas DMSO, L.E. treated interphase cells and the extraction buffer were negative for phospho-Thr320 PP1C $\alpha$ . Together with the results obtained in sections 4.4.1, 4.4.2, 4.4.4 and 4.4.5, we confirmed that rounded cells accumulated after 2 h and 24 h treatment with L.E. were mitotic because they were positive for PH3 signal, contained mitotic spindles, and contained active Cdk1 enzyme.

#### *4.4.6 Mitotic cells that accumulate after treatment with L.E. have high level of securin*

We had established that the L.E. arrests approximately 30% of the treated HT-29 cells in mitosis. However, it seems that anti-mitotic activity of the L.E. does not resemble that of the classic anti-microtubular agents (sections 4.4.2 and 4.4.3). We then considered other possible targets which can be inhibited, such as protein kinases, motor proteins and the proteasome (Chan et al., 2012). We observed that microtubules first assemble into mitotic spindles and that the distorted spindles appeared at later times of arrest (24 h).

This observation led us to investigate proteins that regulate the metaphase-anaphase transition. The sister chromatids are held together by the cohesin proteins, which are degraded by the enzyme separase during the metaphase-anaphase transition (Funabiki et al., 1996; Yanagida, 2000). This enzyme is activated when its natural inhibitor, securin, is degraded (Yanagida, 2000). To determine whether the L.E. inhibits the degradation of the protein securin, we tested its levels of expression in L.E. treated cells by western blotting. DMSO, 200 ng/mL nocodazole, and 300 nM MG132 were used as controls: MG132 is a proteasome inhibitor that impedes the degradation of ubiquitinated proteins, including securin (Lee et al., 1998). As shown in Figure 4.6, the mitotic cells accumulated at 2 and 24 h after treatment with L.E. were positive for securin, whereas the total and the interphase populations contained lower levels of securin, as did the DMSO and nocodazole treated cells. Strikingly, none of the extracts of cells that were treated with the proteasome inhibitor MG132, nor cells treated with nocodazole were positive for securin. This result suggested that the mitotic arrest caused by treatment with L.E. was different from that of nocodazole, as expected, and it was unlikely to be a true proteasome inhibitor like MG132. It remains possible that the L.E. affects one of the components in the ubiquitin-proteasome pathway.

#### *4.4.7 DNA damage accumulates in mitotic cells treated with L.E.*

We had previously determined that the L.E. interferes with two of the three hallmarks of cancer, resisting cell death and enabling replicative immortality. We then explored the last hallmark of cancer, genome instability and mutation, by investigating the capability of the L.E. to damage DNA. HT-29 cells were treated with 50 µg/mL L.E. and analysed for histone  $\gamma$ H2AX staining at 2 and 24 h by immunofluorescence microscopy (Figure 4.7). DMSO and 50 nM CPT were used as controls. CPT is a known

genotoxic agent, which causes the formation of DNA breaks and  $\gamma$ H2AX foci. As expected, few of the DMSO treated cells were positive for histone  $\gamma$ H2AX ( $0.5 \pm 0.2\%$ ), whereas all CPT treated cells exhibited histone  $\gamma$ H2AX staining ( $100.0 \pm 0.0\%$ ) (Figure 4.7A and 4.7B). At 2 h of L.E. treatment, cells rarely displayed histone  $\gamma$ H2AX staining ( $0.5 \pm 0.0\%$ ), however, by 24 h  $11.8 \pm 0.4\%$  of them were positive. During the course of experiments, it became evident that DNA damage caused by the L.E. was occurring in localized foci in cells with condensed DNA (mitotic cells). Furthermore, after 24 h of treatment with L.E., the cells that were positive for  $\gamma$ H2AX displayed small numbers of foci, relative to the hundreds that appear following CPT treatment. We then counted the percentage of cells that were positive for  $\gamma$ H2AX that had either less than or equal to 5 foci, or more than 5 foci and these were  $100 \pm 0\%$  and  $0 \pm 0\%$ , respectively, after 2 h L.E. treatment and  $19.8 \pm 1.8\%$  and  $80.2 \pm 1.8\%$  after 24 h L.E. treatment. We concluded that the L.E. was genotoxic to HT-29 cells, but only in mitotic cells after prolonged treatment and at specific sites.

#### *4.4.8 Confocal microscopy analysis of histone $\gamma$ H2AX phosphorylation pattern in L.E. treated HT-29 cells*

We then analysed the DMSO and 24 h L.E. treated cells that were stained with histone  $\gamma$ H2AX antibody (section 4.4.7) using confocal microscopy (Figure 4.8). The confocal microscope allowed us to create a 3-D image of the desired feature, providing more details on its structure and localization inside the cell. We confirmed that DMSO treatment induces very little phosphorylation of histone H2AX, and not necessarily in correspondence of mitotic cells, as shown in Figure 4.8. The L.E. on the contrary induces DNA lesions in distinct foci. What also emerges is that the number of foci that form after

24 h of treatment is greater than 5 but far fewer than the number in CPT treated cells (see Figure 4.7A).

#### **4.5 Discussion**

We report that a *Gaillardia aristata* L.E. causes mitotic arrest in HT-29 cells. L.E. extract treatment of HT-29 or M059K cells induces a rounded morphology, in a time scale that is consistent with mitotic arrest and supported in part by analysis by flow cytometry (chapter 3). We confirmed that the rounded cells are in mitosis by a number of different experimental approaches. The cells contain condensed chromosomes that are positive for the mitotic marker PH3. The rounded cells also contain mitotic spindles, as determined by staining for  $\alpha$ -tubulin and observation by immunofluorescence microscopy. Furthermore, protein extracts prepared from the rounded cells have relatively high Cdk1 activity and low pTyr15-Cdk1, which are biochemical markers for mitosis. Based on these data, we conclude that L.E. extract prepared from *Gaillardia aristata* can arrest a portion of human cancer cells in mitosis.

Our observation of the mitotic arrest provided insight into possible mechanisms of action by the extract upon the cells. First, as treatment with L.E. was prolonged from 2 to 24 h, the mitotic spindle in cells changed its shape from a standard metaphase arrangement to a distorted shaped spindle, in which chromosomes had not moved to the poles. Concomitant with this timing, cells in mitosis acquired  $\gamma$ H2AX foci, but only in localized regions. By contrast, CPT treatment produces hundreds of foci and even pan-nuclear  $\gamma$ H2AX staining. We do not know of another inhibitor that produces effects similar to these induced by the L.E. We believe there is a relationship between the distorted spindle and the appearance of damaged DNA.

The cells arrested in mitosis had high levels of Cdk1 activity. They also contained high levels of cyclin B, the regulatory component of the Cdk1 complex, and of the protein securin. These observations suggest that the cells were not able to enter anaphase, because if they had done so, cyclin B and securin levels would be relatively low. In addition, the L.E. extract might prevent the degradation of these key proteins, but it would appear to do so differently than a true proteasome inhibitor such as MG132.

Then, we addressed the other hallmark of cancer, genome instability and mutation, and we have found that the L.E. extract was genotoxic on HT-29 cells and induced increasing amounts of damaged DNA as the cells spent more time arrested in mitosis. The L.E. arrests cells in mitosis during which the spindles are deformed and DNA damage is accumulated. We therefore identified two novel activities of *Gaillardia aristata* that were not previously described.

Mitotic cells undergo major structural and biochemical steps to enable the cell to separate its chromosomes into two daughter cells. The duplicated DNA becomes condensed, the nuclear lamina breaks down, the microtubules are assembled into mitotic spindles and the actin cytoskeleton rearranges to confer a spherical shape to the cells (Cadart et al., 2014). Chromosome condensation begins at the onset of mitosis, and is mediated by the phosphorylation of histone H3 on Ser10, which becomes PH3 (Hendzel et al., 1997). Levels of PH3 are maximal in metaphase, start declining during anaphase and are minimal during telophase, making phosphorylation of histone H3 an event unique to mitosis. Staining with PH3 and analysis by immunofluorescence microscopy of HT-29 cells showed that at 2 h post treatment 4% of cells were positive for PH3 and by 24 h, 27% of cells were positive. These data are consistent with the morphology and flow

cytometry data, and provide the demonstration that L.E. accumulates approximately 30% of cells in mitosis.

There are a small number of chemical inhibitors that cause mitotic arrest by directly interacting with tubulin (Jordan et al., 1998; Chan et al., 2012). These agents have one of two effects on microtubule dynamics, either stabilizing microtubule polymerization and preventing spindle disassembly (e.g. paclitaxel), or preventing tubulin polymerization and spindle formation (e.g. nocodazole) (Wani et al., 1971; Vasquez et al., 1997; Weaver, 2014). Thus, the organization of tubulin in mitotic cells elucidates the type of action. We observed the mitotic spindle formation of L.E. treated cells, stained with  $\alpha$ -tubulin antibody and analysed by immunofluorescence microscopy. The results showed that at 2 h post treatment, the majority of mitotic cells (92%) exhibited a regular, bipolar mitotic spindle and condensed chromosomes (DAPI staining). It also appeared that the majority of mitotic cells had condensed chromosomes that were partially aligned on the metaphase plate. However, after 24 h of treatment with L.E. the majority of mitotic cells had highly distorted spindles that in some cases appeared monopolar or multi-polar. Furthermore, the condensed chromosomes were not aligned on the metaphase plate. The L.E. did not mimic the effects of either sub-class of spindle poisons as the mitotic spindle was properly assembled at the onset of mitosis. Therefore, the L.E. likely did not interfere directly with microtubule dynamics and is not a spindle poison.

The main biochemical event that regulates mitotic initiation and early mitotic events until metaphase is the activation of Cdk1 (Malumbres, 2014). This enzyme phosphorylates and activates its targets, when the inhibitory phosphates on the amino acids Thr14 and Tyr15 are removed by the Cdc25 phosphatase (Den Haese et al., 1995; Timofeev et al., 2010), and when Cdk1 is coupled to cyclin B, forming the Cdk1-cyclin B

dimer (Bloom et al., 2007; Gavet et al., 2010). The metaphase-anaphase transition is instead regulated by the anaphase-promoting complex (APC/C), which enables cells to exit mitosis (Vermeulen et al., 2003). The APC/C is a E3 ubiquitin ligase complex that marks target proteins for degradation (Thornton et al., 2006), and the ubiquitinated proteins are degraded by the 26S proteasome (Baumeister et al., 1998; Glickman et al., 2002). The two main targets of this complex are cyclin B and securin, whose degradation allows the cells to progress to anaphase and later to exit mitosis (Cohen-Fix et al., 1996; Thornton et al., 2003). A known inhibitor of the 26S proteasome is MG132, which was used as a control in experiments testing the degradation of cyclin B and securin (Guo et al., 2013). L.E. treated cells contained high levels of cyclin B and high levels of securin after 2 and 24 h of treatment, whereas total and interphase populations did not. Cells treated with MG132 did not contain high amounts of securin, indicating that the L.E. acts upon a different target than MG132. Neither of the targets of the APC/C were degraded, suggesting that the L.E. likely arrested cells at the metaphase-anaphase transition. The observation that treated mitotic cells do not proceed to anaphase led to the conclusion that L.E. arrests cells at a prometaphase-like stage, consistently with the partial alignment of condensed DNA on the metaphase plate.

The third hallmark of cancer is genome instability and mutation. When the DNA is damaged, a cascade of events is activated to repair the damage (Zhou et al., 2000). During activation of the DNA damage response, the histone H2AX variant is phosphorylated on Ser139 1-3 minutes after the damage, becoming histone  $\gamma$ H2AX (Rogakou et al., 1998; Ward et al., 2001; Keogh et al., 2006). The histone  $\gamma$ H2AX is dephosphorylated when the damage is repaired (Nazarov et al., 2003; Chowdhury et al., 2005), making  $\gamma$ H2AX foci a suitable marker for DNA damage (Furuta et al., 2003; Mah

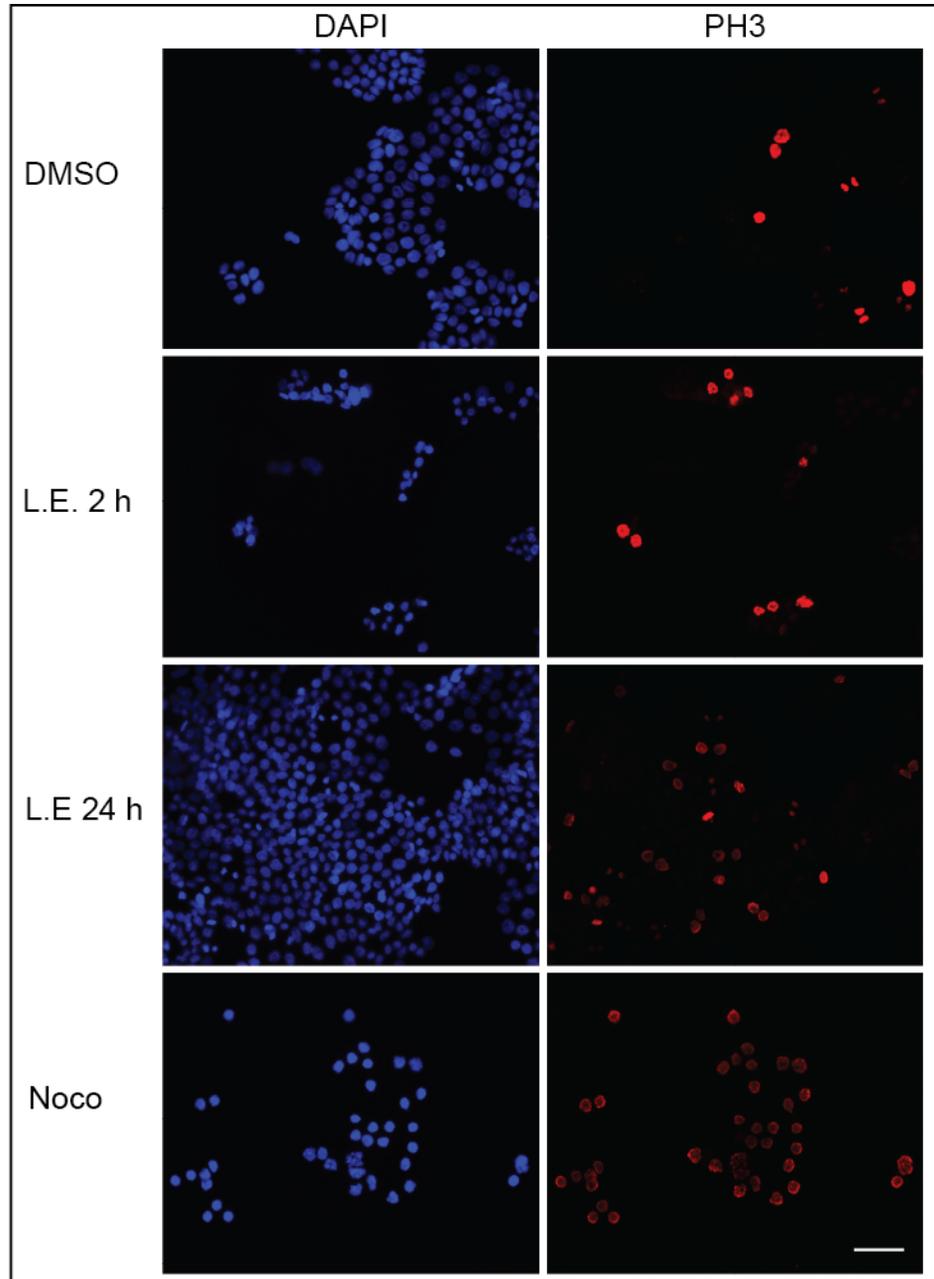
et al., 2010). CPT is a known genotoxic agent, and it acts by inhibiting the topoisomerase I enzyme (Hsiang et al., 1985; Froelich-Ammon et al., 1995). CPT treatment induces the formation of pan-nuclei  $\gamma$ H2AX staining, and was used as a positive control for DNA damage. Immunofluorescence microscopy analysis of HT-29 cells treated with the L.E. and stained with  $\gamma$ H2AX antibody revealed that L.E. treatment induced DNA damage as early as 2 h, but the  $\gamma$ H2AX signal was located in discrete foci and did not exhibit pan-nuclei staining like CPT. Furthermore, at 2 h post treatment there was a small percentage of cells that were positive for  $\gamma$ H2AX staining compared to the 2 h sample. Of these cells, all of them displayed less than or equal to 5 foci, and were mitotic cells. By 24 h however, there were 12 times more mitotic cells that were positive for  $\gamma$ H2AX, and of these the majority had more than 5 foci. These data suggest that the L.E. is genotoxic to HT-29 cells, but it acts through a different mechanism than CPT, judging by the different type of  $\gamma$ H2AX staining pattern. In particular, it seems that DNA damage accumulates as the cells spend more time arrested in mitosis. These observations were supported by the confocal microscopy data, which showed that the discrete  $\gamma$ H2AX foci accumulated after 24 h of treatment with the L.E.

In conclusion, we determined that the *G. aristata* L.E. arrested cells in mitosis. This observation was supported by the increase of cells that were positive for PH3 for the presence of the mitotic spindle. At 2 h post L.E. treatment, mitotic cells contained spindles that were correctly assembled, and condensed chromosomes that partially aligned to the metaphase plate, suggesting a prometaphase-like arrest. By 24 h, the spindles were severely distorted and appeared to be monopolar or multipolar, and the condensed chromosomes were no longer aligned to the metaphase plate. Biochemical analysis also confirmed that L.E. arrests cells in mitosis. Mitotic L.E. treated cells had

active Cdk1 enzyme (not phosphorylated on Tyr15) that was coupled to cyclin B, whose levels were upregulated. In addition, a Cdk1 substrate, PP1 $\alpha$ , was present in its phosphorylated state, meaning that Cdk1 was active in mitotic cells. Furthermore, the L.E. interfered with the degradation of securin and cyclin B as their levels were upregulated in mitotic cells, suggesting a failed activation of the APC/C complex and a metaphase arrest. Finally, as cells arrested in mitosis after treatment with the L.E., they accumulated damaged DNA in discrete foci, whereas the positive control CPT caused the accumulation of many smaller foci. We speculate that the L.E. causes a prometaphase-like arrest induced by the failed degradation of cyclin B and securin, likely due to the interference of the L.E. with the ubiquitin-proteasome pathway. The inhibition of securin degradation could result in an abnormal tension on the kinetochores, which later causes the distortion of the spindle fibers. As the spindles continue to separate the sister chromatids towards opposite poles, the chromatids are still held together by the cohesin proteins. The failed attempt at segregating chromatids could also explain the DNA rupture in correspondence of what are likely kinetochores, staining positive for  $\gamma$ H2AX in specific foci.

There are several important questions that remain unanswered based upon these results. First, what is the precise target that is inhibited by the L.E. extract? Is there a second arrest point in the cell cycle that accounts for the result of 30% mitotic cells in a treated population whereas the remaining 70% arrested elsewhere? Before these questions can be answered, it would be essential to purify the chemical(s) responsible for the biological phenomena that we observed. We decided to attempt to isolate the chemical by biology-guided fractionation, using the rounded morphology as an assay.

A



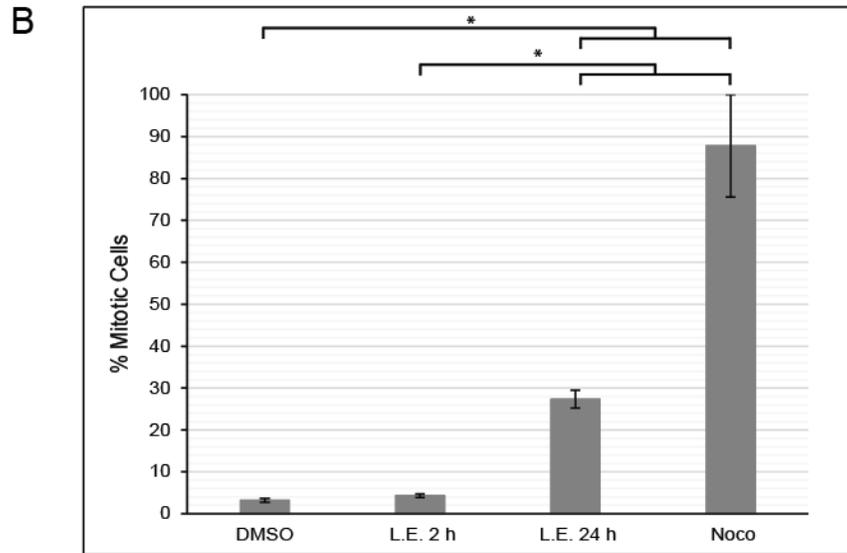
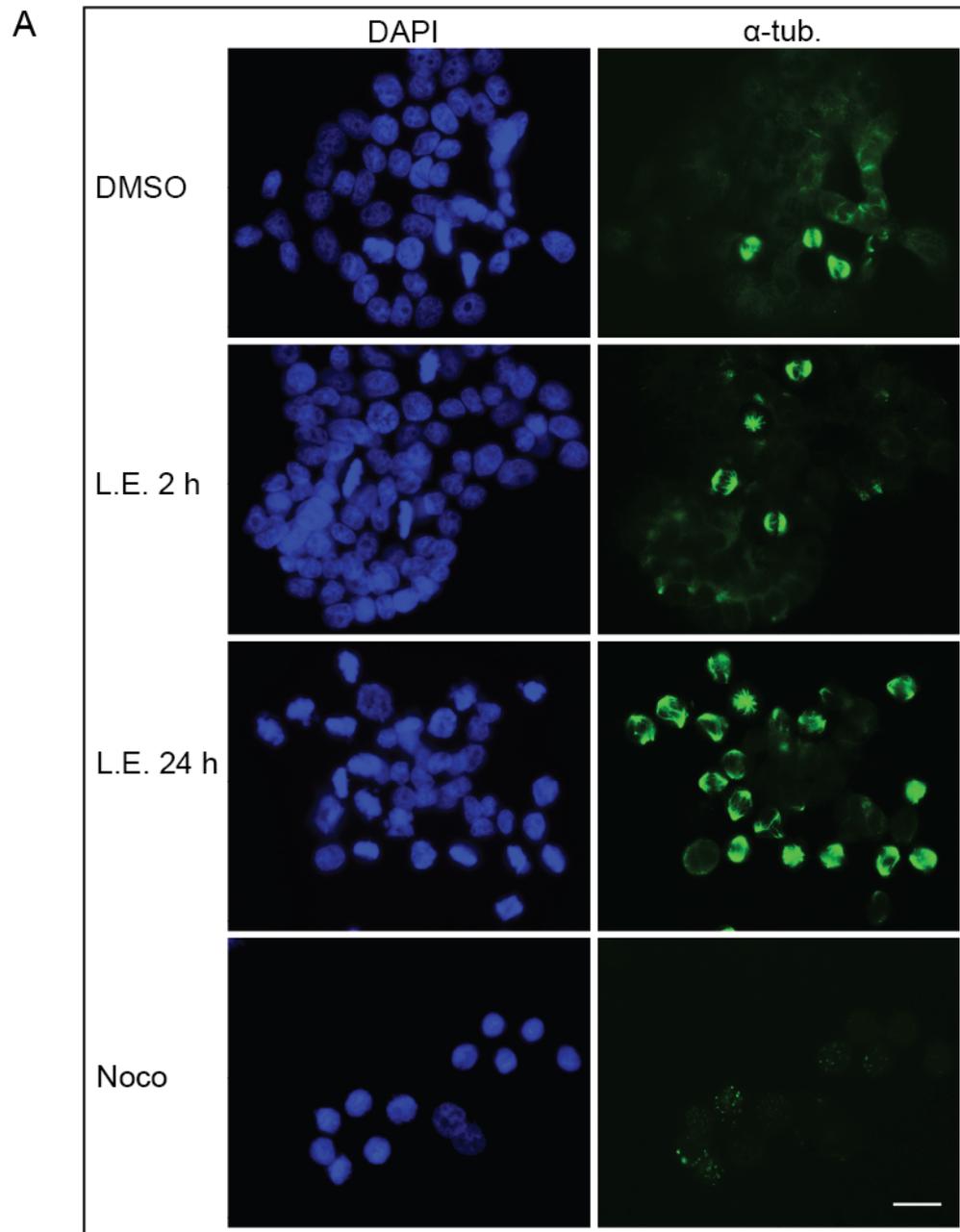


Figure 4.1: Rounded HT-29 cells that were treated with L.E. for 2 or 24 h are positive for PH3, indicating that they are in mitosis. A) HT-29 cells were treated with either DMSO, 50  $\mu\text{g}/\text{mL}$  L.E. for 2 or 24 h, or 200  $\text{ng}/\text{mL}$  nocodazole and stained with DAPI (blue) to detect DNA and with anti-phospho-Ser10 histone H3 antibodies (PH3) (red). Cells were analysed by immunofluorescence microscopy and representative images are shown. Scale bar equals 20  $\mu\text{m}$ . B) The percentages of cells staining positive for phospho-Ser10 histone H3 (PH3) after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells staining positive for PH3 were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .



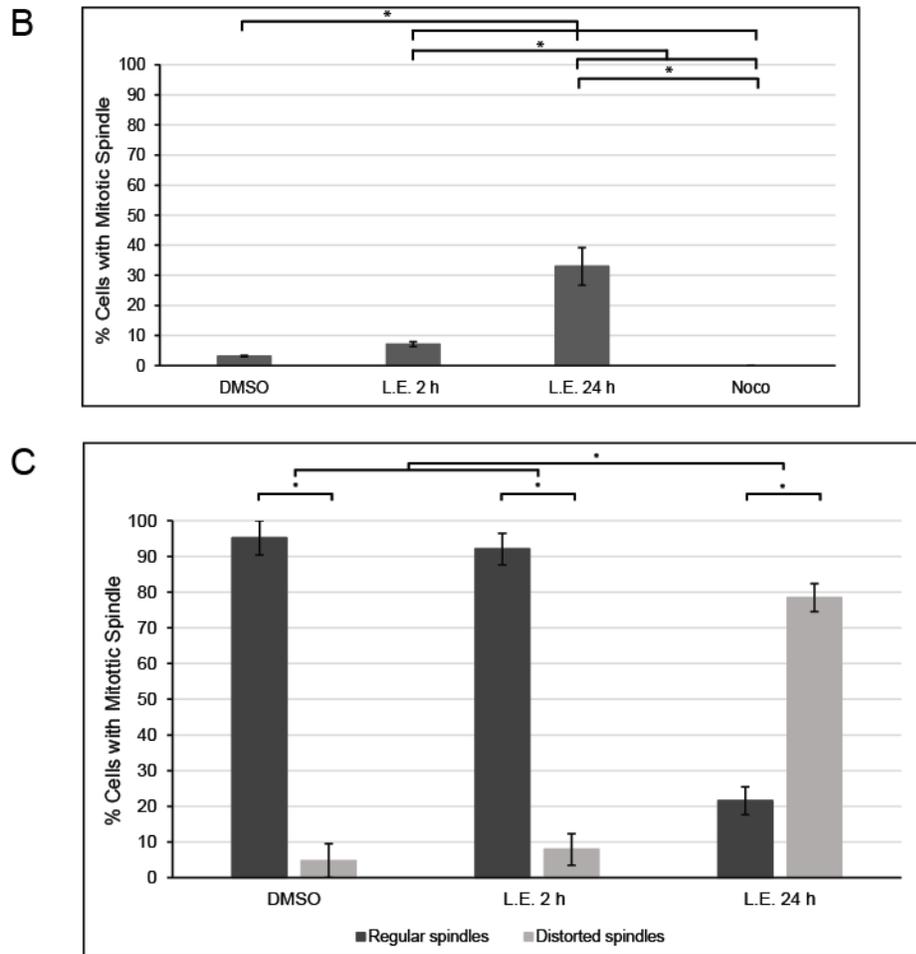


Figure 4.2: Rounded HT-29 cells treated with L.E. for 2 h exhibit regular mitotic spindles, whereas cells that were treated for 24 h exhibit distorted, irregular mitotic spindles. A) HT-29 cells were treated with either DMSO, 50  $\mu\text{g}/\text{mL}$  L.E. for 2 or 24 h, or 200  $\text{ng}/\text{mL}$  nocodazole and stained with DAPI (blue) to detect DNA and with anti- $\alpha$ -tubulin antibodies to detect microtubules (green). Cells were analysed by immunofluorescence microscopy and representative images are shown. Scale bar equals 20  $\mu\text{m}$ . B) The percentages of cells exhibiting a mitotic spindle after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells positive for a mitotic spindle were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ . C) The percentages of cells exhibiting regular or distorted mitotic spindle after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells with a regular or distorted mitotic spindle were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .

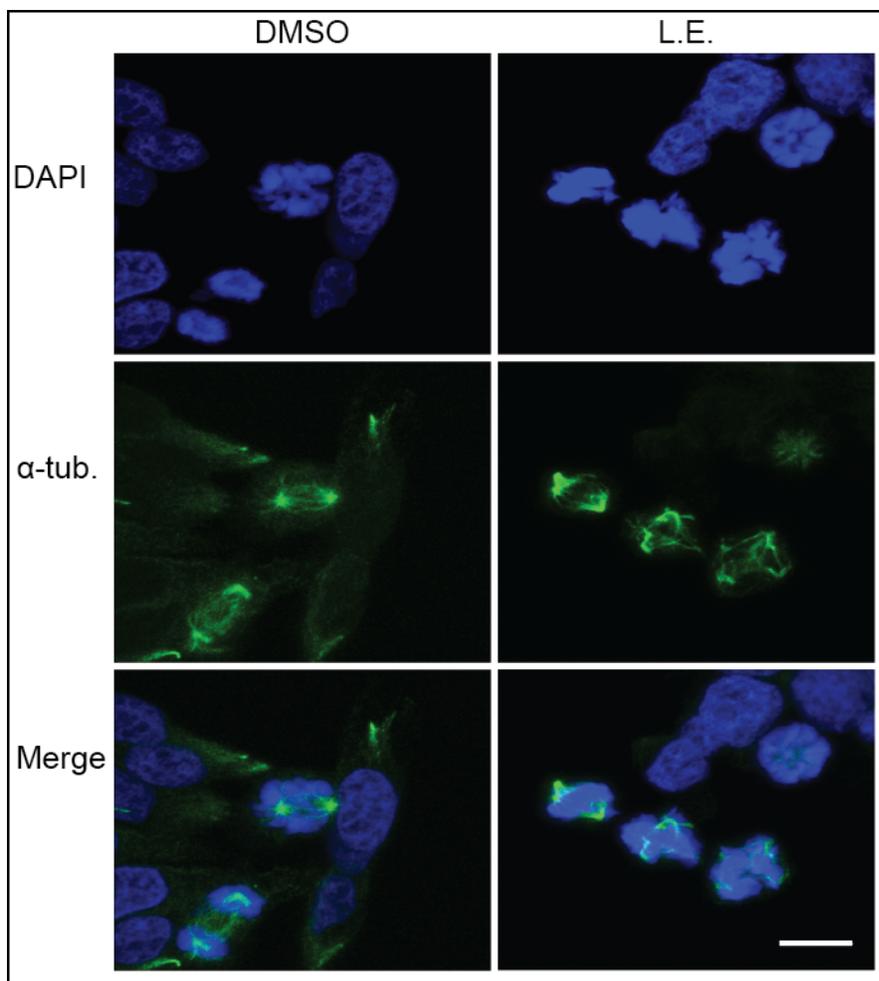


Figure 4.3: The L.E. causes a distortion of the mitotic spindles over 24 h of treatment. HT-29 cells were treated with DMSO or 50  $\mu\text{g}/\text{mL}$  L.E. and stained with DAPI (blue) to detect DNA and with anti- $\alpha$ -tubulin antibodies to detect microtubules (green). Cells were analysed by confocal microscopy and representative images are shown. Scale bar equals 10  $\mu\text{m}$ .

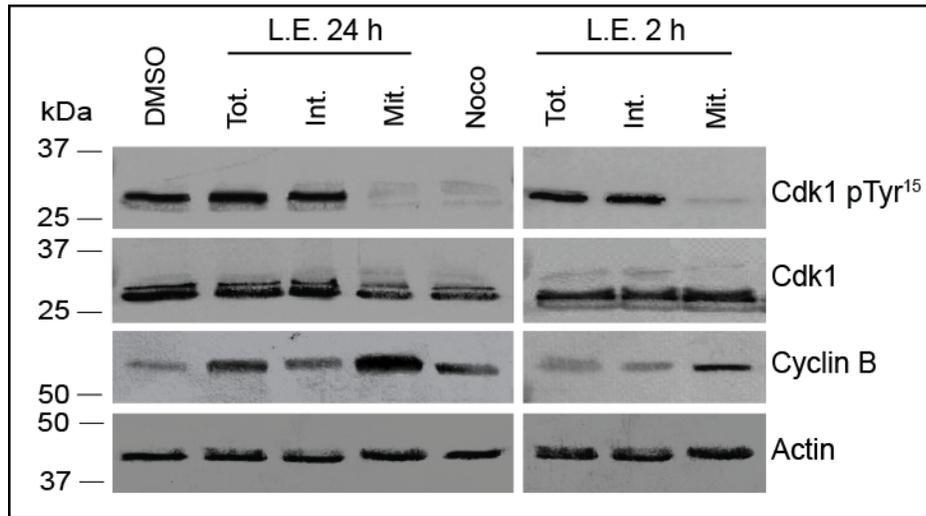


Figure 4.4: L.E. causes a downregulation of pTyr15-Cdk1 and an upregulation of cyclin B in rounded cells, indicating that these cells are mitotic. HT-29 cells were treated with either DMSO, 50  $\mu\text{g}/\text{mL}$  L.E. for 2 or 24 h, or 200  $\text{ng}/\text{mL}$  nocodazole. Mechanical shake-off was used to separate treated rounded (mitotic) cells from flattened interphasic cells and DMSO treated, L.E. treated total, interphasic and mitotic and nocodazole treated mitotic cell extracts were prepared. Cell extracts were analysed by western blotting using anti-Cdk1, anti-phospho Tyr15 Cdk1, anti-cyclin B and anti-actin antibodies. Actin is used as a loading control. Molecular masses are indicated in kDa.

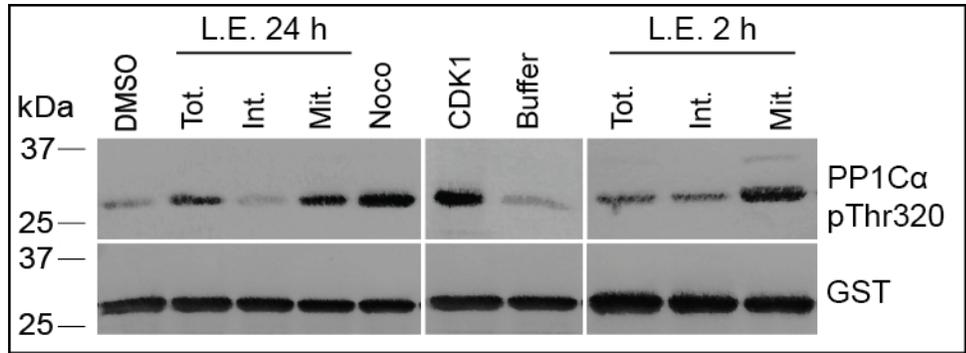


Figure 4.5: Rounded HT-29 cells treated with L.E. contain active Cdk1. HT-29 cells were treated either with DMSO, with 50  $\mu\text{g}/\text{mL}$  L.E. for 2 or 24 h, or with 200  $\text{ng}/\text{mL}$  nocodazole. Mechanical shake-off was used to separate treated rounded (mitotic) cells from flattened interphasic cells and DMSO treated, L.E. treated total, interphasic and mitotic and nocodazole treated mitotic cell extracts were prepared. Cell extracts were incubated with an artificial GST-Thr320-PP1C $\alpha$  substrate. Extraction buffer was used as a negative control, and purified Cdk1 was used as a positive control. Samples were analysed by western blotting using anti-phospho Thr320 PP1C $\alpha$  and anti-GST antibodies. Molecular masses are indicated in kDa.

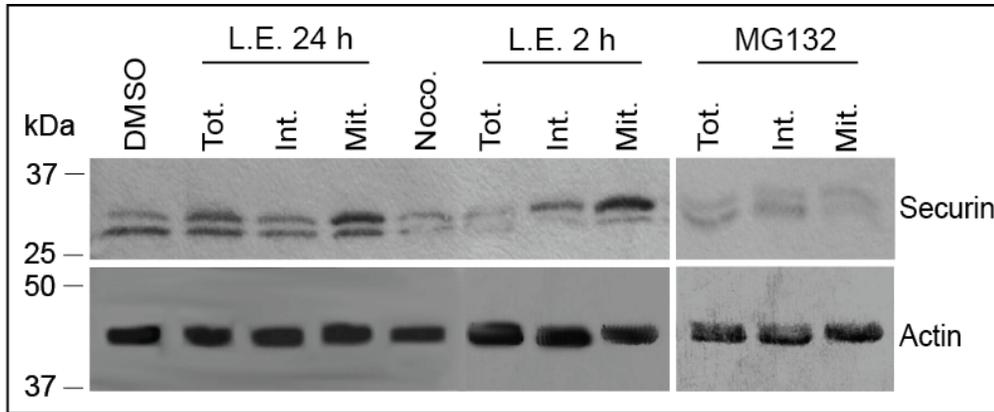
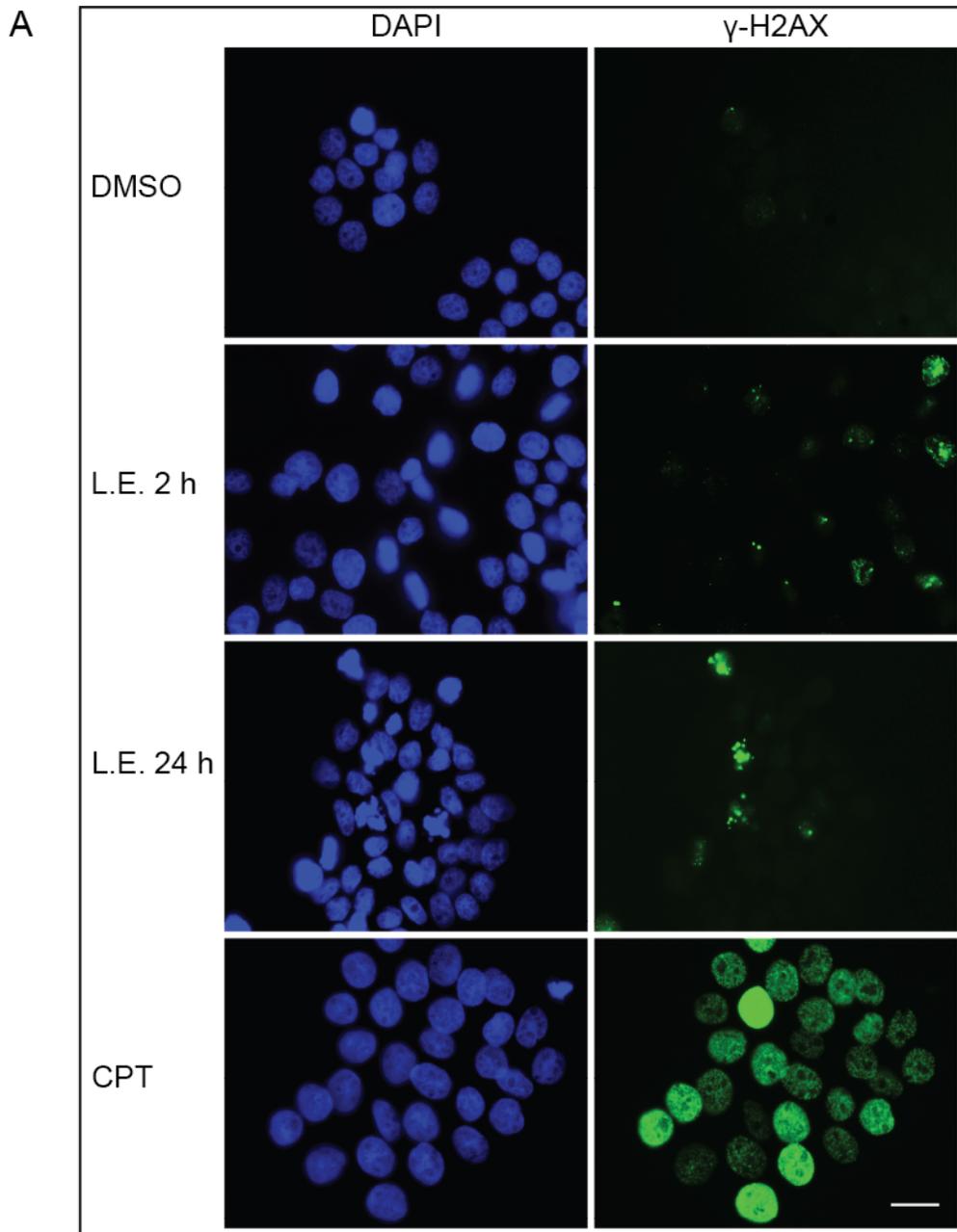


Figure 4.6: Rounded HT-29 cells that were treated with L.E. for 2 or 24 h contain securin, whereas cells treated with MG132 or nocodazole do not. HT-29 cells were treated either with DMSO, with 50  $\mu\text{g}/\text{mL}$  L.E. for 2 or 24 h, or with 200  $\text{ng}/\text{mL}$  nocodazole. Mechanical shake-off was used to separate treated rounded (mitotic) cells from flattened interphasic cells and DMSO treated, L.E. treated total, interphasic and mitotic, MG132 treated total, interphasic and mitotic and nocodazole treated mitotic cell extracts were prepared. Cell extracts were analysed by western blotting using anti-securin and anti-actin antibodies. Actin is used as a loading control. Molecular masses are indicated in kDa.



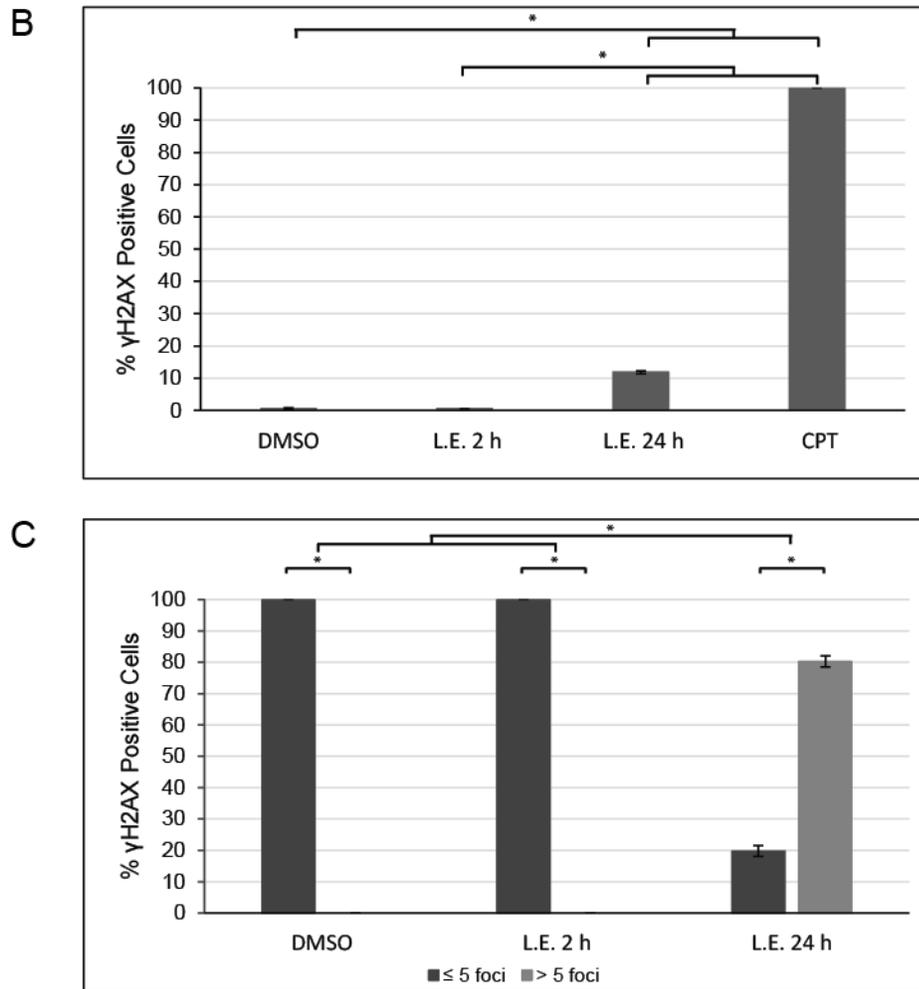


Figure 4.7: L.E. induces damaged DNA in treated mitotic HT-29 cells that accumulates over 24 h of treatment. A) HT-29 cells were treated with either DMSO, 50  $\mu$ g/mL L.E. for 2 or 24 h, or 50 nM CPT and stained with DAPI (blue) to detect DNA and with anti-histone  $\gamma$ H2AX antibodies to detect damaged DNA (green). Cells were analysed by immunofluorescence microscopy and representative images are shown. Scale bar equals 20  $\mu$ m. B) The percentages of cells containing damaged DNA after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells positive for histone  $\gamma$ H2AX were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ . C) The percentages of cells containing less than or equal to 5  $\gamma$ H2AX foci or more than 5 foci after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells with less than or more than 5 foci were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .

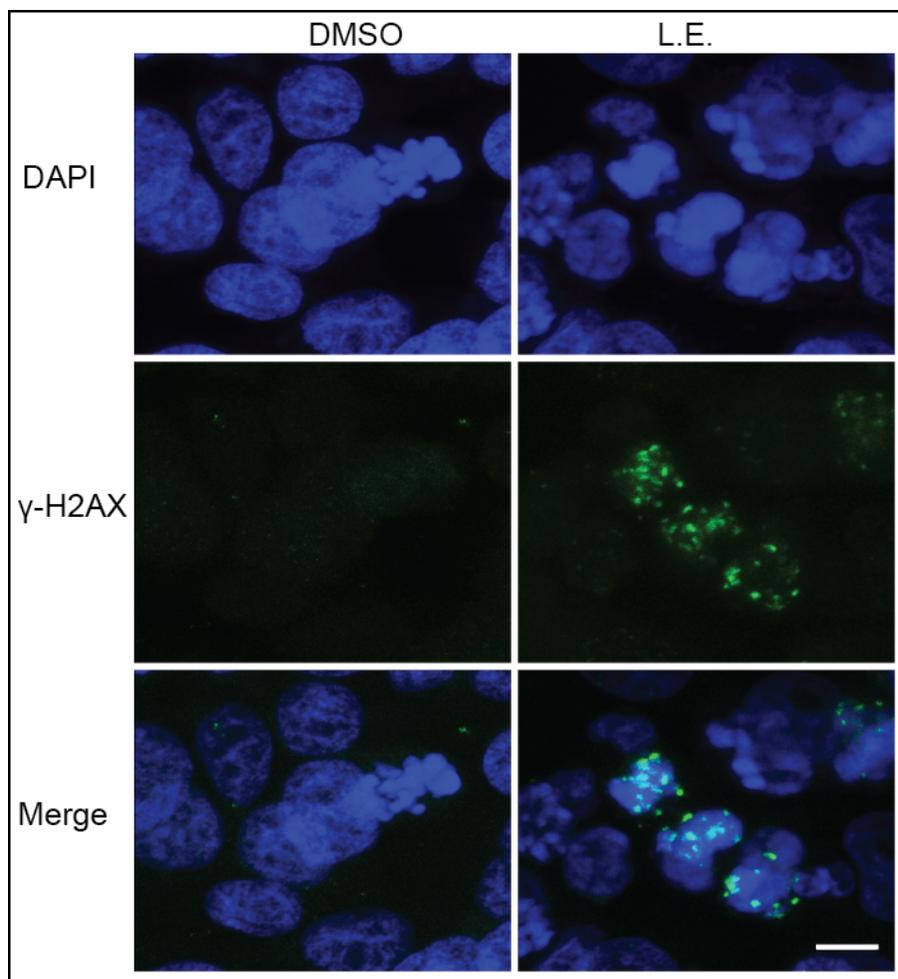


Figure 4.8: L.E. causes an accumulation of damaged DNA in treated mitotic HT-29 cells. HT-29 cells were treated with DMSO, or 50  $\mu\text{g}/\text{mL}$  L.E. for 24 h and stained with DAPI (blue) to detect DNA and with anti-histone  $\gamma\text{H2AX}$  antibodies (green) to detect damaged DNA. Cells were analysed by confocal microscopy and representative images are shown. Scale bar equals 10  $\mu\text{m}$ .

## CHAPTER 5

### **Identification of a chemical from *Gaillardia aristata* leaves that induces a mitotic arrest**

#### **5.1 Abstract**

We had characterized an extract prepared from *Gaillardia aristata* leaves that had anti-mitotic and genotoxic properties against a colon carcinoma cell line. We wanted to isolate the compound(s) that were responsible for the mitotic phenotype that we observed. The isolation of the bioactive compound(s) can lead to the determination of its structure, and identification of the protein(s) that interact with it. In addition, further characterization of the cellular response would be best done with pure compounds to ensure that we are studying one or few pathways at a time. In collaboration with Dr. Raymond Andersen, a natural product chemist, we used the bioassay-guided fractionation approach. By this method, we successfully isolated and identified the compound Puchelloid A, which induces the mitotic phenotype when added to HT-29 cells. This is the first description of this type of activity for this compound, and may represent a novel activity to a subclass of sesquiterpene lactone molecules, of which Pulchelloid A is a member.

#### **5.2 Introduction**

A major challenge in the field of natural products is to identify the compounds with bio-activities of scientific interest. Isolation of compounds can lead to the

identification of the structure of the compounds and their bioactivity, including their molecular targets. Isolation of the active compound(s) allows their structural determination, also opening the possibility of synthesizing them or using the compounds as sources for structural modifications and rationalization of the mechanism of action (Colegate et al., 2007). The bioactivity of the compound can be investigated in relation to its 3D structure, determining which chemical group is responsible for the inhibition of a target(s) (structure-activity relationship studies, SAR). Once the SAR of the bioactive compound is known, the development of new compounds with similar or more desirable properties can also be achieved, reducing dependency on the original source. Bioassay-guided fractionation is aimed at isolating substances that show activity in a particular bioassay or set of bioassays, and ultimately determining the structure of the biologically relevant small molecules (Mander et al., 2010). It integrates the processes of separation of compounds in a mixture, using different analytical methods, with results obtained from biological testing (Koehn et al., 2005). Specifically, it consists of sequential cycles of biological testing to evaluate the activity followed by the resolution of compounds in a separation step. It is a successful technique that has been extensively used in drug discovery and in many other different fields, such as microbiology (Jamil et al., 2012), medical research (Colombo et al., 2015), and ultimately cancer (Mehta et al., 2002; Kinghorn et al., 2003).

Sesquiterpenes are a large and diverse class of plant-derived compounds and are one of the most biologically relevant classes of secondary metabolites, and are comprised of approximately 5000 members (Heinrich et al., 1998; Wedge et al., 2000; Chadwick et al., 2013). These compounds are present in many plant families but are produced mainly by the Asteraceae species, which are the source of over 3000 reported structures (Robles

et al., 1995; Heinrich et al., 1998; Zhang et al., 2005). Sesquiterpene lactones are characterized by an  $\alpha,\beta$ -unsaturated carbonyl group found in an  $\alpha,\beta$ -unsaturated cyclopentenone or  $\alpha$ -methylene- $\gamma$ -lactone (Zhang et al., 2005). This group is highly reactive with nucleophiles (e.g. sulfhydryl groups in cysteines), and interacts with several cellular proteins, causing the inhibition of several of cellular functions, ultimately leading to apoptosis (Dirsch et al., 2001). Due to their high reactivity, sesquiterpene lactones were reported to be toxic to various organisms such as fungi and bacteria and repel or poison grazing herbivores and insects, defending the plant that produces them (Burnett et al., 1974; Lee et al., 1977; Picman, 1986; Rossiter et al., 1986; Barrero et al., 2000; Barrero et al., 2005). Sesquiterpene lactones are active on humans as well, and were reported to cause irritation and contact dermatitis when people had physical contact with plants that produced them, such as *Gaillardia aristata* (Burry, 1980; Arlette et al., 1981; Paulsen, 1992). On a smaller scale, these sesquiterpenes have anti-inflammatory effects through inhibition of the NF- $\kappa$ B complex and reduction of the inflammatory response (Rüngeler et al., 1999). This class of compounds also has anti-cancer properties showing cytotoxicity, inhibition of telomerase activity, induction of apoptosis, disruption of cell cycle, inhibition of the NF- $\kappa$ B pathway and consequent inhibited transcription of genes downstream of NF- $\kappa$ B and downregulation of proteins involved in the control of cell proliferation and survival (Lee et al., 1971; Huang et al., 2005; Baud et al., 2009).

The antitumour activities of sesquiterpenes were reviewed in chapter 2. One emerging anti-cancer property of this class of compounds is the anti-mitotic activity. However, of the 5000 sesquiterpenes, only 17 of them were shown to inhibit proliferation of cancer cells at the G<sub>2</sub>/M-phase of the cell cycle.

We found that the active compound from the L.E. is Pulchelloid A, a sesquiterpene lactone. Pulchelloid A is a pseudoguaianolide-type of sesquiterpene lactone with a central 7-member ring fused to a 5-member ring and an  $\alpha$ -methylene- $\gamma$ -lactone. This compound was first isolated with a chloroform extraction from the dried whole *Gaillardia pulchella* plant, along with the related compounds Puchelloid B and neopulchellin (Inayama et al., 1982).

The initial *G. aristata* L.E. was fractionated by our collaborator Dr. Raymond Andersen and his team at the University of British Columbia (the fractionation steps will not be described here). After the purified fractions were separated, we tested them to confirm the anti-mitotic activity and identified the fraction that contained it, until a purified active compound was isolated, Pulchelloid A. The bioactivities of Pulchelloid A have not been previously investigated, and in this chapter we report that we have identified and characterized these bioactivities. We then repeated the tests from chapter 3 and chapter 4 to investigate whether Pulchelloid A showed the same activity as the crude extract or it was the result of the interaction between different chemicals that produced the mitotic arrest.

## **5.3 Materials and Methods**

### *5.3.1 Second collection of Gaillardia aristata*

We had established a collaboration with Dr. Raymond Andersen to purify the active molecule from the leaves of *Gaillardia aristata*. We agreed to send plant material to prepare new extracts and fractionate them. In summer 2015 we went on a second harvest and collected 16 kg of wet plant. *Gaillardia aristata* aerial plant parts were collected at an undisturbed site on the Porcupine Hills, with the following GPS

coordinates: 49°57'14.3"/ -114°04'21.4" (Porcupine Hills, at 1683 meters, 08/07/2015). During this harvest the amount of wet plant collected was 16 kg. The harvest of plants was undertaken by sustainable practice; one every two plants was collected when they were abundant, whereas only one in five was collected when they were scarce. Plant taxonomy was confirmed to species based on documents of the Flora of southern Alberta (Kuijt, 1982; Moss et al., 1983; G.P.F.A. et al., 1986), and verified by Dr. John Bain, the director of the University of Lethbridge Herbarium. Information including collection date, site description, associated species and location were stored with a voucher specimen in the Herbarium database. Following the harvest, plants were dried (at room temperature for the first day, then in an oven at 40-50°C for three or four days) and the different parts were separated (flower heads, stems and leaves), catalogued and stored in paper bags in a dry environment at room temperature until extraction.

### 5.3.2 Preparation of a dichloromethane extract from *Gaillardia aristata* leaves

Plant extracts were prepared by grinding of dried material with a mortar and pestle or a blender to a fine powder. The powder was suspended to 10% (w/v), with dichloromethane (DCM), and stirred. After overnight incubation at room temperature the suspension was filtered to collect the soluble fraction, which was dried with a roto evaporator, then placed in an oven at 40°C for eight hours. The dried material was collected, weighed, labeled, given a code number and stored in darkness at room temperature. The *G. aristata* leaf extract was shipped to Dr. Raymond Andersen's laboratory at the University of British Columbia to be fractionated.

### 5.3.3 Cell culture

The human cell line HT-29 (ATCC HTB-38) was obtained from the American Type Culture Collection (ATCC). The colorectal carcinoma cell line HT-29 was

maintained in RPMI 1640 medium (Gibco; 21870-092) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco; 12484028) and 1.6 mM GlutaMAX (Gibco; 35050-061). Cells were grown at 37°C in 5% CO<sub>2</sub> and the media were changed every two-three days. HT-29 cells were plated at a density of 3.0 x 10<sup>5</sup> cells/25 cm<sup>2</sup> flask and cultured for 48 h prior to treatment. The compounds CPT (Sigma; 7689-03-4) and nocodazole (Sigma; M1404-10MG) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D2438) to a concentration of 10 mM and 200 µg/mL respectively. The extracts and the fractions were suspended in DMSO at 100 mg/mL and at 1 or 5 mg/mL, respectively, and all extracts and other compounds listed were stored at -20°C. In not-treated (NT) cells DMSO was added to a final concentration of 0.1% (v/v) as a solvent vehicle control.

#### *5.3.4 Chemical Fractionation*

The chemical fractionation was performed by Dr. Andersen and his colleagues at the Department of Chemistry, University of British Columbia, Vancouver, BC. The procedure will not be described here. The original extract was fractionated a first time, and tested in a morphology assay. The active fractions underwent a second and third cycle of fractionation and testing.

#### *5.3.5 Light microscopy*

HT-29 cells were seeded at 1.0 x 10<sup>5</sup>/well in a 6 well culture plate and incubated at 37°C for 48 h prior to treatment. Images were captured at room temperature with an Infinity 1 camera operated by Infinity Capture imaging software (Lumenera Corporation) on an Olympus CKX41 inverted microscope using an Olympus LUCPlanFLN 20x objective with 0.45 numerical aperture. Images were processed using Adobe Photoshop

(CC 2014.1.0). Rounded cells were counted using Image J software (IJ 1.46r). All experiments were performed three times, except where otherwise specified.

### *5.3.6 Immunofluorescence microscopy*

HT-29 cells were plated on glass coverslips at  $1.0 \times 10^5$ /well in a 6 well culture plate and incubated at 37°C for 48 h prior to treatment. After treatment, cells were fixed at room temperature for 20 min in 3% (v/v) formaldehyde (Ted Pella Inc; 18505), diluted in PBS. Fixation was quenched with 50 mM NH<sub>4</sub>Cl in PBS and cells were permeabilised for 5 minutes using 0.2% (v/v) Triton X-100 in PBS and blocked for 30 minutes with 3% (w/v) BSA in PBS-T (0.1% (v/v) Tween-20 diluted in PBS). Cells were then incubated with primary antibodies as described: anti-histone  $\gamma$ H2AX (Millipore; 05-636; 1:400) for 1 h at room temperature; anti-phospho-Ser10 histone H3 (Millipore; 06-570(CH); 1:1000) for 18 h at 4°C, and anti- $\alpha$ -tubulin (Santa Cruz Biotechnology; sc-53030; 1:200) for 2 h at room temperature. After washing with PBS-T, cells were incubated with secondary antibodies for 1 h at room temperature as follows: Alexa Fluor 488-conjugated anti-mouse (Life Technologies; A11059; 1:400) for anti-histone  $\gamma$ H2AX, Texas Red-conjugated anti-rabbit (Jackson ImmunoResearch; 111-075-003; 1:400) for anti-phospho-Ser10 histone H3 and Alexa Fluor 488-conjugated anti-rat (Life Technologies; A11006; 1:400) for anti- $\alpha$ -tubulin. Nuclei were stained with 300 nM DAPI (4',6-diamidino-2-phenylindole) in PBS for 15 minutes and coverslips were mounted onto microscope slides using ProLong Gold Antifade reagent (Molecular probes; P36934). Cells were observed at room temperature on an Olympus BX41 microscope using either an Olympus UPlanFL N 20x objective with 0.50 numerical aperture or an Olympus UPlanFL N 60x objective with 1.25 numerical aperture. Images were captured using an Infinity 3 camera operated

by Infinity Capture imaging software (Lumenera Corporation), within the linear range of the detectors. Images were prepared using Adobe Photoshop (CC 2014.1.0) software. Cells positive for histone  $\gamma$ H2AX, phospho-Ser10 histone H3 and  $\alpha$ -tubulin were counted using Image J (IJ 1.46r) software. At least 20 cells were counted for each treatment, unless otherwise stated, and experiments were performed three times.

### *5.3.7 Statistics*

Data and statistical analysis were performed using Microsoft Excel 2010 software. Data were plotted as means from three separate experiments  $\pm$  standard errors of the means. Statistical significance was calculated using the student's t-test for two paired sample means and values were considered significantly different when  $p < 0.05$ .

## **5.4 Results**

### *5.4.1 Two fractions are active in a morphology assay*

We collaborated with Dr. Raymond Andersen to isolate and identify chemicals that induced mitosis from the original L.E. using biology-guided fractionation. We first provided a dichloromethane extract, which we confirmed had mitotic activity as described in previous chapters. In the first round the dichloromethane extract (see section 5.3.3) was sent to Dr. Andersen, who performed a first separation of the different groups of chemicals using High Performance Liquid Chromatography (HPLC), and we were able to identify, at a first screen, a fraction with anti-mitotic activity. Then, the active fraction underwent a second round of fractionation, and all subfractions were tested on HT-29 cells (Figure 5.1). A concentration curve was performed to find which concentrations could be used, and 4.5  $\mu$ g/mL was chosen as the concentration that induced the highest number of cells to become rounded.

HT-29 cells were then treated with 4.5  $\mu\text{g}/\text{mL}$  of the fractions (only the most representative fractions were shown, which were fraction 9, 10, 11, 12, and 13). DMSO, 50  $\mu\text{g}/\text{mL}$  L.E. and 200  $\text{ng}/\text{mL}$  nocodazole were used as controls, and photos were taken at 24 h. Figure 5.1 shows all the active fractions and those that were contiguous when separated. The most active fractions were fraction 10 and fraction 11, which induce a rounded cell morphology, whereas fraction 12 is less active and fraction 9 and 13 did not have any cell rounding activity. Therefore, we pursued our investigation using fractions 10 and 11.

A second cycle of biology-guided fractionation was performed, to obtain more material and identify the compound(s) present in fraction 11. After *Gaillardia aristata* leaves were dried and separated from the other plant parts, 714 g of dried material were sent to Dr. Raymond Andersen. Dr. Andersen performed the fractionation and isolated the sesquiterpene lactone Pulchelloid A, which was later confirmed to be the active molecule present in fraction 11, in a third round of biology-guided fractionation.

#### 5.4.2 Fraction 10 and 11 display similar activities

Having observed a mitotic activity in fractions 10 and 11, we compared their activities to determine whether they induced similar percentages of cells to become rounded. HT-29 cells were treated with concentrations of ranging from 0.05 to 15  $\mu\text{g}/\text{mL}$  of each subfraction, and the number of rounded cells was counted at 24 h post treatment (Figure 5.2). The number of rounded cells in fraction 11 increased in a concentration-dependent manner and it reached a maximum at 4.5  $\mu\text{g}/\text{mL}$  with approximately 50% of rounded cells. At 15  $\mu\text{g}/\text{mL}$  the number of rounded cells declined to  $22.2 \pm 2.3\%$ . The percentage of rounded cells treated with different concentrations of fraction 10 reached

31.5 ± 3.5% at 4.5 µg/mL and remained at similar values. The number of rounded cells after treatment with fraction 10 at 4.5 µg/mL (31.5 ± 2.5%) was lower compared to fraction 11 at 4.5 µg/mL (51.5 ± 1.9%) and remained similar to this value with higher concentrations, whereas the percentage of rounded cells treated with fraction 11 decreased at concentrations higher than 4.5 µg/mL. The most active fraction was fraction 11, thus we performed all the remaining experiments using this fraction. An interpretation of the activity in fraction 10 is that this fraction might contain compounds that are related to Pulchelloid A, and have a lower activity. Another possibility is that this fraction contained contaminants that partially inhibited the activity of the compounds.

#### *5.4.3 HT-29 cells treated with fraction 11 express a mitotic marker*

To determine whether fraction 11 maintained the same anti-mitotic activity that we identified in the L.E., we investigated if the rounded cells were mitotic. HT-29 cells were treated with DMSO, 4.5 µg/mL fraction 11 for 2 and 24 h, and 200 ng/mL nocodazole, and were analysed for PH3 staining by immunofluorescence microscopy (Figure 5.3). The percentages of cells that were positive for PH3 staining in DMSO, 2 and 24 h fraction 11 and nocodazole treated cells were, respectively, 3.2 ± 0.4%, 9.2 ± 1.6%, 28.4 ± 1.7% and 87.8 ± 12.2% (Figure 5.3A and 5.3B), indicating that the rounded cells previously observed by light microscopy were mitotic.

#### *5.4.4 Fraction 11 induces a mitotic arrest and distortion of mitotic spindle in HT-29 cells*

We next verified if fraction 11 would induce distorted mitotic spindles, in a manner similar to that observed with L.E. treated cells. Cells were treated with 4.5 µg/mL fraction 11 for 2 and 24 h and analysed for  $\alpha$ -tubulin staining by immunofluorescence microscopy (Figure 5.4). DMSO and 200 ng/mL nocodazole were used as controls. DMSO treated cells displayed 3.2 ± 1.2% of cells with a mitotic spindle (Figure 5.4A and

5.4B) and among these,  $95.2 \pm 4.8\%$  had regular, bipolar spindles, whereas  $4.8 \pm 4.8\%$  featured a slightly distorted mitotic spindle (Figure 5.4C). At 2 h post treatment with fraction 11,  $11.1 \pm 1.4\%$  cells exhibited a mitotic spindle,  $93.0 \pm 1.9\%$  of which had a regular spindle and  $7.0 \pm 1.9\%$  of which displayed a slightly distorted spindle. By contrast, 24 h after treatment with fraction 11  $28.5 \pm 1.7\%$  cells had a mitotic spindle and  $89.4 \pm 3.6\%$  of mitotic cells had a highly distorted spindle. Overall, the number of mitotic cells accumulated after treatment with fraction 11 was consistent with the light microscopy morphology assay and the PH3 staining. In addition, fraction 11 treated cells had a distorted mitotic spindle, a result that was similar to that of the L.E. treated cells. These data suggest that the anti-mitotic activity of the L.E. is maintained in fraction 11, thus fraction 11 most likely contains the active molecule(s).

#### *5.4.5 Mitotic cells that are treated with fraction 11 contain damaged DNA*

We investigated if cells treated with  $4.5 \mu\text{g/mL}$  fraction 11 were positive for histone  $\gamma\text{H2AX}$ . HT-29 cells were treated with  $4.5 \mu\text{g/mL}$  fraction 11 for either 2 or 24 h, and analysed for histone  $\gamma\text{H2AX}$  staining by immunofluorescence microscopy (Figure 5.5). DMSO and  $50 \text{ nM}$  CPT were used as controls. As expected, DMSO and CPT treated cells had  $0.5 \pm 0.3\%$  and  $100.0 \pm 0.0\%$ , respectively, of cells that were positive for  $\gamma\text{H2AX}$ . The percentage of cells that were positive for  $\gamma\text{H2AX}$  were  $2.5 \pm 0.1\%$ , and  $16.9 \pm 7.2\%$ , respectively, after treatment with 2 and 24 h fraction 11 (Figure 5.5A and 5.5B). We observed that the number of damaged DNA foci changed with time: at 2 hours post treatment with fraction 11 the majority of cells had few foci. More precisely,  $100.0 \pm 0.0\%$  of the cells that were positive for histone  $\gamma\text{H2AX}$  staining showed less than or equal to five foci. However, at 24 h after treatment with fraction 11, this percentage decreased

to  $12.8 \pm 7.2\%$ , and  $87.2 \pm 7.2\%$  of  $\gamma$ H2AX positive cells exhibited more than five foci. To conclude, fraction 11, similarly to the L.E., arrests cells in mitosis and induces DNA lesions in mitotic cells. By 24 h the majority of cells with damaged DNA exhibited  $\gamma$ H2AX staining in more than five foci, whereas by 2 h treatment cells had fewer foci.

#### *5.4.6 Pulchelloid A is the active compound responsible for the anti-mitotic activity*

Dr. Andersen and his laboratory performed a first fractionation, and the fractions were tested in our biological assays. After the active fraction(s) were identified, this step was repeated twice, until we purified the compound that was in fraction 11. The entire process was repeated a second time, starting with 714 g of dried material that were sent to Dr. Raymond Andersen. Dr. Andersen prepared the crude extract, performed the fractionation three times and isolated the sesquiterpene lactone Pulchelloid A, which was later confirmed to be the active molecule present in fraction 11. Therefore, we identified the compound present in fraction 11 as a sesquiterpene lactone with the name of Pulchelloid A (Figure 5.6). We then repeated the process of biology-guided fractionation with a second batch of material (see section 5.4.1), and isolated Pulchelloid A, confirming our first observation.

## **5.5 Discussion**

In this chapter by the method of biology-guided fractionation, we isolated and identified a compound from *Gaillardia aristata* leaves that induces a mitotic arrest in human cancer cells. We performed the entire process three times, from plant material that was collected at different times in different years, confirming that the compounds and the bioactivity are reliably present in this species. The compound is Pulchelloid A, which is a member of the sesquiterpene lactone family of natural products. Pulchelloid A's structure

had been determined in 1982 when it was isolated from the species *Gaillardia pulchella* (Inayama et al., 1982). There are no reports of its activity upon any biological system, therefore, we appear to be the first to describe its anti-mitotic activity.

The activity of Pulchelloid A upon cells was very similar to that of the crude extracts that we prepared from plant leaves. These activities include a mitotic arrest as demonstrated by cell rounding, PH3 signals, and formation of a mitotic spindle. Importantly, we also observed two distinguishing features of Pulchelloid A upon cells, formation of distorted spindles and acquisition of  $\gamma$ H2AX foci that are correlated with the mitotic spindle distortion. We do not know of any other compound that induces this phenotype when applied to human cells. These data suggest that Pulchelloid A induces a previously undescribed mitotic arrest. It will be of great interest to identify the cellular target of this molecule.

To purify the active compound, the L.E. extract from *Gaillardia aristata* (see section 5.3.2) was fractionated twice by Dr. Andersen and the fractions were tested for morphology. We identified two active subfractions that displayed the rounded morphology phenotype, which were fraction 10 and fraction 11. Fraction 11 was more active than fraction 10, suggesting that fraction 10 might contain different compounds. After a subsequent and independent fractionation of *G. aristata* leaves extract, we isolated the compound Pulchelloid A. Structural analysis of Pulchelloid A revealed that it corresponded to the compound present in fraction 11.

The identification of the Pulchelloid A by biology-guided fractionation provided insight into its structure, and guided the investigation of its activity. By knowing the chemical structure we could pursue our studies in three directions: knowledge of sesquiterpene activity, the target, and the structure-activity relationship of the compound.

Sesquiterpene lactones are natural compounds commonly produced by several plants, but are predominant in the Asteraceae taxonomical family (Robles et al., 1995; Heinrich et al., 1998). There are more than 5000 compounds that belong to this class of natural products, and more than 3000 are synthesized by Asteraceae plants (Heinrich et al., 1998; Chadwick et al., 2013). These compounds have several activities, including anti-cancer activities (Picman, 1986; Chadwick et al., 2013). One emerging anti-cancer property of sesquiterpene lactones is the anti-mitotic activity. However, only 14 sesquiterpene lactones have been reported to arrest human cells in mitosis (see chapter 2), suggesting that this type of activity is little characterized for this class of natural products. These compounds arrest human cells at a prometaphase-like stage and cause a distortion of the mitotic spindle, similarly to the L.E. (chapter 2).

We then confirmed Pulchelloid A anti-mitotic activity by morphology assay. Pulchelloid A caused approximately 50% of cells to become rounded, indicating that the compound is more active than the original L.E. However, unlike tubulin toxins, neither the L.E. nor Pulchelloid A induce 100% of rounded cells. This observation suggests that there could be another cell cycle arrest that prevents a portion of cells to enter mitosis. Other experiments to investigate Pulchelloid A's anti-proliferative activity included immunofluorescence microscopy with the mitotic marker PH3 (Hendzel et al., 1997), and  $\alpha$ -tubulin staining. Pulchelloid A caused an increased number of cells to express PH3, and exhibit a mitotic spindle. At 2 h post treatment with Pulchelloid A, cells had condensed chromosomes partially aligned to the metaphase plate, and a regular, bipolar arrangement of the mitotic spindles. By 24 h, the condensed chromosomes were not aligned as they were at 2 h post treatment, and the mitotic spindles were distorted. These results were similar to those obtained with the L.E., and suggest that Pulchelloid A is the active

molecule isolated from *Gaillardia aristata* leaves that arrests cells at a prometaphase-like stage.

Pulchelloid A was tested for genotoxicity by immunofluorescence microscopy with staining for the damaged DNA marker histone  $\gamma$ H2AX (Kuo et al., 2008). At 2 h after treatment, few mitotic cells exhibited low levels of damaged DNA, which was located on discrete foci, unlike the genotoxic agent CPT, which exhibited a higher number of smaller foci. As the duration of the treatment increased, more mitotic cells accumulated damaged DNA, and in bigger amounts. These results confirmed that Pulchelloid A has a similar activity to the L.E. However, the phosphorylation pattern of histone H2AX was not similar to that of CPT, as the foci were bigger and located in fewer, discrete spots. Pulchelloid A does not seem to be a classic genotoxic agent, and it might induce DNA damage as a consequence of the mitotic arrest and the spindle distortion.

Therefore, Pulchelloid A has a similar activity to some of the sesquiterpene lactones reviewed in chapter 2, and it arrests human cells at a prometaphase-like stage and causes a distortion of the mitotic spindle. Although the DNA damage aspect has not been previously linked to this type of mitotic arrest, our data suggest that some sesquiterpene lactones, including Pulchelloid A, arrest human cells in mitosis by interfering with similar mechanisms, and that this type of arrest has not been characterized before.

The anti-mitotic activities of the sesquiterpene lactones we reviewed are dual and act upon different mechanisms. A subset of sesquiterpene lactones directly act upon tubulin, affecting tubulin stability, and causing microtubule hyperpolymerization and/or cytokinesis defects. On the contrary, another subset of these compounds indirectly acts

upon tubulin, arresting cells at a stage that resembles prometaphase, with disorganized mitotic spindles. 6-OAP, in particular, was demonstrated to interfere with the ubiquitin-proteasome pathway by inhibiting the SCF complex, an E3 ubiquitin ligase, and arresting cells in mitosis (Liu et al., 2011; Liu et al., 2015). The SCF complex ligates ubiquitin to proteins that are degraded by the 26S proteasome (Pagano et al., 2004). The target proteins are recognized by the F-box proteins, which are part of the SCF complex. The similarity of 6-OAP structure and anti-proliferative activity to that of Pulchelloid A suggests that Pulchelloid A might have a similar mechanism of action and inhibit the SCF complex.

6-OAP inhibits binding of the Skp1 protein to the NIPA and Skp2 complexes which cause an upregulation of their targets (cyclin B, P27, E-Cadherin, and I- $\kappa$ B $\alpha$ ), and the authors do not show how 6-OAP perturbs mitosis and the mitotic spindles. We believe that Pulchelloid A might inhibit the SCF complex conjugated to the  $\beta$ -TrCP F-box protein. The SCF $^{\beta$ -TrCP targets the protein early mitotic inhibitor 1 (Emi1) for destruction, which is a natural inhibitor of the APC/C $^{Cdc20}$  complex (Margottin-Goguet et al., 2003). Once Emi1 is degraded, the APC/C $^{Cdc20}$  is active and drives the cells to anaphase and to exit mitosis. If Emi1 were not degraded, the APC/C $^{Cdc20}$  would be inhibited and not be able to mark its targets, cyclin B and securin, for destruction.

The SCF complex can interact with other F-boxes that target proteins involved in the regulation of different cell cycle stages. There is the possibility that Pulchelloid A prevents binding of the SCF complex to different F-boxes, affecting other stages of the cell cycle. Therefore, Pulchelloid A might cause other cell cycle arrests. This prediction could explain why the percentage of cells that arrest in mitosis never reaches 100%, and it might be because a portion of treated cells are arrested at the S phase. Furthermore, this

prediction could explain the results obtained with the L.E. (chapter 4): if the APC/C is inhibited, cyclin B and securin cannot be degraded (the L.E. caused an upregulation of these two proteins) (Thornton et al., 2003).

Securin, which is an inhibitor of the separase enzyme, would continue to inhibit this enzyme preventing the degradation of the cohesin proteins (Yanagida, 2000; Waizenegger et al., 2002). The cohesins hold the sister chromatids together at the centromere, and when they are cleaved, the chromatids are free to move to the opposite poles. Therefore, if the cohesins are not degraded, the chromosomes cannot segregate. The mitotic spindle, which is already attached to the chromosomes, continues to exert a tension to separate the chromosomes. This tension applied by the spindle could cause distortion of the spindle fibers, as well as a rupture of the chromosomes in correspondance of the centromeres, as shown by the  $\gamma$ H2AX staining.

Knowing the structure of Pulchelloid A, and its anti-mitotic activity, it is possible to do SAR studies to understand which reactive group on the molecule causes the activity, and how this activity changes in relation to different structural modifications. It is postulated that the reactive chemical group responsible for sesquiterpene lactones activity is the lactone component, which is characterized by an  $\alpha$ -methylene- $\gamma$ -lactone (Rodriguez et al., 1976). This unsaturated carbonyl structure reacts by a Michael-type addition with nucleophiles in biological systems, such as the sulfhydryl group in the amino acid cysteine (Kupchan et al., 1970; Scotti et al., 2007). By modifying the reactive group, the compound is inhibited, and loses its activity. Psilostachyin A, a sesquiterpene lactone with anti-mitotic activity (chapter 2), was modified by addition of a 2-mercaptoethanol group on the  $\alpha$ -methylene- $\gamma$ -lactone (Sturgeon et al., 2005). The new adduct was not active in mitotic arrest assays, indicating that the unsaturated carbonyl on the lactone was

necessary for psilostachyin A activity. Therefore, we predict that if we change the structure of Pulchelloid A by inactivating the lactone, Pulchelloid A will lose its activity and not arrest cells in mitosis. This observation opens new possibilities for future studies to identify the reactive group and modify the structure with the goal of obtaining a very active molecule of pharmaceutical interest.

In summary, in this chapter we describe the isolation of the active compound from the leaves of *Gaillardia aristata*. This compound, the sesquiterpene lactone Pulchelloid A, had been previously described (Inayama et al., 1982), but was not investigated for biological activities in animals cells. We tested Pulchelloid A in a morphology and immunofluorescence assay to investigate its activity. Our results confirm that Pulchelloid A has a similar anti-proliferative activity to the original leaf extract and that it likely interferes with the ubiquitin-proteasome degradation of proteins. Other sesquiterpene lactones were demonstrated to inhibit mitosis, although no precise mechanism was elucidated. Based on previous literature, we predict that Puchelloid A's reactive group is the methylene on the lactone, and that by modifying its structure the reactive group is inhibited, and does not have any anti-mitotic activity. Furthermore, during the biology-guided fractionation, we isolated other active molecules that might be related to Pulchelloid A. With this information, it is then possible to modify Pulchelloid A to do structure-activity relationship studies and to create new, more active derivatives. Future studies are therefore aimed at characterizing Pulchelloid A's reactive groups and its mechanism of action upon human cells.

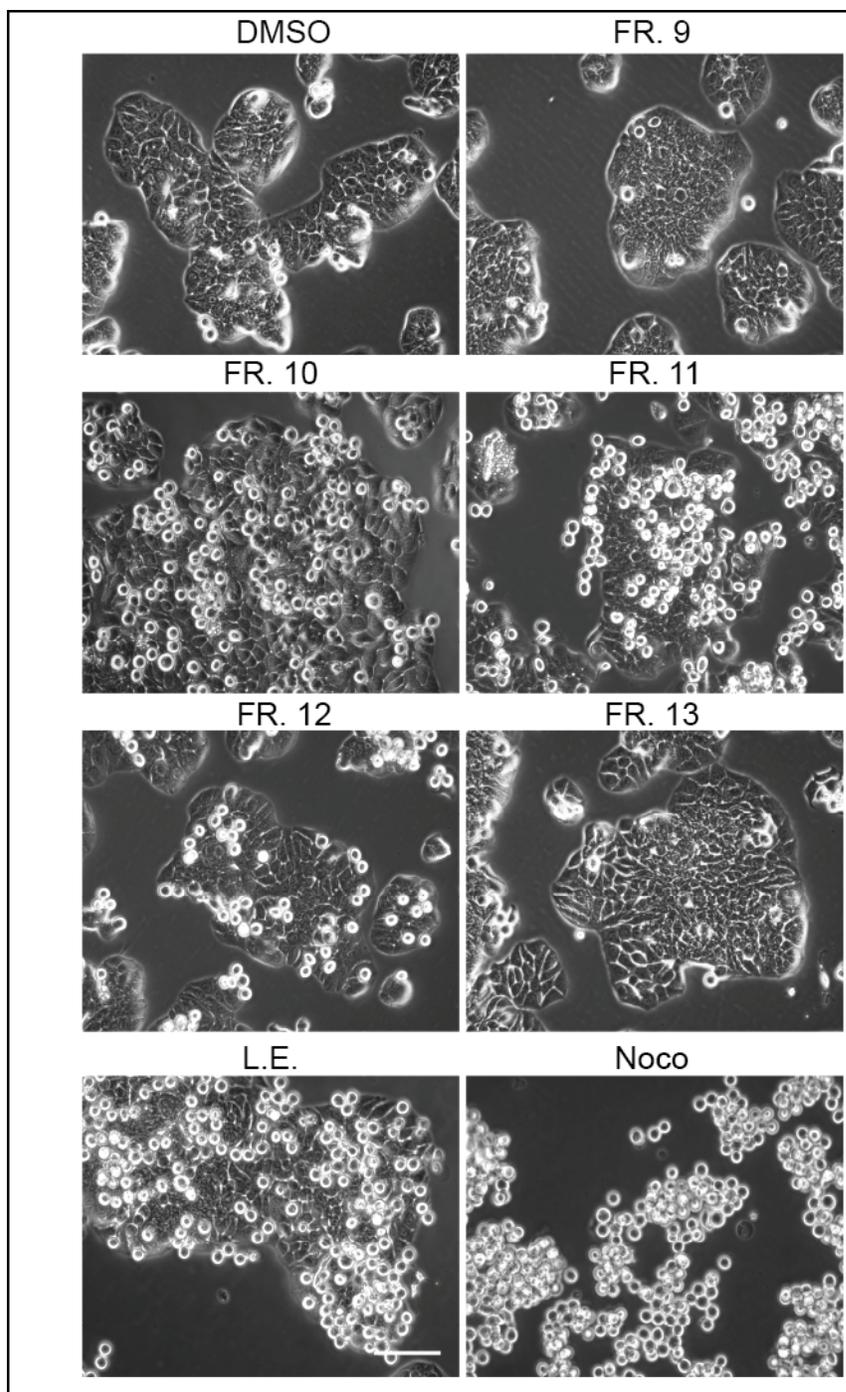


Figure 5.1: HT-29 cells treated with FR.10, FR.11 or FR.12 become rounded, whereas cells treated with FR.9 or FR.13 do not. HT-29 cells were treated with 4.5  $\mu\text{g}/\text{mL}$  FR. 9- FR.13, or with 50  $\mu\text{g}/\text{mL}$  L.E. for 24 h. DMSO and 200  $\text{ng}/\text{mL}$  treated cells were used as controls. Cells were observed by phase-contrast light microscopy. Scale bar equals 100  $\mu\text{m}$ .

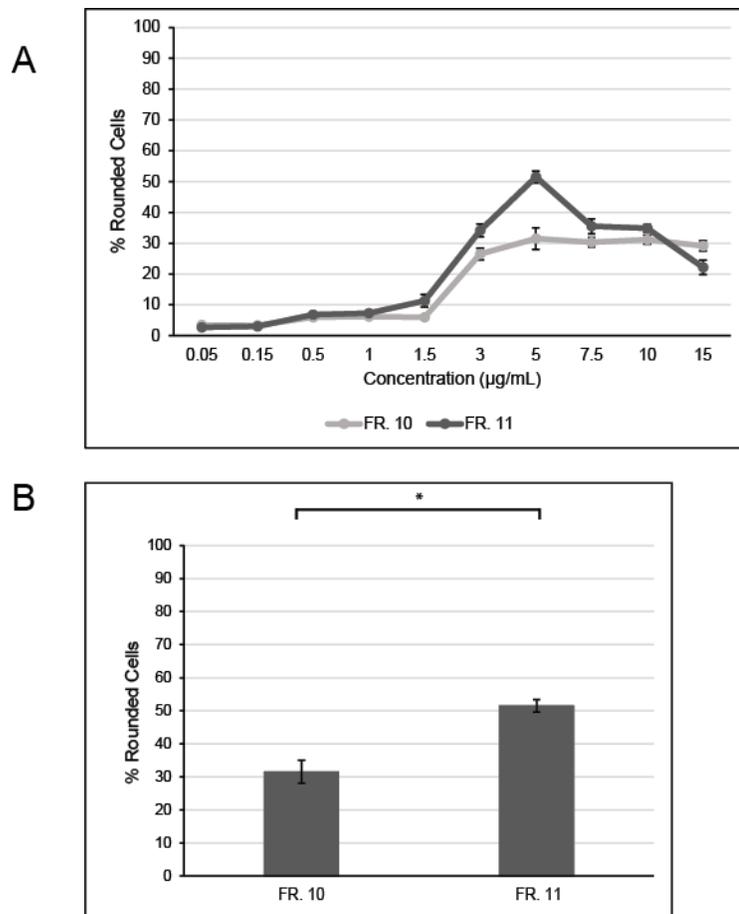
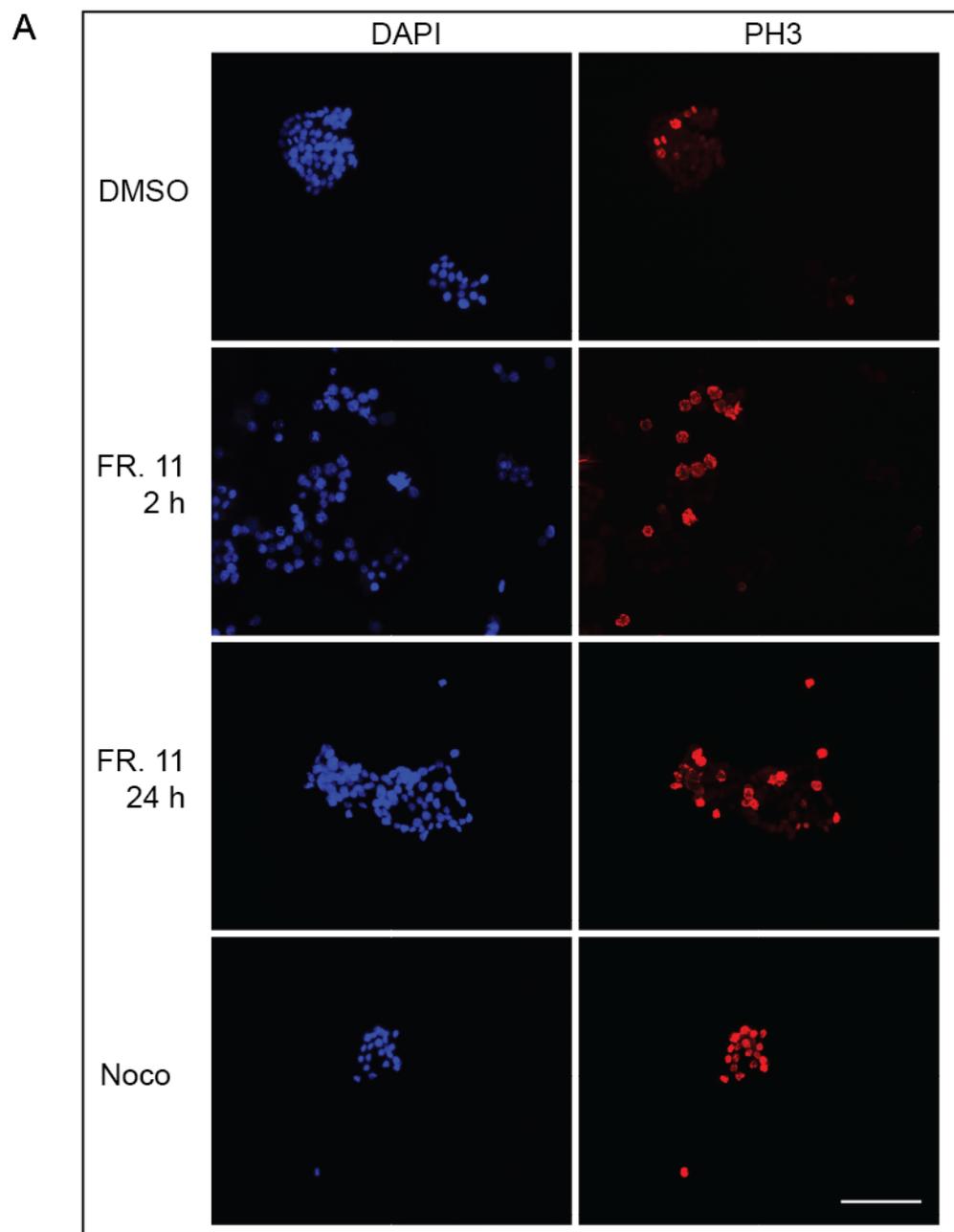


Figure 5.2: FR.11 causes an accumulation of a higher number of rounded cells compared to FR.10, suggesting the compound(s) in these fractions are different. A) HT-29 cells were treated with FR.10 or FR.11 at concentrations ranging from 0.05 µg/mL to 15 µg/mL, and cells were observed by phase-contrast light microscopy. Percentages of rounded cells were manually determined using Image J software at 24 h. At least 200 cells were counted for each treatment per experiment. Mean percentages from three separate experiments and standard errors of the means are shown. B) Percentages of rounded cells treated with 5 µg/mL FR.10 or FR.11 were determined as in A). Mean percentages from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .



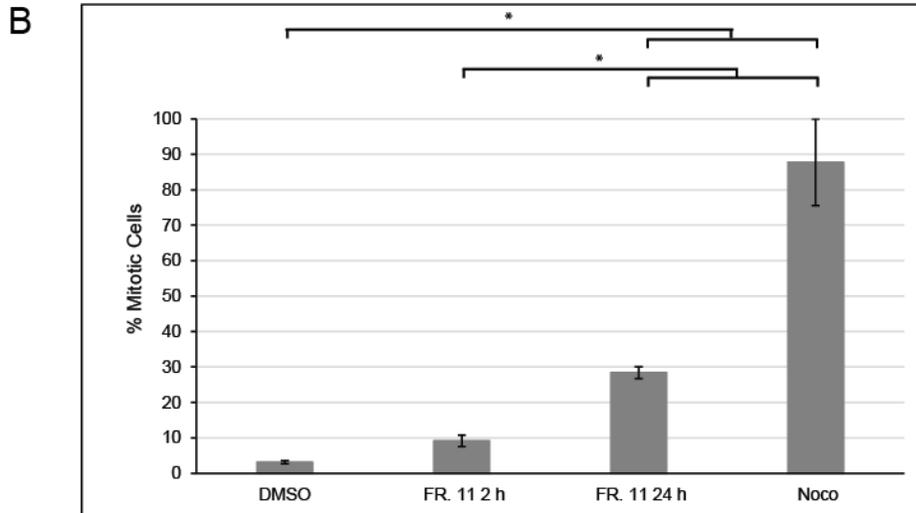
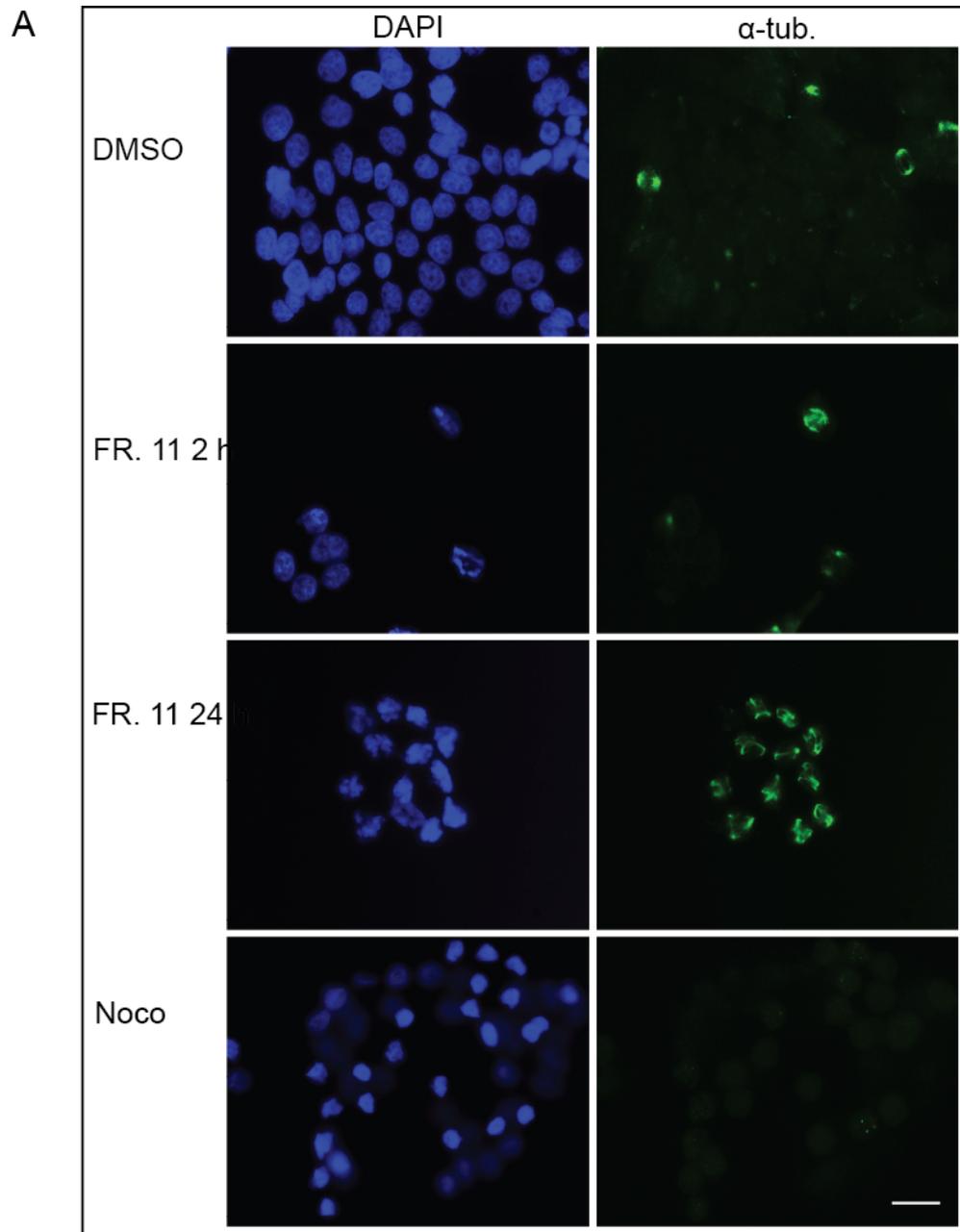


Figure 5.3: Rounded HT-29 cells that were treated with FR.11 for 2 or 24 h are positive for PH3, indicating that they are in mitosis. A) HT-29 cells were treated with either DMSO, 4.5  $\mu\text{g}/\text{mL}$  FR.11 for 2 or 24 h, or 200  $\text{ng}/\text{mL}$  nocodazole and stained with DAPI (blue) to detect DNA and with anti-phospho-Ser10 histone H3 antibodies (PH3) (red). Cells were analysed by immunofluorescence microscopy and representative images are shown. Scale bar equals 20  $\mu\text{m}$ . B) The percentages of cells staining positive for phospho-Ser10 histone H3 (PH3) after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells staining positive for PH3 were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .



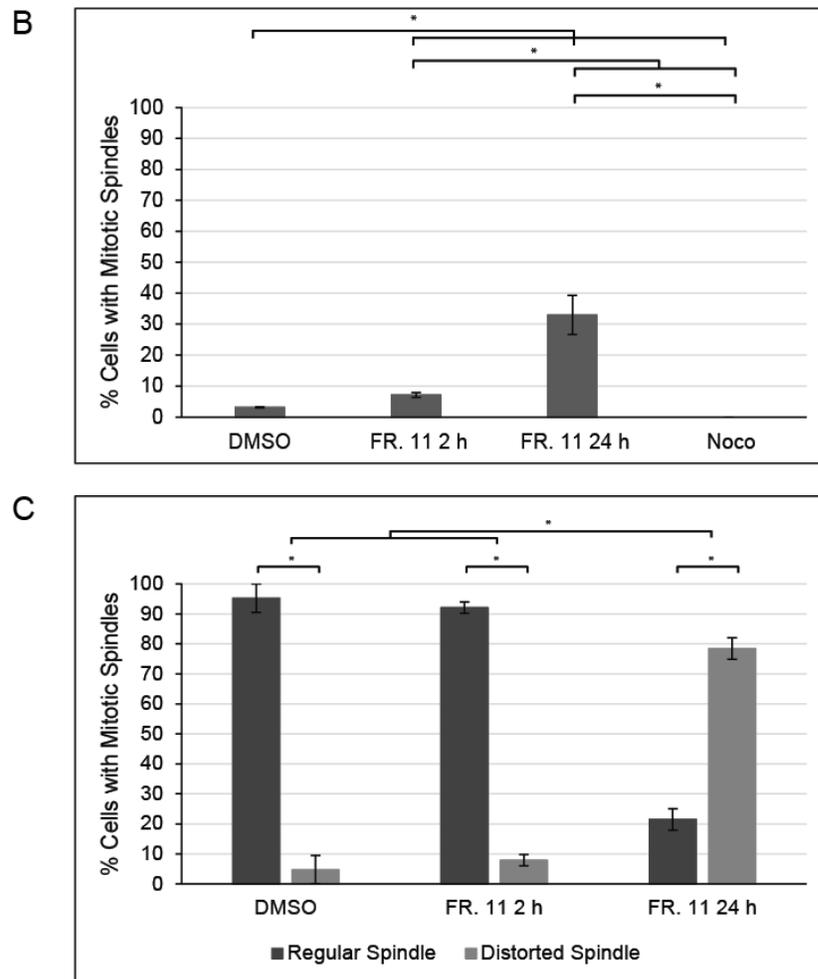
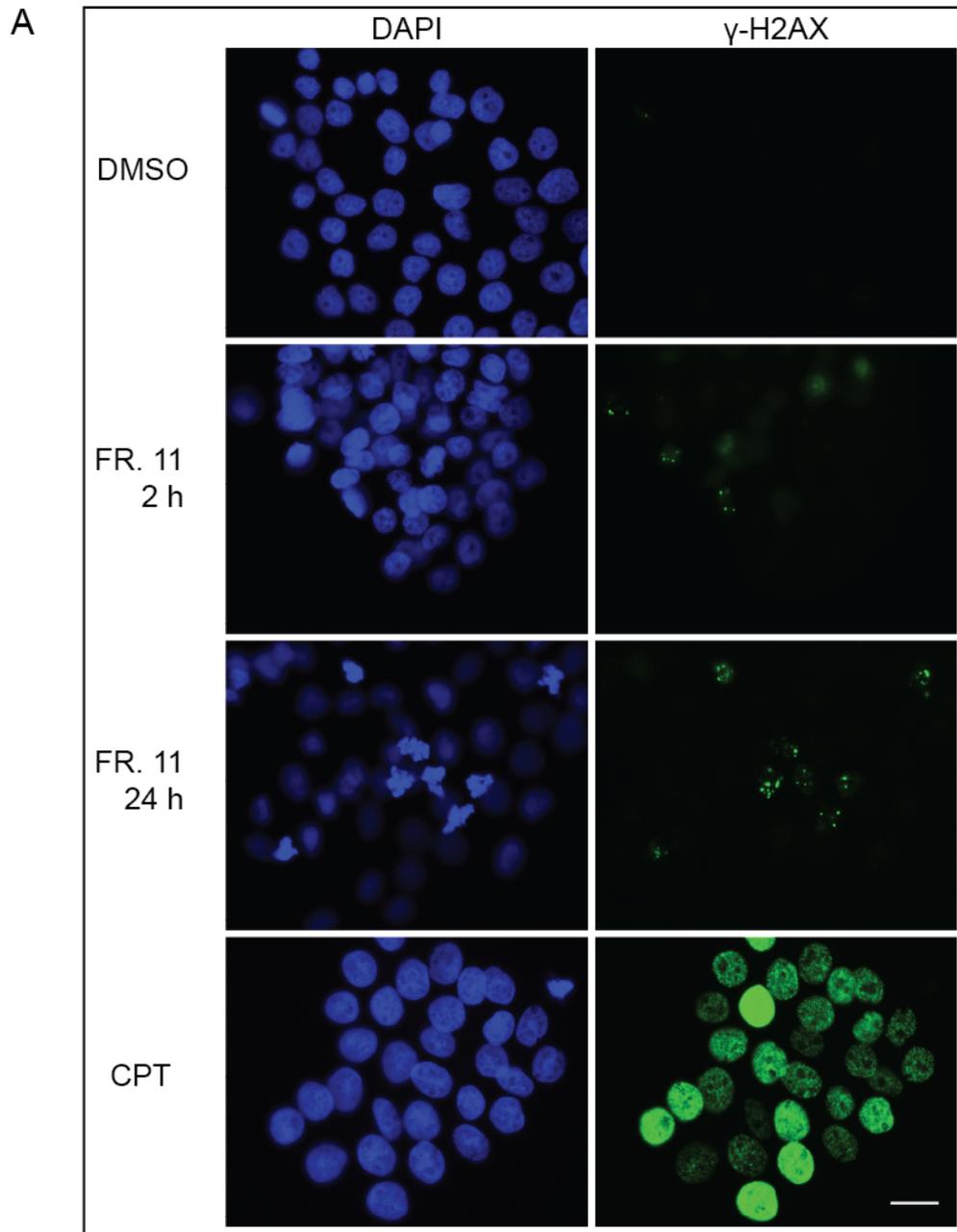


Figure 5.4: Rounded HT-29 cells treated with FR.11 for 2 h exhibit regular mitotic spindles, whereas cells that were treated for 24 h exhibit distorted, irregular mitotic spindles. A) HT-29 cells were treated with either DMSO, 4.5  $\mu\text{g}/\text{mL}$  FR.11 for 2 or 24 h, or 200  $\text{ng}/\text{mL}$  nocodazole and stained with DAPI (blue) to detect DNA and with anti- $\alpha$ -tubulin antibodies to detect microtubules (green). Cells were analysed by immunofluorescence microscopy and representative images are shown. Scale bar equals 20  $\mu\text{m}$ . B) The percentages of cells exhibiting a mitotic spindle after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells positive for a mitotic spindle were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ . C) The percentages of cells exhibiting regular or distorted mitotic spindles after treatment were determined as in B). Mean percentages of cells with a regular or distorted mitotic spindle were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .



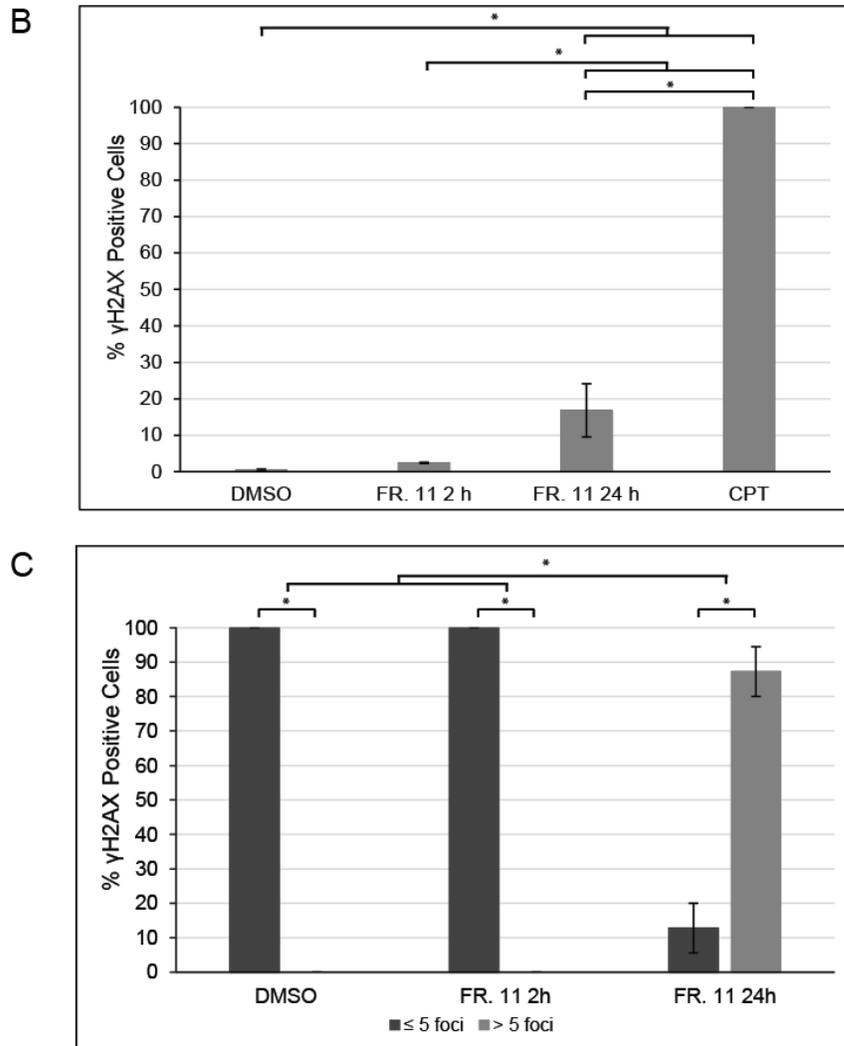


Figure 5.5: Fraction 11 induces damaged DNA in treated mitotic HT-29 cells over 24 h of treatment, similarly to the L.E. (see chapter 4). A) HT-29 cells were treated with either DMSO, 4.5  $\mu\text{g}/\text{mL}$  FR.11 for 2 or 24 h, or 50 nM CPT and stained with DAPI (blue) to detect DNA and with anti-histone  $\gamma\text{H2AX}$  antibodies to detect damaged DNA (green). Cells were analysed by immunofluorescence microscopy and representative images are shown. Scale bar equals 20  $\mu\text{m}$ . B) The percentages of cells containing damaged DNA after treatment were determined using Image J software. At least 20 cells were counted for each treatment per experiment. Mean percentages of cells positive for histone  $\gamma\text{H2AX}$  were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ . C) The percentages of cells containing less than or equal to 5  $\gamma\text{H2AX}$  foci or more than 5 foci after treatment were determined as in B). Mean percentages of cells with less than or more than 5 foci were calculated from three separate experiments and standard errors of the means are shown. Asterisks show significant differences, paired student's t-test, 2 degrees of freedom,  $p < 0.05$ .

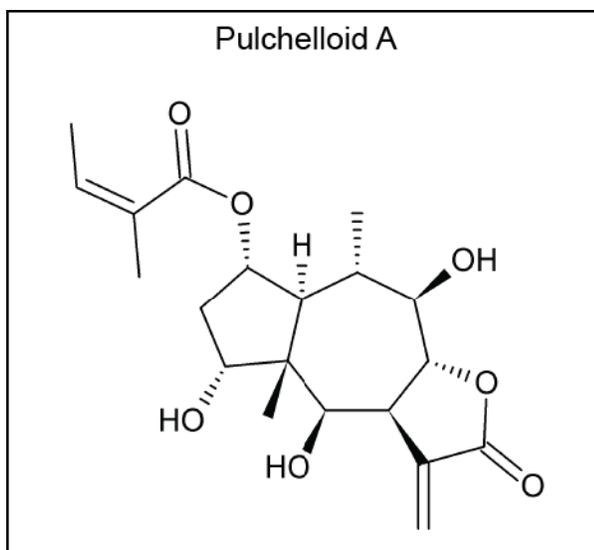


Figure 5.6: The structure of the sesquiterpene natural product Pulchelloid A.

## CHAPTER 6

### **General discussion**

In this this thesis we investigated the prairie plant *Gaillardia aristata* for natural products with bioactivities on human cells. We described the activity of *Gaillardia aristata* extracts on human HT-29 colon carcinoma, M059K glioblastoma and the non-cancerous WI-38 lung fibroblast cells. *G. aristata* leaves extract (L.E.) were toxic to all cell lines at different concentrations, and induced rounded cells in HT-29 and M059K cells, but not on WI-38 cells. We then characterized the activity of the L.E. and found that it has an anti-mitotic activity on HT-29 cells, which might be related to the ubiquitin-proteasome pathway. In collaboration with Dr. Raymond Andersen, we isolated and characterized the compound from the leaves of *G. aristata*, which was the source of the mitotic activity. The compound is the sesquiterpene lactone, Pulchelloid A. This compound had been previously isolated and characterized (Inayama et al., 1983); however, its effects on human cells had not been described.

The discovery of Pulchelloid A as the active compound, and the notion that it is a sesquiterpene lactone, opened new avenues for scientific investigation. Sesquiterpene lactones are common in different plant families, but are prevalent in the Asteraceae family. Of approximately 5000 sesquiterpenes, only a few were reported to display anti-mitotic activity on human cells (chapter 2). Furthermore, various studies on the chemistry of sesquiterpene lactones predicted that the activity of these compounds is due to the reactive lactone group (Kupchan et al., 1970; Rodriguez et al., 1976; Picman, 1986). Indeed, Pulchelloid A contains an  $\alpha$ -methylene- $\gamma$ -lactone that is anticipated to be the reactive group. We predict that if this group is inhibited, the molecule will lose its

activity. In addition to finding a cellular activity for Pulchelloid A, we might have isolated novel molecules with anti-mitotic activity, and opened the possibilities for future structure-activity relationship studies.

We selected the prairie plant species *Gaillardia aristata* for investigation, because it is native to the prairies, and because there were several records of interesting activities of the plant. Although this plant was not studied in modern medicine, there are records of dermatitis caused by contact with *G. aristata*, indicating that it might contain irritating chemicals (Burry, 1980). *G. aristata* in fact is listed as a toxic plant in the F.D.A database of poisonous plants (F.D.A., 2016), it is considered as poor forage value for cattle (Pahl et al., 1999), and there is anecdotal evidence that it is not consumed by grazing herbivores when mature. *G. aristata* chemical profile had been studied and this plant had been demonstrated to be cytotoxic to different cancer cells, but its activity was not investigated further (Salama et al., 2012).

To test *G. aristata* activity we used phenotypic screens based on the hallmarks of cancer (Hanahan et al., 2011; Schumacher et al., 2011). In this thesis, the three hallmarks of cancer that were investigated were enabling replicative immortality, resisting cell death and genome instability. We first needed to select a solvent and the plant parts from which extract the chemicals. We found that the dichloromethane extract from the leaves of *G. aristata* had the highest cytotoxicity and cell rounding activity in a morphology assay. Further tests were made with extracts prepared from the leaves of *Gaillardia aristata* using dichloromethane.

We then pursued the phenomenon of cell rounding with the hypothesis that it was caused by cells in mitosis. We investigated a leaf extract (L.E.) in a flow cytometric analysis and confirmed that there was a G<sub>2</sub>/M-phase arrest, consistent to the morphology

data. The L.E. was tested on another cancer cell line, glioblastoma M059K cells, and on a non-cancerous cell line, WI-38 lung fibroblast cells. The extract was toxic to both cell lines, but induced cell rounding only in M059K cells, indicating that the mechanism it acts on might be selective to cancer cells.

In chapter 4, we investigated into detail the phenomenon of rounded cells. The L.E. was demonstrated to arrest cells in mitosis, as indicated by the increase of cells that were positive for PH3 and for mitotic spindles. Furthermore, configuration of the condensed chromosomes and the spindle suggested that cells arrested at a prometaphase-like stage. However, as the duration of treatment increased, the mitotic spindles became severely distorted, and the condensed chromosomes were not aligned on the equator of the cells. Most mitotic spindle poisons cause microtubule depolymerization, preventing formation of mitotic spindles (such as nocodazole), or stabilize microtubule polymerization, preventing the dissolution of the spindle apparatus (such as Paclitaxel) (Vasquez et al., 1997; Jordan et al., 1998; Weaver, 2014). The L.E. did not cause either of these effects, suggesting that it might act elsewhere in mitosis. Rounded treated cells were analysed for biochemical mitotic markers, and were found to contain low amounts of phospho-Tyr15 Cdk1, high amounts of cyclin B, high amounts of phosphorylated PP1 $\alpha$ , a Cdk1 substrate, and high amounts of securin. These data were consistent with the morphology assay, flow cytometry and immunofluorescence microscopy for PH3 and  $\alpha$ -tubulin. In addition, treated rounded cells arrested at metaphase, as indicated by the failed degradation of cyclin B and securin.

Another novel feature of the mitotic arrest was the genotoxicity of the L.E. In a genotoxicity assay, the L.E. induced damaged DNA when cells entered mitosis, which accumulated during the arrest. At 2 h post treatment with L.E., mitotic cells accumulated

DNA damage in few distinct foci. At 24 h post treatment the signal was present in a larger portion of mitotic cells and was also amplified in every single cell as the majority of  $\gamma$ H2AX positive cells contained 5 or more foci of damaged DNA. Our results indicate that the L.E. is genotoxic to HT-29 cells, but is not similar to a classic genotoxic agent like CPT. CPT induces damaged DNA in interphase cells that arrest in S phase. Furthermore, the damage caused by CPT accumulates  $\gamma$ H2AX signal in a higher number of smaller foci that are located evenly throughout the nuclei (Hsiang et al., 1985). This result is unique as there are no scientific publications that report the occurrence of damaged DNA in mitosis caused by natural extracts, in conjunction with the distorted spindle morphology we observed.

Furthermore, the number of rounded cells accumulated after treatment with the L.E. did not reach 100%, but ranged approximately between 25 and 40%. It is possible that a fraction of cells does not enter mitosis, for example in case these cells were arrested in another cell cycle phase following treatment with L.E.

Taken together, these results suggest that the L.E. causes a prometaphase arrest, during which the spindles are distorted and the cells gradually acquire damaged DNA. We believe that the spindle morphological changes and the occurrence of damaged DNA are a consequence of the initial arrest. To our knowledge, there are very few scientific studies that describe this phenotype and mitotic arrest, and it has never been characterized in detail. It is not clear why the percentage of mitotic cells never reaches 100%. We speculate that the L.E. might cause another cell cycle arrest, which prevents cells from entering mitosis, as suggested by the S-phase arrest observed in the flow cytometric analysis (chapter 3). In chapter 3 and 4 we have therefore established *Gaillardia aristata*

effects upon human cells and how these effects are related to the biochemical pathways involved in the hallmarks of cancer.

Purification of the activity provided insight into the structure of the active compound and the anti-mitotic activity. The original crude extract was fractionated, and the fractions were tested for anti-mitotic activity using bioassay-guided fractionation. In collaboration with Dr. Raymond Andersen, after three rounds of fractionation we identified a compound, Pulchelloid A, which scored positive in a morphological assay for rounding activity. Pulchelloid A is a member of the class of sesquiterpene lactones. These are natural compounds found predominantly in the Asteraceae family, and can cause an allergic reaction or toxicity upon exposure or ingestion. Several sesquiterpene lactones have been reported to have anti-cancer properties, among which is the emerging anti-mitotic activity (chapter 2). In particular, 6-OAP, was reported to have an anti-mitotic activity that could be linked to the inhibition of the proteasomal degradation by the ubiquitin-proteasome pathway (Liu et al., 2015). This compound caused effects similar to those of Pulchelloid A, and had a related chemical structure. This type of activity has not been characterized into detail, and might lead to novel mechanisms that regulate mitosis.

We propose that Pulchelloid A was the major active compound present in the L.E. that caused the mitotic arrest. Pulchelloid A's anti-mitotic activity was investigated by immunofluorescence microscopy with PH3,  $\alpha$ -tubulin and  $\gamma$ H2AX staining. Pulchelloid A induced a prometaphase-like mitotic arrest during which the mitotic spindle was distorted, the condensed chromosomes spread from the metaphase plate across the cell, and concomitantly damaged DNA started to accumulate in distinct foci. These results were consistent with the data obtained with the L.E. and indicated that Pulchelloid A had a similar anti-mitotic activity as the crude extract, confirming our prediction that it was the

bioactive compound that caused the mitotic arrest. This approach was successful in isolating an active compound from the original crude extract.

Similarly to the sesquiterpene lactone 6-OAP, Pulchelloid A might inhibit cell proliferation by interfering with components of the ubiquitin-proteasome pathway. In addition, a second fraction (fraction 10) was found to be active in a cell rounding assay, albeit not as active as Pulchelloid A. This fraction might contain compounds that are structurally related to the sesquiterpene lactone, suggesting the possibility for future studies and to identify novel compounds.

In our review of the anti-proliferative activities of the sesquiterpene lactones we identified 18 compounds that are structurally similar to Pulchelloid A and that cause a mitotic arrest (chapter 2). Few of these compounds induced similar morphologies on cancer cells and caused a disorganization of the mitotic spindle. However, none of these sesquiterpenes were shown to induce damaged DNA in relation to the mitotic arrest. Furthermore, the molecular mechanism behind this type of arrest has not been characterized in detail. 6-OAP, a sesquiterpene lactone reviewed in chapter 2, showed a similar morphological and biochemical activity characterized by the prometaphase-like arrest (Liu et al., 2015). The authors found that this compound targets a component of the E3 ubiquitin ligase SCF complex, preventing the degradation of its targets, which are in turn involved in the degradation of cyclin B and securin, and in the transition from metaphase to anaphase. However, they do not provide a mechanism responsible for the distortion of the spindle and for the failed degradation of the APC/C targets. We postulate that Pulchelloid A could be an inhibitor of the SCF complex that binds to the  $\beta$ -TrCP F-box protein. The SCF <sup>$\beta$ -TrCP</sup> complex degrades the early mitotic inhibitor 1 (Emi1) (Pagano et al., 2004). Emi1 is a natural inhibitor of APC/C<sup>Cdc20</sup>, and if its degradation is prevented,

the APC/C complex is not activated, leading to accumulation of cyclin B and securin, and to metaphase arrest (Yamashita et al., 1999; Pagano et al., 2004; Vodermaier, 2004). Securin mediates the attachment of the duplicated sister chromatids at the kinetochore, and when not degraded, chromosome segregation is impaired (Funabiki et al., 1996). The tension applied by the mitotic spindle on the chromosomes might eventually cause a distortion of the microtubule fibers, and rupture of the chromosomes. The SCF complex ubiquitinates proteins that regulate other phases of the cell cycle, such as S-phase (Pagano et al., 2004). If Pulchelloid A inhibits one of the components of the SCF complex, it might be possible that some targets in S-phase are not degraded, causing a second cell cycle arrest and preventing a portion of cells to enter mitosis. This prediction could explain why the percentage of mitotic cells never reaches 100%, even with the purified compound.

The unsaturated carbonyl group on the lactone is highly reactive with sulfhydryl groups of cysteines through Michael addition, covalently modifying the protein or enzyme that contains the cysteine (Kupchan, 1970; Rodriguez et al., 1976; Chadwick et al., 2013). Inhibition of this group might inactivate the molecule, as shown by Sturgeon et al. when they reacted a 2-mercaptoethanol group to psilostachyin A's lactone (Sturgeon et al., 2005). The psilostachyin new adduct did not arrest cells in mitosis, indicating that the reactive group of the sesquiterpene lactone had been inhibited. In a similar experiment, it is possible to inhibit the lactone in Pulchelloid A and test the activity of the modified compound. The outcome of this experiment will elucidate whether it is possible to modify the compound, and how the activity is affected. These modifications could lead to the synthesis of a new version of Pulchelloid A, giving it more appealing pharmaceutical properties.

We have discovered an anti-mitotic activity of Pulchelloid A that, to our knowledge, has not been previously characterized. Therefore, a future goal for this project is to identify the target of Pulchelloid A, to do structure-activity relationship studies to produce a more potent molecule, and to determine if a subset of sesquiterpene lactones are novel inhibitors of cell cycle phases, such as mitosis.

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