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2008-07-14

Comparative Cytotoxicity of an FDA-approved Cancer Drug to Extracts of Atriplex confertifolia on Human Breast and Cervical Cancer Cells

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Comparative Cytotoxicity of an FDA-approved Cancer Drug to Extracts

of *Atriplex confertifolia*

on Human Breast and Cervical Cancer Cells

by

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A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Plant and Wildlife Sciences

Brigham Young University

August 2008

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the thesis of Christopher James Capua in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

Comparative Cytotoxicity of an FDA-Approved Cancer Drug to Extracts of *Atriplex confertifolia* on Human Breast and Cervical Cancer Cells

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Master of Science

The severity and number of people affected by cancer is a worldwide problem with millions of people affected annually. The search for treatment and cures of cancer continues to be a global effort. As part of this global effort, many natural products have been tested against cancer cell lines, most from plants located in tropical regions. However, this study reports that extracts of *Atriplex confertifolia,* a native North American plant, has significant bioactivity against human breast cancer cell lines MCF-7, 435, and 231, and HeLa cells (cervical cancer cells). The bioactivity of *A. confertifolia* extracts of these cells lines was compared to an FDA-approved cancer drug and an industry-standard leukocyte control cell line. Active portions of the extracts were found primarily in the polar fractions of the plant. A dose-response curve of the extracts clearly showed significant cell death similar to the FDA-approved drug. The plant extracts did not inhibit the viability of the leukocyte cell line. Cancer cell death was followed as a function of time and concentration. Cell death appears to be a result of apoptosis.

ACKNOWLEDGEMENTS

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I.   INTRODUCTION

In 2008 565,000 Americans are expected to die of cancer. Cancer is the second most common cause of death in the US, exceeded only by heart disease (American Cancer Society 2008). Pollner (1993) reported that cancer in the United States has more than doubled in the last 30 years, from 1 in 20 in 1960 to 1 in 9 today. Although cancer is not the number one cause of mortality in the United States it is often painful, and is the most feared of diseases (CQ Researcher 1995). Therefore, the search for cancer treatments will continue until a cure is found.

Cancer has been treated for thousands of years, though treatments were largely ineffective until the 19th century. One of the most dated surgeries to remove cancer is known to have been tried as early as 1660 B.C. Near the end of the 19th century physicians realized that cancer often recurred when only the tumor was removed. Radiation treatment began in 1899 along with surgery, as an additional approach to treat cancer. Chemotherapy was discovered during World War II as part of a continuing investigation of the mode of action of toxic gases. More than half of the cases seen each year will be curable with surgery and/or radiation. "Of the remaining patients… a significant fraction can be cured with chemotherapy" (CQ Researcher 1995).

The remainder of patients will only receive partial alleviation and palliative benefit from such treatments. Hence, continuing research is needed to find alternative treatments for these patients. In the past 10 years new technology has provided

additional therapies. For example, imaging technology has delivered tomosynthesis (Port 2003) and advances in genetics have produced a variety of anti-angiogenesis drugs. However, as Bettelheim (1998) noted, "The war on cancer isn't just fought with bioengineered drugs and souped-up genes. Scientists also utilize ornamental shrubs, tree bark, sea horses and thousands of other natural products that serve as the basis for new cancer drugs".

 Plants have been used for medicinal purposes for centuries. In recent years natural products have provided such medicines as morphine and opium, which are derived from the poppy flower. Penicillin was first discovered and produced from bread mold. Each year thousands of plant extracts are screened for bioactivity against cancer (Bettelheim 1998). Most botanical investigations have come from rainforest or tropical plants, yet there are many untested non-tropical plants and a few have shown bioactivity (Shekhawati and Anand 1985, Sallal and Alkofahi 1996).

Taxol, the number one selling cancer drug, is derived from Pacific yew tree bark. Though, its initial discovery was not enthusiastically endorsed by the medical community, its success has precipitated an intensive search for new natural product treatments. Other plant-derived drugs that have been discovered including; topotecan, vincristine and vinblastine (Bettelheim 1998).

Atriplex confertifolia

A Brigham Young University (BYU) study was completed in 2001 to screen more than 40 plants of North America for their cytotoxicity (Malmstrom 2001). One of the plants, *Atriplex confertifolia*, showed much greater bioactivity than others. This plant is

widely distributed throughout North America from Texas to North Dakota and west to the Pacific Ocean, (Figure1).

Figure 1.
Geographic distribution of *Atriplex confertifolia* (Courtesy of USDA).

The majority of studies on *A. confertifolia* have been focused on its distribution (Sanderson 1994), lifespan (Bowers 1995), botanical and ecological characteristics (Banner 1992) and how it has been affected by grazing (Angelo 1998). No studies had been performed on the bioactivity of *A. confertifolia* until 2004, when Welch (2004) determined the cytotoxicity effects of *A. confertifolia* on human cervical cancer cells (HeLa cells).

A. confertifolia (Figure 2) is known to provide a source of palatable, nutritious forage for a wide variety of wildlife and livestock. Specifically, the fruits and leaves are a food source for deer, desert bighorn sheep, pronghorn, small rodents, jackrabbits, game birds, and songbirds (Hanson 1962). Of all *Atriplex* genera in North America, *A. confertifolia* is ecologically the most important and can grow on a greater variety of soils. Welch (2004) found that the most bioactive

Figure 2.
Atriplex confertifolia.

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fraction of *A. confertifolia* killed more than 94% of the HeLa cells in a laboratory
bioassay. "The fact that *A. confertifolia* is edible but still kills cancer cells may be very
important. It suggests that the cytotoxic agents in the plant may show specificity only
towards cancerous cells, making it an excellent candidate for pharmacological use"
(Welch 2004). From the positive cytotoxicity results of Welch's study using HeLa cells,
it was thought that *A. confertifolia* may have bioactivity on other human cancer cells.

Objectives

The objectives of this research were to (1) determine the cytotoxicity of the polar
and non-polar fractions of *A. confertifolia* against selected cancer cell lines, (2) determine
the cytotoxicity of the most active fractions of *A. confertifolia* against various cancer cells
compared to those of a normal leukocyte cell culture, (3) develop dose-response curves
for each cell line of the active fractions compared to the FDA-approved drug Onxol®,
(4) determine the cytotoxicity of *A. confertifolia* against selected cancer cell lines over
time, and (5) to determine if cell death is via apoptosis or necrosis.

II.   METHODS AND MATERIALS

Source of Plant Material

All procedures used the same samples of *Atriplex confertifolia* that were taken west of Lehi, Utah (40° 13' 51" N, 112° 11' 33" W) and stored at 4°C in a cold room at BYU.

Isolation

Extraction and Bilayer Separation

The leaves, stems, and branches of *A. confertifolia* were cut or chopped into 2.5 cm or smaller pieces and then further homogenized using a common mortar and pestle. Approximately 23g of crude, dry plant material were added to a 250 ml Erlenmeyer Flask. Then 130 ml of methanol were added to the flask and the mixture was stirred on a stir plate for 24 hours. This methanol solution was then filtered using Whatman No. 30 (11.0 cm) filter paper and the supernatent was retained.

Approximately 3 ml of the methanol/*Atriplex* extraction were placed into a 15 ml screw-cap conical test tube. This was followed by the addition of 3 ml of distilled water to the test tube and then 3 ml of hexane. The test tube was then tightly capped and shaken vigorously for 20 seconds. This was usually done with a sequence of four test tubes at a time. These test tubes were then centrifuged for 5 minutes at 1500 rpm. The polar methanol/water portions dissolved the polar compounds, while hexane dissolved the non-polar compounds, resulting in an aqueous hexane bi-layer. The hexane fraction formed the upper phase in the test tube. The hexane was then pipetted from the methanol /water portion using a standard Pasteur pipette.

Cell Culture Lines

From the positive cytotoxicity results of Welch's study using HeLa cells, it was thought that *A. confertifolia* may have bioactivity on other human cancer cells. The following cell lines were used in the current study; MCF7 (human breast cancer cells) were established from pleural effusion from a 69 year female with adenocarcinoma (Zhang 1993). MCF7 cells were recommended as target cells because of their widely acknowledged estrogen sensitivity (Villalobos 1995). Included in the study of breast cancer are two other breast cancer lines 435 and 231, which are ductal carcinomas and adenocarcinomas respectively (Siciliano 1979). Finally, a line of leukocyte (monocyte) cells from a healthy 28 yr old male was used as a control.

Non – Polar and Polar Extracts

A bioassay was then performed using each cell line to determine which portion showed cytotoxicity. The bioassay was performed in the following manner:

A 2 ml volume of the methanol/water portion was added to a 2 ml Eppendorf tube (microcentrifuge tube) and 2 ml of the hexane were added to another 2 ml Eppendorf tube. These tubes were allowed to evaporate to dryness. 300 µl of Roswell Park Memorial Institute 1640 (RPMI1640), which is the cell growth medium (see Appendix A for information about the growth medium and cell culturing details), was then added to each Eppendorf tube. These tubes were then capped and thoroughly mixed using a sonicator (Cole-Parmer 8851) and a deluxe mixer (Scientific Products).

A volume of 40 µl of each sample was then added to each of three wells in a 96-well flat-bottom plate that was prepared with a cell solution with a concentration of 0.8-$1x10^5$ cells/ml. Volumes of 50 µl each of RPMI1640 were also added to a total of 9 wells in the plate to serve as controls. (see Appendix A for information concerning the preparation of a 96 well plate). The plate was allowed to incubate for 24 hours and then stained with a sulforhodamine stain (see Appendix A for information on sulforhodamine staining).

Living cells were stained while dead cells were washed away. Once the cells were stained they were read using BioTek® EL800 a spectrofluorometer at 570 nm. After the number of viable cells in the control wells was then compared to the number in the wells treated with the methanol/water and with the hexane portions of the *A. confertifolia* extraction/separation; the cytotoxic fraction was detected by finding which portion showed the lowest cell viability.

Dose-Response

The dose response curve was obtained by the following procedure:

Once the fraction from the column that showed the highest degree of cytotoxicity was identified using the cell bioassay described in the isolation procedure above, it was placed in a pre-weighed 2 ml Eppendorf tube and allowed to evaporate. Small portions of the cytotoxic fraction were then added to the Eppendorf tube and allowed to evaporate in this fashion until approximately 9 mg of the *A. confertifolia* extract were dried in the bottom of the Eppendorf tube.

1 ml of RPMI1640 was added to the completely dry *A. confertifolia* extract in the Eppendorf tube and thoroughly mixed with a sonicator (Cole-Parmer 8851) and a deluxe mixer (Scientific Products) so that all of the dry *A. confertifolia* extract was in solution, resulting in an *A. confertifolia* concentration of 9 mg/ml. 50 µl of normal RPMI1640 was then added to three wells in a previously prepared 96-well flat-bottomed plate where each well had 150 µl of a 0.8-1x10^5 cells/ml solution. This represented a dosage of 0 mg per ml. Normal RPMI1640 was also added to 9 other wells on the plate as the control for the experiment.

45 µl of the normal RPMI1640 was then added to three wells of the plate. To these same three wells, 5 µl of the treated RPMI1640 were added. This gave these three wells a total concentration of 0.23 mg/ml. The calculated concentration takes into account that 150 µl of RPMI1640 had been added to each well when the cells were originally added to the plate. Thus, the total liquid volume in each well was now 200 µl. In the next three wells, 40 µl of normal RPMI1640 were added, 10 µl of the treated RPMI1640 was also added to create a concentration of 0.46 mg/ml.

This pattern was continued until 50 µl of treated RPMI1640 was placed in each of three wells with no normal RPMI1640. Those wells resulted in a concentration of 2.28 mg/ml. Three more wells containing 45 µl of the normal RPMI1640 were prepared. To these same three wells, 5 µl of diluted treated RPMI1640 were added. This step was continued until concentrations of 0.12, 0.06, and 0.03 mg/ml were obtained. This 96-well plate was then incubated for 24 hours and then subjected to the sulforhodamine staining procedure so that the viability of the cells could be measured. These data were then plotted graphically as dose-response curves. The data were transformed to the log scale

and analyzed using a linear mixed model program (SAS Institute Inc., 1999). A first order model, second order model and a separate means model were fitted.

FDA-Approved Drug Comparison

Dose-response curves from the *A. confertifolia* extracts were then compared to the chemotherapy drug Onxol® dose response curves obtained by the following procedure:

Onxol® drug comes in liquid form at a concentration of 4 mg/ml. 50 µl of normal RPMI1640 was then added to three wells in a previously prepared 96-well flat-bottomed plate where each well had 150 µl of a $0.8-1 \times 10^5$ cells/ml solution. This represented a dosage of 0 mg per ml. Normal RPMI1640 was also added to 9 other wells on the plate as a control for the experiment.

45 µl of the normal RPMI1640 was added to three wells of the plate. To these same three wells, 5 µl of the Onxol® were added. This gave these three wells a total concentration of 0.15 mg/ml. The calculated concentration takes into account that 150 µl of RPMI1640 had been added to each well when the cells were originally added to the plate. The total liquid volume in each well was now 200 µl.

40 µl of normal RPMI1640 were added to the next three wells, 10 µl of the Onxol® was added to create a concentration of 0.30 mg/ml. This pattern was continued until 50 µl of Onxol® were placed in each of three wells with no normal RPMI1640. This gave those wells a concentration of 1.52 mg/ml.

Three more wells of 45 µl of the normal RPMI1640 were added. To these same three wells, 5 µl of diluted Onxol® were added. This step was continued until concentrations of 0.08, 0.04, and 0.02 mg/ml were obtained. This 96-well plate was then

incubated for 24 hours and then subjected to the sulforhodamine staining procedure so that the viability of the cells could be measured.

Timed Response

The timed response curve was obtained by the following procedure:

Once the fraction from the column that showed the highest degree of cytotoxicity was identified using the cell bioassay described in the isolation procedure above, it was placed in a pre-weighed 2 ml Eppendorf tube and allowed to evaporate. Small portions of the cytotoxic fraction were then added to the Eppendorf tube and allowed to evaporate in this fashion until approximately 9 mg of the *A. confertifolia* extract were dried in the bottom of the Eppendorf tube.

1 ml of RPMI1640 was added to the completely dry *A. confertifolia* extract in the Eppendorf tube and thoroughly mixed with a sonicator (Cole-Parmer 8851) and a deluxe mixer (Scientific Products) so that all of the dry *A. confertifolia* extract was in solution, resulting in an *A. confertifolia* concentration of 9 mg/ml. 50 µl of normal RPMI1640 was then added to three wells in a previously prepared 96-well flat-bottomed plate where each well had 150 µl of a $0.8\text{-}1\text{x}10^5$ cells/ml solution. This represented a dosage of 0 mg per ml. Normal RPMI1640 was also added to 9 other wells on the plate as the control for the experiment.

15 µl of the normal RPMI1640 was added to three wells of the plate. To these same three wells, 35 µl of the treated RPMI1640 were added. This gave these three wells a total concentration of 1.59 mg/ml. The 96-well plate was then incubated for 1 hour and then subjected to the sulforhodamine staining procedure. Another 96-well plate that had

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been prepared in the same fashion was incubated for 2 hours and then subjected to the sulforhodamine staining procedure. And a third 96-well plate prepared in a similar manner was incubated for 4 hours and then subjected to the sulforhodamine staining procedure. This procedure was continued at increments of 2 hours up to 24 hours from the time the first treated RPMI1640 was added. These data were then plotted graphically as time-response curves. The data were transformed to the log scale and analyzed using a linear mixed model program (SAS Institute Inc., 1999). A first order model was used to fit the data.

Cell Preparation for Scanning Electron Microscopy

The scanning electron microscopy images were obtained by the following procedure:

Once the fraction from the column that showed the highest degree of cytotoxicity was identified using the cell bioassay described in the isolation procedure above, it was placed in a pre-weighed 2 ml Eppendorf tube and allowed to evaporate. Small portions of the cytotoxic fraction were then added to the Eppendorf tube and allowed to evaporate in this fashion until approximately 9 mg of the *A. confertifolia* extract were dried in the bottom of the Eppendorf tube.

1 ml of RPMI1640 was added to the completely dry *A. confertifolia* extract in the Eppendorf tube and thoroughly mixed with a sonicator (Cole-Parmer 8851) and a deluxe mixer (Scientific Products) so that all of the dry *A. confertifolia* extract was in solution, resulting in an *A. confertifolia* concentration of 9 mg/ml. 3 ml of solution of $0.8\text{-}1\mathrm{x}10^5$ cells/ml was pipetted onto on each of two microscope slides. 1 ml of normal RPMI1640

was added to one slide as a control for the experiment. One ml of treated RPMI1640 was

added to the other slide. These were left to incubate for 6-8 hours. The slides were then

subjected to a SEM preparation (see Appendix A).

HeLa cells were prepared for imaging using a Scanning Electron Microscope

(SEM) model Philips XL30 ESEM FEG located at the Cluff Building, BYU.

III. RESULTS AND DISCUSSION

Atriplex confertifolia was first shown to contain bioactive compounds during a

cooperative study
between BYU and the
New York Botanical
Garden (Welch 2004).
Figure 3 shows in a
similar manner that the
bioactive component(s)
of the *A. confertifolia* are
found primarily in the polar
methanol/water portion of the
extraction. The polar fraction killed

Figure 3. Bioactivity of the non-polar and polar extracts of *Atriplex confertifolia* against three human breast cancer cell lines (435, 231, and MCF-7) and a human cervical cancer cell line (HeLa).

about 90% of the cells on all cell lines, while the non-polar hexane fraction only reduced

cell viability by less than 20%.

These results are similar to the study reported by Welch (2004), but are in contrast

to the work done by Donaldson (2000) who, while doing HeLa cell bioassays, found that

Atriplex canescens showed activity against HeLa cells with hexane fractions (48.7% cell

inhibition), but no activity with methanol fractions (0.9% cell inhibition). However,

Davis (2002) clearly demonstrated that methanol fractions were more toxic than hexane

fractions when tested against a wide variety of plants.

When the *A. confertifolia* extracts from the active fraction were administered at

different concentrations to the cell lines, cell viability showed a dose-response. The doses
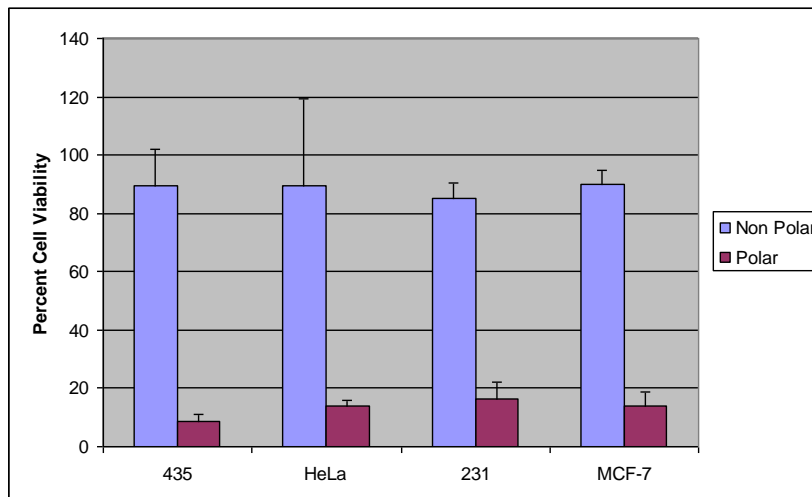
ranged from 0.03 mg/ml to 2.28 mg/ml. Cancer cell viability ranged on average from 95 to 10% after exposing the cell lines to varying concentrations of the *A. confertifolia* compounds for 24 hours. The extract was apparently highly selective since the monocyte control cells were affected very little by the extract (Figure 4). Comparing full and 2nd order log linear data models by a lack of fit test gives a $\chi^2 = 12.1$ and a $p \leq 0.001$. Demonstrating the 2nd order log linear model is a preferred model. Similar data were found comparing 1st order log linear and 2nd order log linear $\chi^2 = 63.3$ and a $p \leq 0.05$.
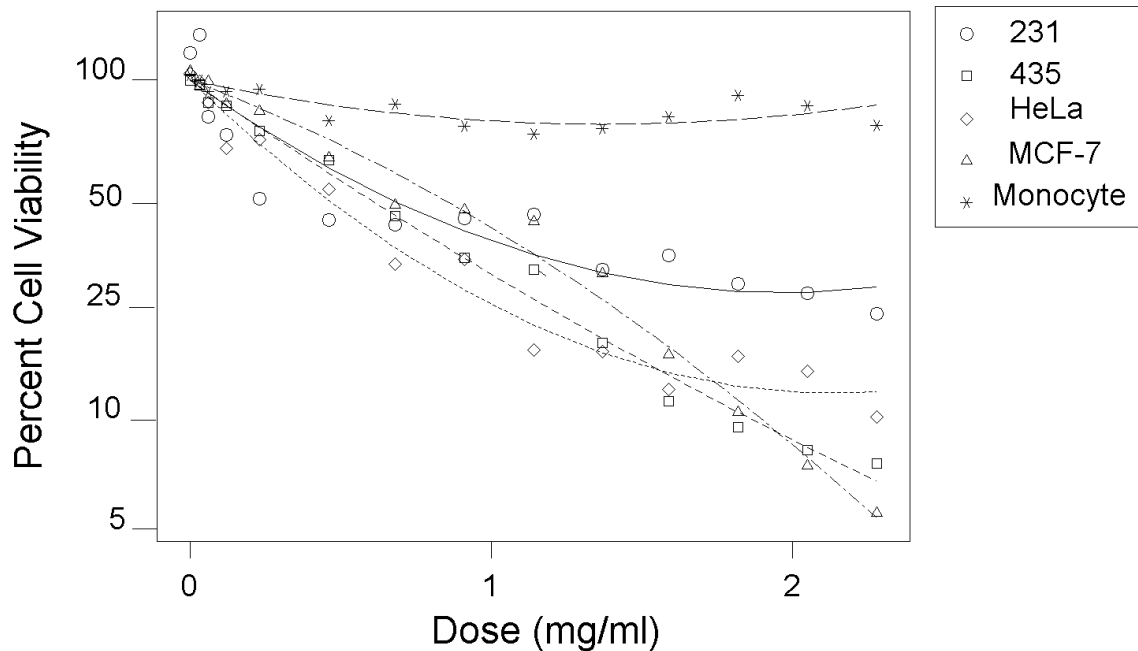
Figure 4. Comparison of the dose-response curves for extracts of *Atriplex confertifolia* against three human breast cancer cell lines (425 231, MCF-7), a human cervical cell line (HeLa), and a human monocyte cell line (control).

Overall there were significant differences among the curves F=16.97, p=<0.0001. Among the four cancer cell lines, there were significant differences for all pairs of lines, with the smallest F=5.50, and largest p=0.0043. These dose-response curves are similar

to those reported by Lau, Lin, Zhao and Leung (2008). They used multiple pancreatic adenocarcinoma cell lines to study the anticancer effects of the fruit *Brucea javanica*. They reported a dose range of 0.1 μg/ml to 1000 μg/ml and a cell survival rate of 90% to 20% after exposing the cells to the active compound for 72 hours. Another study by Medina-Hoguin (2008) studies of a desert plant *Anemopsis californica* to show root oils demonstrated anti-proliferative activity against AN3CA and HeLa cells in vitro but no activity against lung, breast, prostate or colon cancer cells.

After the dose reached a concentration of approximately 1.59 mg/ml, the cell viability leveled off at approximately 10% percent. This plateau is seen in other dose-responses listed in the literature also. For example, the dose-response curves of Aliabadi and Ahmadi (2000) started to plateau at about 20% cell viability.
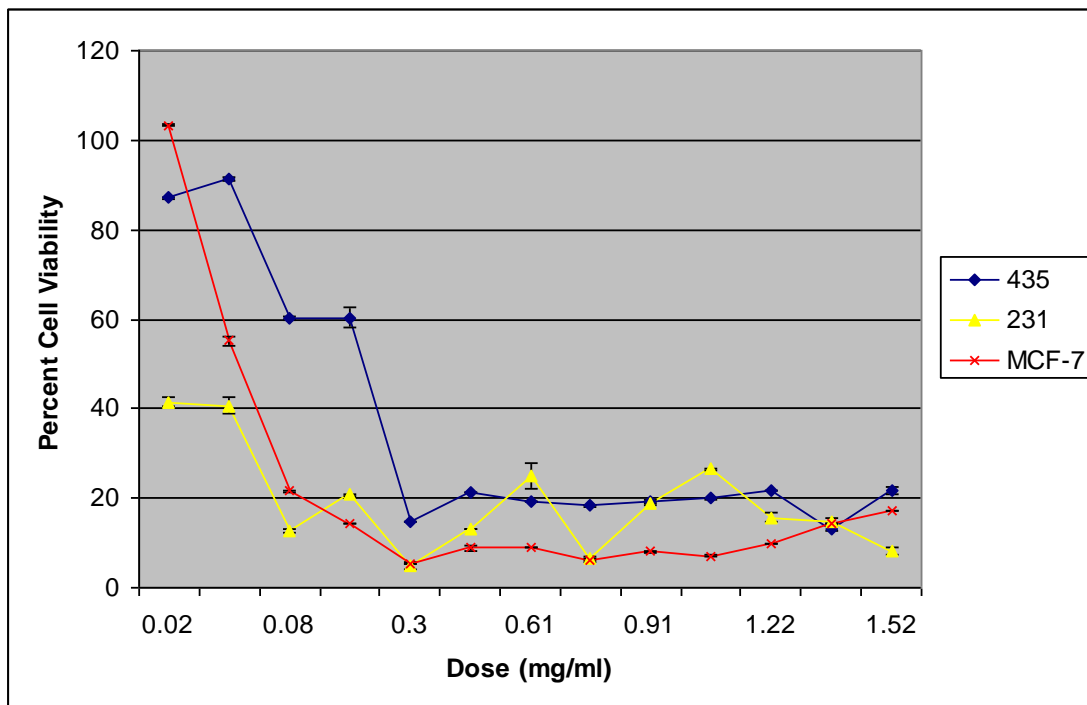

Figure 5. Toxicity of Onxol® to three human breast cancer cell lines (435, 231, MCF-7).

The toxicity data from the *A. confertifolia* extracts (Figure 4) are similar to the dose-response curves generated using the chemotherapeutic drug Onxol® (Paclitaxel) (Figure 5). These data clearly show Onxol® is an excellent anti-cancer drug, with a significant dose response (F=8.51, p<0.0001) that differed among cell lines (F=1.71, p=0.0315). For Onxol, lower concentrations (0.3 mg/ml) cause near 100% mortality. Gangadevi and Muthumary (2007) have shown similar findings, in their study using Paclitaxel they viewed about 80% apoptosis in each of five cell lines. Although the *A. confertifolia* extract did not elicit the exact same dose-response curves, it did show a similar degree of toxicity in the higher doses.

We may also take into consideration that extracts of *A. confertifolia* have not been purified to single compounds which are causing cell death. With future isolation procedures a more potent concentration of *A. confertifolia* might be obtained resulting in even higher levels of toxicity at even lower doses than the currently FDA-approved drugs.

To examine cell viability over time, a dose of 2.05 mg/ml was administered to a breast cancer cell line (435), a cervical cancer cell line (HeLa), and the control monocyte cell line. These cultures were allowed to incubate from 1 to 24 hours. Figure 6 shows that cancer cell viability decreased precipitously after 8 hours of incubation, especially for the breast cancer cells. Again, monocyte control cells did not appear to be greatly affected. After 18 hours of incubation, both cancer cell cultures were reduced to approximately 20% viability. There were significant differences among all three curves F=168.89, p<0.0001. However, there is no statistical difference between the cancer cell lines T =-0.9, p =0.93.
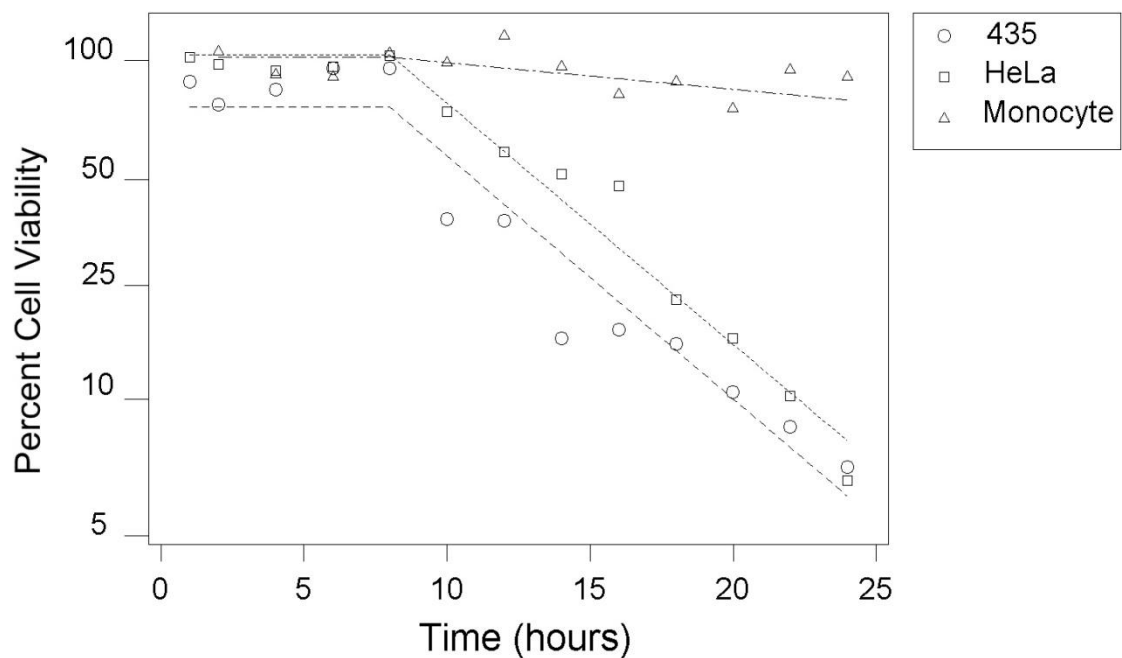
Figure 6. Timed toxicity response of a breast cancer cell line (435), a cervical cancer cell line (HeLa), and a monocyte control cell line to 2.05 mg/ml of extract from *Atriplex confertifolia*.

Many chemo-therapy drugs, such as paclitaxel and colchicines, interfere with the normal function of microtubule breakdown. Colchicine causes the depolymerization of microtubules whereas paclitaxel arrests their function by having the opposite effect; it hyper-stabilizes their structure. This destroys the cell's ability to use its cytoskeleton in a flexible manner and does not have the ability to disassemble. This adversely affects cell function because the shortening and lengthening of microtubules (termed dynamic instability) is necessary for their function as a mechanism to transport other cellular components (Kumar, 1981).

The delay in toxicity shown in Figure 6 suggests that it takes approximately 8 hours for the toxic compounds to enter the cell, eventually disrupting microtubule function and resulting in death either by apoptosis or necrosis. Apoptosis is controlled

cell death while necrosis is the immediate complete breakdown of the plasma membrane, resulting in the release of intercellular proinflammatory molecules (Edinger, 2004).

Figure 7 shows an SEM of two normal HeLa cells, while Figure 8 shows a HeLa cell treated with 2.05mg/ml of *A. confertifolia* for 6-8 hours. The contorted-looking state of the treated cell in Figure 8 shows cellular blebbing and the formation of apoptotic bodies. The blebbing is an irregular bulge in the plasma membrane typical of a cell undergoing apoptosis.

These bulges noted in Figure 8 often separate from the cell, taking a portion of the cytoplasm with them (Coleman, 2001; Edinger, 2004). Hence, these data strongly suggest that *A. confertifolia* kills HeLa cells by apoptosis and not by necrosis.
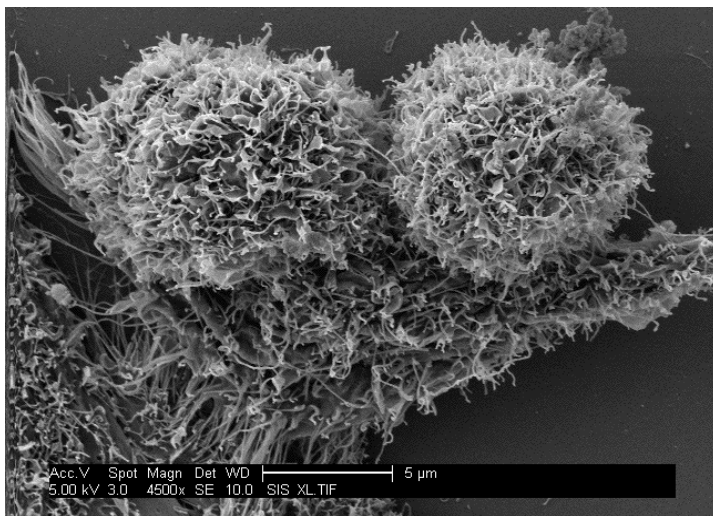
Figure 7. Scanning electron micrograph (SEM) of two normal HeLa cancer cells.

Overall, these data clearly indicate that extracts of *A. confertifolia* contain compounds that are toxic to several cancer cell lines that approaches the toxicity of even Onxol®, an FDA-approved breast cancer drug. Hence, isolation and testing of these potent compounds needs to be further tested.
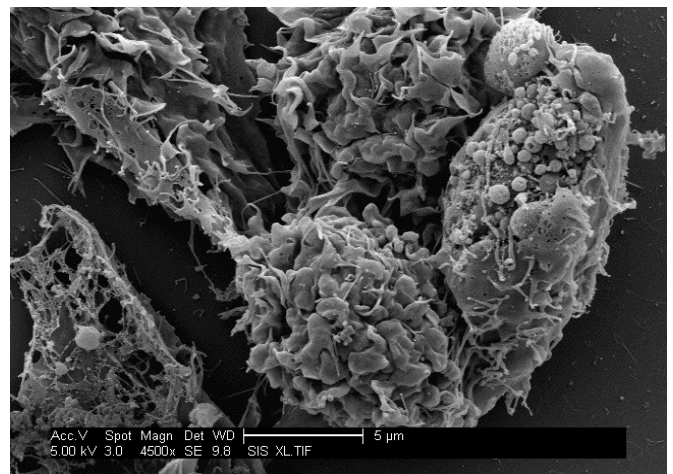
Figure 8. Scanning electron micrograph of a HeLa cell treated with extracts of *Atriplex confertifolia*, showing the formation of apoptotic bodies.

IV. CONCLUSIONS

These data provide documentation that extracts of *A. confertifolia* selectively kills three types of breast cancer cells but does not affect monocyte control cells. It is clear that the majority of toxic compounds are found in the polar fractions of the plant extract. The toxicity of *A. confertifolia* and the concentration of the extracts were generally comparable to those of the FDA-approved drug Onxol® especially at the highest doses, although Onxol® killed the cells more completely at lower doses. In addition, it takes approximately 8 - 10 hours before cell mortality can be detected. This was observed for both breast and cervical cancer cells. The monocyte control culture was again not affected by the 24 hour incubation period with *A. confertifolia*. It is concluded that extracts of *A. confertifolia* causes cell death via apoptosis and not by necrosis. *A. confertifolia* is one of the few non-tropical desert species that shows selective bioactivity against a variety of human cancer cells.

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APPENDIX A

Normal Maintenance (Passaging) of the Cells

Procedure:

1) Withdraw all medium from 250 ml, 75 cm^2 tissue culture flask and discard.

2) Wash cells with pre-warmed 37 ºC Hank's Balanced Salt Solution (HBSS), aspirate and discard. The volume of HBSS should be approximately the same as the volume of medium used for culturing cells. This will remove the acting ions from serum that will inhibit trypsin enzymatic activity.

3) Repeat above procedure.

4) Add enough 1x trypsin-EDTA solution to cover the monolayer and rock the flask 4-5 times to coat the monolayer. Solution is 10x trypsin-EDTA solution. Dilute to 1x concentration using HBSS. Trypsin should be warmed no longer than 45-60 minutes, otherwise refrigerate.

5) Loosen the flask cap and place the flask in the incubator for 3-5 minutes.

6) Remove flask from incubator, tighten flask cap and firmly rap the side of the flask with palm of hand to assist detachment. If cells have not dislodged, loosen the flask cap and return the flask to the incubator for a few minutes. Note: do not leave the cells in trypsin for extended periods of time as trypsin will kill the cells. Overly confluent cultures, senescent cells and some cell lines may be resistant. Some cells lines may detach when repeatedly pipetteing the cells up and down in media. This should be done as gently as possible to avoid damaging the cells.

7) Once dislodged, resuspend the cells in pre-warmed growth medium containing serum. The magnesium and calcium ions found in the serum will deactivate the trypsin.

8) Add 10 ml of RPMI1640 and triturate 20 times. Transfer 10 ml from incubation flask into a sterile 14 ml vial, leaving 1.0 ml in flask for cells, 0.5-0.7 ml for B16F10 and 1-1.5 ml 3T3 to reseed the culture.

9) Add RPMI1640 to a final volume of 14 ml in incubation flask. Replace incubation flask every 5 passages.

10) Place back in the incubator (37 ºC with 5% CO2) until cells are confluent (3-4 days, never to exceed 5 days). Leave cap loose for CO2 transfer.

Preparation of Leukocyte Cells

1) Draw determined amount of blood into EDTA tubes.

2) After filling and removing each tube, invert each tube multiple times to homogenously spread the EDTA.

3) Fill 50ml conical vials with up to 21ml of blood.

4) Double blood volume with Hank's. IE: if you have 21ml of blood, top the tube off at 42ml. If you draw more blood, need to work with more than one tube.

Fill another 50ml conical vial with about 7ml of Lymphocyte Separation Media (LSM).

Layer your blood/hank's solution on top of the LSM. You'll need to pipette the blood very slowly down the side of the tube so that it doesn't break or dip into the LSM layer. Once you've got a few mls of blood you can begin to pipette faster and faster, but I would go very slowly until you have 10 to 15ml of blood already layered.

Spin the LSM/blood/Hank's solution for 25 minutes, with the centrifuge brake off, at 1500rpm.

You'll have 4 easily identifiable layers if you look at the vial from the side, there are actually 5, here they are in order; you'll be removing the white buffy coat.

1) Red Blood Cells
2) Thin layer of granulocytes directly on top of the blood (basophils, neutrophils, eosinophils)
3) LSM
4) Buffy Coat - Try to pipette from above the coat to minimize LSM in your pipette. Also try to do in one continuous suction, as each time you stop the suction some will drip out of the pipette back into your LSM and become less recoverable.
5) Blood serum

Add buffy coat to clean 50ml conical vial. Top off using COLD Hanks (must be cold) to about 50ml.

Spin for 10 minutes at 1280 rpm.

Discard supernatant.

Reconstitute pellet with about 50ml of cold Hanks

Spin for 15 minutes at 860 rpm.

Discard supernatant.

Reconstitute pellet with about 50ml of cold Hanks

Spin for 10 minutes at 1160rpm.

Discard supernatant.

Reconstitute pellet with 5 to 10ml of cold media.

Suction media+buffy coat cells and place in flask.

Let incubate for 90 to 120 minutes (120 strongly recommended).

Without disturbing the monolayer, remove media. This media will contain your lymphocytes, the monolayer will contain monocytes.

Your lymphocytes should be good for about 24 hours, of course the sooner you work with them the better.

Preparation of the "Complete" Growth Medium

1) RPMI1640 Balanced Salt Solution
    450 ml medium (RPMI1640)
    50 ml Cosmic Calf Serum, heat inactivated. (Store in inner cold room).
    5 ml L – Glutamine 200 mM solution. (Store in inner cold room).
    5 ml Sodium Pyruvate 100 mM solution, (Keep refrigerated).
    5 ml MEM Nonessential Amino Acids 100 x solution. (Keep refrigerated).
    0.5 ml Gentamicin 50 mg/ml solution. (Keep refrigerated).

Preparation of a 96-Well Flat-Bottom Plate for Cell Assay

Procedure:

1) In a sterile trough:
    a. Add 500 μL of cell solution.
    b. Add 15 mL of RPMI1640 warmed to 37 ºC.
2) Triturate 15-20 times.
3) Add 150 μL to each well of 96 well plate (with flat bottom).
4) Incubate for 24 hours at 37 ºC with 5% CO2.

"Drugging" the Cells

Procedure:

1) Add a total volume of 50 μL to each well. This contains RPMI1640 warmed to 37 ºC and varying concentrations of drug solution. Return plate to incubator for 24 hours.

2) Use a random number generator to produce chart where you will place the controls (B-2,3,4; D-5,6,7; F-8,9,10) and the extract—don't use the edges of the wells and use double digits from the randomization table (3 sets of 3 per drug).

3) Add 50μL of <u>control</u> ά-MEM Growth Media to designated wells (C) and 50μL <u>experiment</u> extract + media to designated wells (50) in 3 sets of 3.

4) The following is an example of a chart used for drugging the cells.

Date: Project: Plate #:   Plants: Initials:

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	X	X	X	X	X	X	X	X	X	X
B	X	C	C	C	15	15	15		45	45	45	X
C	X		5	5	5							X
D	X				C	C	C					X
E	X			50	50	50	20	20	20			X
F	X	10	10	10				C	C	C		X
G	X			25	25	25	30	30	30			X
H	X	X	X	X	X	X	X	X	X	X	X	X

Sulforhodamine Staining Protocol

Procedure:

1) Aspirate off medium:
    a. Add 75 μl of 0.4 M perchloric acid .
    b. Let sit for 15 minutes.
    c. Aspirate off perchloric acid.
    d. Rinse wells 4 times with distilled water.
2) Add 100 μl of 4% sulforhodamine B in 1% acetic acid.
    a. Let sit for 30 minutes at room temperature.
    b. Aspirate off sulforhodamine B.
    c. Rinse wells 4 times with 1% acetic acid (150 μl per rinse).
    d. Allow to dry overnight.
3) Add 75 μl of 10 mM tris base (unbuffered) to wells.
    a. Read plates at 570 nm.

Sulforhodamine Preparation

Weigh out 400 mg of sulforhodamine B and add 100 ml of 1% acetic acid.

0.4 M perchloric acid
Add 4.5 ml of 20% perchloric acid to 120.5 ml H_2O.

10 mM tris base (unbuffered)
Add 4 ml of 1 M tris base (unbuffered) to 396 ml of H_2O.

Counting the Cells with a Hemocytometer

1) Cut out a small piece of parafilm.

2) Place a thin glass slide over the hemacytometer.

3) Shake the test tube (from the passage procedure above) to mix the cells. Using a pipette place 20 μl of cell solution on the parafilm. With another pipette tip place 20 μl of trypan blue on the drop of cell solution and triturate to mix them.

4) Using the pipette inject the mix into the groves on each side of the hemocytometer until the liquid barely fills the entire grid.

5) Focus the microscope on the hemocytometer until the cells in the one of the corner area grids can easily be viewed.

6) Count the number of cells in the 16 squares in each of these 4 regions for both grids getting a total of 8 values.

7) Calculate the concentration of cells in the solution.
    a.   Average the number from the 8 regions.
    b.   Multiply by 20,000 this = # cells/ml solution.

Note: Cells should be in ~ 1 or 2 x 10^6 in concentration when diluted.
         After dilution of protocol of 500μL and 15mL of media, cell concentration will have a concentration of something similar to 0.8-$1\text{x}10^{5}$ cells/ml.

Grid Example:

Small square = 1/400 sq. mm.   1/25 sq. mm.

Counting grid (central area)
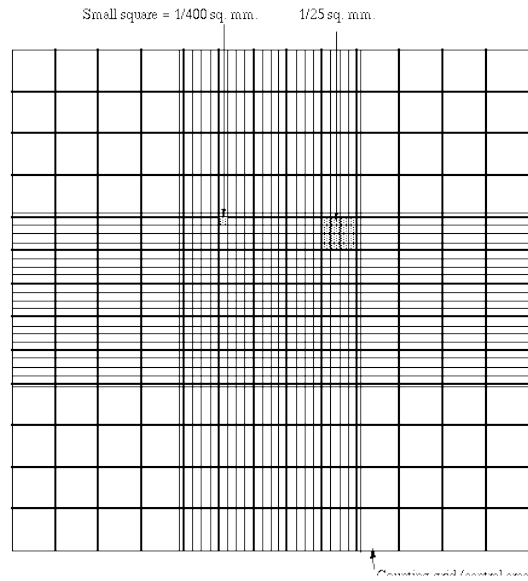
Freezing Cells

Procedure:

1) Trypsinize cells, re-suspend in cell culture medium, and transfer to a 15 ml centrifuge tube.

1b) Centrifuge and re-suspend in fresh medium (5 ml or less).
    Add DMSO, put 1 - 1.2 ml in each vial.

2) To a separate 15 ml tube, add 1 ml of cell suspension for every tube or vial to be frozen. For every 1 ml of cell suspension, add 0.05 ml of sterile DMSO. Mix well and place in ice.

3) Add 1 ml cell suspension to cryogenic vial, using care sterile technique. Cap, label and place in ice.

4) Transfer vials to neck of liquid N2 tank, using 2 gauze squares to plug the hole in the neck. Allow to freeze overnight. Transfer vials to wands. Record addition to Frozen Cell Inventory, Cap #, date, and cell line. N2 freezer in (775775) 841 WIDB, closest to sink with white tape.

Thawing Frozen Cells

Procedure:

Remove vial from liquid N_2 tank. Put in ice to transport. Thaw rapidly in 37 ºC water bath with agitation. Spray complete vial with 70% ETOH. Using a 1 ml pipette, add to 20 ml of prewarmed growth medium. Incubate 2-3 days until confluent.

Preparation of Cell Culture Medium from Powder

Procedure:

1) One bottle makes one liter. We usually prepare 4 liters. Using a 2 L cylinder, and the cell culture prep 4 L beaker, get MQ H_2O from Dr. Simmons's cell culture lab (around C-260 BNSN). Put 2 L in the beaker and retain 2 L in the cylinder.

2) While stirring, add the 4 bottles of powdered media, rinsing out each bottle, into 3 liters of H_2O. Stir until dissolved.

3) Add buffers according to the medium being prepared, add more H_2O to 3.8 L. Allow to stir well to dissolve all powder. Use a Pasteur pipette to wash down powder clinging to the sides of the beaker.

4) Test the pH of the medium. RPMI = 7.2 pH, RPMI1640 = 7.0 pH. Adjust if necessary with NaOH or HCl.

5) Measure the medium using another 2 L cylinder, to 4 L exactly. Add back to the 4 L beaker and stir briefly. The medium is now ready to filter through the Gelman Vac-cap 0.2 μm filter for sterilization.

6) Fill medium bottles to 450 ml. Catch first 40 ml in a sterile 100 ml bottle.

7) Test for sterility by incubating in the 37 ºC incubator.

Preparing Trypsin:EDTA 1x

Add 11 ml of thawed trypsin: EDTA (10x) solution to 100 ml of sterile PBS.

PBS 4L (.01 M) = 1.04g NaH_2PO_4-H_2O, 4.6 g Na_2HPO_4, 34 g NaCl. Filter at 0.2 μm to sterilize and adjust pH to 7.2

Scanning Electron Microscope Preparation
Procedure:

Day 1
1) Wash with PBS (3 – 4x 10 min each)
2) Discard PBS
3) Soak cells in 2% Buffered Gluteraldehyde (30 minutes – 1 hr or can leave over night)

Day 2
4) Sodium Cacodylate Buffer (6x 10 min each)
5) Fix with OsO_4-Osmium Tetraoxide (1 - 2 hrs)
6) Wash with DI water (6x 10 min each)
7) Dehydrate
   a. 10%
   b. 30%   EtOH (3x 10 min each)
   c. 50%
   d. 70%   (can leave in 70% up to 3 days)

Day 3
8) Dehydrate
   a. 95 %   EtOH (3x 10 min each)
   b. 100%
9) 100% Acetone (3x 10 min each)
10) Critical Point Dry

Day 4
11) Gold sputter coat the slide
12) Observe slide in electron microscope.