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Antimicrobial activity of d-lenolate®

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ANTIMICROBIAL ACTIVITY OF D-LENOLATE®

by

Andy Phui

Bachelor of Science
University of Nevada, Las Vegas
2007

A thesis submitted in partial fulfillment of
the requirements for the

Master of Science Degree in Biochemistry
Department of Chemistry
College of Sciences

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THE GRADUATE COLLEGE

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Andy Phui

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ABSTRACT

Antimicrobial Activity of D-lenolate®

by

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Olive trees are one of the most important fruit trees in the Mediterranean. Although not validated by research, olive leaves are traditionally believed to fight off fever and infections. It has been shown that olive leaf extracts possess antimicrobial activity. Olive leaf extracts contain polyphenols. One of the major phenolic compounds is oleuropein. Oleuropein and other polyphenols have been shown to exhibit antimicrobial activity.

East Park Research (EPR) developed an extraction process that they claim does not alter the chemical composition of the olive leaves. The extract is known by the commercial name d-lenolate®. Studies have provided evidence that d-lenolate®, like other olive leaf extracts, has antimicrobial activity against different pathogens. However, the active antimicrobial compound(s) of d-lenolate® has not been identified.

The purpose of this study was to purify the active compounds of d-lenolate® and evaluate their antimicrobial activity. Different chromatographic methods were used to fractionate d-lenolate®. Disk diffusion and growth inhibition tests were performed to test for antimicrobial activity.

We found that fractions of d-lenolate® extracts devoid of oleuropein were significantly more active than purified oleuropein. Interestingly, compounds from extracts that possess antimicrobial activity based on our results were shown to lose the trait when purified. Whether the d-lenolate® extracts or purified compounds provide

greater antimicrobial properties in this study is not conclusive. However, the data demonstrated that d-lenolate® extracts at 1 mg/ml do not possess 100% antimicrobial activity, but only delays the growth of *Bacillus anthracis* and *Escherichia coli*.

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CHAPTER 1

INTRODUCTION

1.1 Background and Significance

In the United States alone, some 14,000 individuals die each year from drug-resistant microbes acquired in hospitals. Of the resistant microbes now proliferating around the world, none carries more potential for destruction than the emergence of hospital-acquired "super-infections". The resurgence of these "super-bugs" has created the need for the discovery of novel anti-infective compounds. Thus the development of new antibiotics is a primary concern, due to the antimicrobial resistance of microbes to current antibiotics (1). The interest of pharmaceutical industries in natural drugs, especially plant sources, is on the rise. Natural medicine has become a focal point because the public views these drugs as harmless. These plant sources have been reported to possess a wide spectrum of antimicrobial activity (2, 3). One particular plant source widely used as a source of natural drug stems from the olive tree.

Olive trees (*Olea europea*) are a type of evergreen trees that produce the fruit olive. They are one of the most important fruit trees in the Mediterranean (4). These trees grow very slowly, ranging between 12-36 feet tall. They are characterized with twisted trunks and branches, green silvery leaves, and white flowers. Olive trees have been documented to have a long life span. They have been known to be resistant to microbes and insect attacks, due to their antimicrobial properties (5).

People living in the Mediterranean practice a healthy life style. A significant portion of their daily diet consists of olive oil, which is a contributor to their well-being (6, 7). One of the main components of olive oil are polyphenols, which is believed to exhibit

antimicrobial activity. Due to the phenolic content, olive products are regarded as an important part of a healthy diet (8). It was reported that phenolic compounds extracted from olives inhibited *Bacillus cereus* T spore germination, delaying spore outgrowth (9).

When olives are physically pressed, virgin olive oil is produced. The result of the pressing is an olive paste rich in phenols, contributing to the pungent and bitter taste of virgin olive oil (7, 10). On the other hand, when olives go through a different refining process, olive oil is produced. This results in a decrease in polyphenol count. An increase in polyphenol count in refined olive oil occurs when it is mixed with virgin olive oil (10, 11). A study performed by a research group in Spain helped support the fact that polyphenols in olive oil possess antimicrobial activity. Olive oil and virgin olive oil were tested to determine the inhibition of growth of foodborne pathogens. The results indicated that both oils exhibit antimicrobial properties, but virgin olive oil was determined to be the stronger inhibitor (12).

One of the major phenolic compounds possessing antimicrobial activity is oleuropein (4). Oleuropein can be found throughout the entire olive tree but the highest amount is present in the leaves, approximately 60-90 mg per gram in dry weight (7, 13)

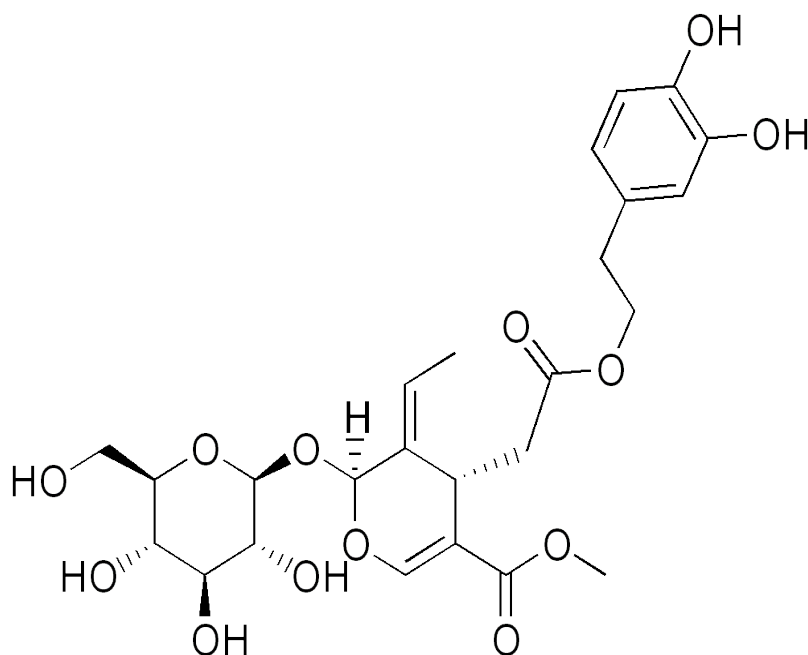


Figure 1. Structure of Oleuropein. Oleuropein is an ester of 2'-(3',4'-dihydroxyphenyl)-ethanol (hydroxytyrosol) with a molecular weight of 540.5 (1, 14).

Studies have reported that at different concentrations of oleuropein, antimicrobial activity was seen. At low and intermediate concentrations of oleuropein, an extended lag phase and a delay of bacterial growth was observed in *Staphylococcus aureus*, *Salmonella enteritidis*, and yeast cultures (15, 16, 17). However with high concentrations of oleuropein, complete inhibition of growth was seen in *Staphylococcus aureus* (15).

Another study with human erythrocytes showed that oleuropein at low concentrations caused lysis of red blood cells. Addition of oleuropein at 0.5, 1.0, and 2.0 mg per ml of suspension caused lysis of erythrocytes up to 40, 48, and 60%, respectively (18). On the contrary, others have reported that high concentrations of olive oil phenolic compounds, including oleuropein, appear to have no cytotoxic effects on human cells from the oral cavity (19).

A different study suggested that oleuropein can interfere with the peptidoglycan layer of *Lactobacillus plantarum*, a rod-shaped, Gram-positive bacterium (20). The interference of the peptidoglycan layer prohibited the cell from retaining the crystal violet stain that would indicate a Gram-positive bacterium, thus they observed a Gram-negative profile. Also, when treated with oleuropein, the morphology of the bacteria became longer and wider. The disruption of *Lactobacillus plantarum* peptidoglycan layer promoted by oleuropein could lead to cell death by destruction of the cell envelope, which is composed of the cell wall and cell membrane. The mechanism is still unknown, but there are assumptions of how oleuropein exhibits antimicrobial activity. Oleuropein is suggested to be a surface-active compound that can cause leakage of the cell. They are assumed to have the ability to denature enzymes and cause the release of cell constituents, resulting in the killing of the cell (5, 21).

In addition to oleuropein, extracts of olive leaves has been shown to inhibit bacterial growth. Although not validated by research, the olive leaves were traditionally believed to fight off fever and infections (22). Current studies have shown that extracts inhibit bacterial growth of *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas solanacearum* in a paper disk assay (23). Though a zone of inhibition was observed, bacterial growth was seen after several days, suggesting an extended lag phase (28).

Extracts of olive leaves has been reported that inhibition of bacterial growth may be due to synergistic properties (4, 24). A study done in Portugal showed that olive leaf extracts inhibited bacterial growth of Gram-positive and Gram-negative bacteria and fungi. They speculated that the mixture of components worked well together and that individual compounds would not be as effective (4). A research group from California

supported the assumption of synergistic activity. Preliminary screenings of a methanol crude extract of olives against *Bacillus subtilis* exhibited antimicrobial activity. But the attempt to isolate the active compound(s) was not successful (24).

D-lenolate® is an olive leaf extract produced by East Park Research, a company headquartered in Las Vegas. They claim that their patented extraction process does not alter the chemical composition of the olive leaves. Studies have provided evidence that d-lenolate®, like other olive leaf extracts, has antimicrobial activity against different pathogens (25, 26).

A study done in Japan tested the therapeutic effects of d-lenolate® from East Park Research on infections in immuno-compromised mice. The d-lenolate® supplements were given to the immuno-deficient mice in order to investigate any reversal of leukocytopenia, a decrease of white blood cells. Mice that have been exposed to X-ray irradiation and infected with microbes such as, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, had a significantly lower survival rate when compared to that of irradiated and infected mice with d-lenolate® treatment (25).

The same research group from Japan tested the survival rates of mice when treated with d-lenolate® against influenza virus. The results indicated that d-lenolate® possess the ability to restore peripheral polymorphonuclear leukocytes (PMN) levels. As a result d-lenolate® can help the host with early protection against the virus infection (26).

1.2 Specific Aims of the Project

The goal of this project was to separate and purify individual antimicrobial active compounds from d-lenolate®. Although it is believed that phenolic compounds of olive leaf extracts possess antimicrobial properties, a direct separation, purification, and individual compound analysis of d-lenolate® has not been attempted. Different chromatographic methods were applied to fractionate d-lenolate®. Testing for antimicrobial activity against different pathogens was also performed. The d-lenolate® mixture was not used as a whole; instead components obtained through purification were tested to determine the existing antimicrobial compound(s).

These compounds were tested against *Bacillus anthracis* and *Escherichia coli* bacteria to determine the presence of any antimicrobial activity. *B. anthracis* is a Gram-positive, rod-shaped bacterium that can be grown in aerobic or anaerobic conditions. They are also spore forming cells, which are capable of surviving harsh conditions. These conditions may be dehydration or the lack of nutrients. Spores are much more heat resistant than vegetative cells and cannot be killed by mere boiling (27, 28). Only when optimal conditions are presented that the *B. anthracis* spores will germinate into vegetative cells.

Escherichia coli is a Gram-negative, rod-shaped bacterium. Just like *B. anthracis*, *E. coli* can also grow in both aerobic and anaerobic conditions. These are not spore forming cells, usually only Gram-positive bacteria can form spores. *E. coli* are primarily located in the intestines of mammals. These cells are usually the cause of gastric discomfort such as diarrhea (29).

There were two reasons why *B. anthracis* and *E. coli* were used to test for antimicrobial activity. The first reason was that they were easily obtained due to the use of both *B. anthracis* and *E. coli* in the lab. The second reason was that *B. anthracis* is Gram-positive and *E. coli* is Gram-negative bacterium. Testing these two bacteria will provide an understanding of which category of bacterium, Gram-negative or Gram-positive, is more susceptible to d-lenolate®.

A toxicity test against mammalian cells was also performed to determine if these antimicrobial compounds are cytotoxic. The mammalian cell line used for this part of the experiment is the J774a.1 murine macrophages. This cell line was used in this experiment as opposed other human cells because these cells were readily available to us and our lab is familiar with the procedures of maintaining and growing these cells. The stability of oleuropein in artificial gastric juice was determined. The binding of oleuropein to serum was also tested.

CHAPTER 2

MATERIALS AND METHODS

2.1 Solubility of D-lenolate®

Samples of d-lenolate® (100 mg) were weighed and placed in 10 individual vials. Each vial was independently treated with 10 ml of hexane, dichloromethane, methanol, tetrahydrofuran, ethyl acetate, acetonitrile, DMSO, water, 5% HCl, and 5% NaOH, respectively. The samples were vortexed for one minute. Vials were then centrifuged for five minutes. Solvents were decanted and vials dried at 50 °C under vacuum. The weight of the remaining d-lenolate® was determined. The percentage of dissolved d-lenolate® was calculated by dividing the weight of the remaining d-lenolate® by the weight of the original d-lenolate® sample.

2.2 Qualitative Determination of Phenolic Compounds in D-lenolate®

D-lenolate® was dissolved in methanol and the solution was applied to thin layer chromatography (TLC) plates. The plates were individually developed in 0%, 1%, 2%, 3%, 5%, 10%, 12.5%, 15% and 20% methanol/dichloromethane solvent solutions. TLC plates were observed under ultraviolet (UV) light and stained with iodine crystals to find spots corresponding to phenolic compounds.

2.3 Purification of Oleuropein from D-lenolate® and Competitor Olive Leaf Extract

Chemical and physical characterization of purified oleuropein from Indofine Chemical Company, Inc. was initially determined. This commercial product was used to ensure that oleuropein is soluble in methanol. Methanol was then used to extract soluble compounds from d-lenolate®. TLC was performed with the methanol extracted d-

lenolate® sample and pure oleuropein as the control to help determine the presence of oleuropein in the d-lenolate® extracted sample. The MeOH extracted sample was dried under pressure producing a crude extract. The crude extract was then separated and purified by column chromatography. Column chromatography was developed using a solvent elution step gradient of 100% CH₂Cl₂, 5 % MeOH/CH₂Cl₂, 10 % MeOH/CH₂Cl₂, 15 % MeOH/CH₂Cl₂, and 20 % MeOH/CH₂Cl₂. The fractions collected were then analyzed by TLC to determine the presence of pure oleuropein. The column was repeated twice to ensure pure oleuropein was extracted. The above protocol was also used to separate oleuropein from a different olive leaf extract labeled “C” produced by a competitor company.

2.4 Binding of Commercial Oleuropein to Serum

Oleuropein was dissolved in double distilled water to a final concentration of 1 mM. The UV-visible spectrum of oleuropein in water shows strong absorption peaks at 235 nm and 275 nm. A weaker absorption peak was also detected at 330 nm as seen in figure 2. The molar absorptivity for each of the three peaks according to Beer’s Law is 3830 M⁻¹ cm⁻¹, 2920 M⁻¹ cm⁻¹ and 860 M⁻¹ cm⁻¹, respectively. A dialyzed fetal bovine serum (Sigma) sample, on the other hand, showed absorbance peaks at 220 nm and 280 nm. Due to overlap of the absorbance spectra in the 200-280nm range, the oleuropein peak at 330 nm was used to determine serum-oleuropein binding. To test for serum binding, a 1 mM solution of oleuropein in water was mixed with an equal volume of dialyzed fetal bovine serum (FBS). The mixture was partitioned using a centrifuge size-exclusion filter (Pall).

The filter (3K) was big enough to only allow oleuropein to flow through, but too small to allow the proteins of FBS to cross through.

Flow-through and retentate samples were analyzed by UV-visible spectroscopy to determine the amount of free and serum-bound oleuropein, respectively. Each sample was normalized by dividing each spectrum by the corresponding spectrum of a serum-free oleuropein sample. If oleuropein does not interact with serum, it is expected that the both the flow-through spectra will have maximum normalized value of 1. On the other hand, if serum proteins can bind oleuropein, it is expected that the retentate will have a positive peak with a normalized value >1 . Correspondingly, the flow-through spectra will show a negative peak with a normalized value <1 .

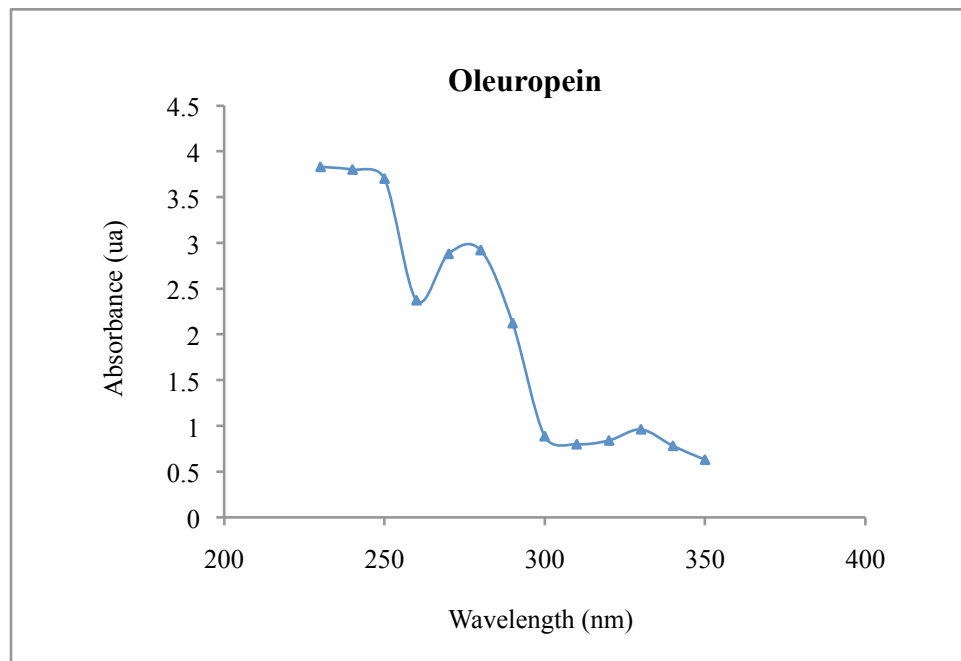


Figure 2. Absorbance spectrum of oleuropein.

2.5 Testing of Oleuropein Binding to BSA

Oleuropein purified from d-lenolate® and oleuropein purified from extract “C” were tested for serum binding. However, in this instance, complete dialyzed fetal bovine serum could not be used due to the high background in the absorbance spectra. High background was a problem even after serum was re-dialyzed. Due to this problem, bovine serum albumin (BSA) was used as surrogate for complete serum. Albumin is the most abundant protein in serum. The oleuropein peak at 330 nm was also used to determine serum-oleuropein binding because there was no peak observed in the BSA absorbance spectrum as seen in figure 3. To determine binding of oleuropein samples to BSA, the equilibrium dialysis procedure was used. The apparatus used in this experiment was the Spectrum Equilibrium Dialyzer from SpectrumLabs. Different millimolar (0.5 mM, 0.25 mM, and 0.125 mM) concentrations of both extracted oleuropein, d-lenolate® and “C” were tested during this part of the experiment. The procedure included testing 0.5 mM extracted oleuropein against 0.1 mM BSA, 0.25 mM extracted oleuropein against 0.25 mM BSA and 0.125 mM extracted oleuropein against 0.1 mM BSA.

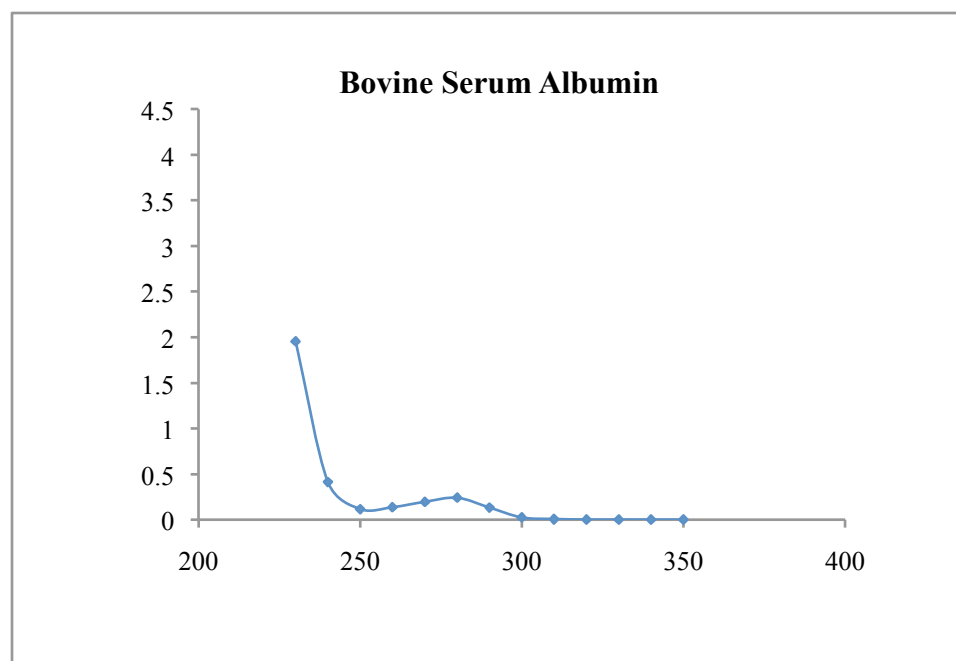


Figure 3. Absorbance spectrum of Bovine Serum Albumin.

The basis of this test is that in each cell of the apparatus, there are two chambers. One chamber is loaded with oleuropein along with BSA and in the second chamber, only oleuropein (same concentration and volume as the first chamber). A membrane disc that only allows the diffusion of oleuropein is placed in between the two chambers. The equilibrium dialysis will run for 24 hours. In that 24-hour span, free (nonbinding) oleuropein will reach equilibrium between the two chambers. Absorbance readings of each chamber are then taken using the InfiniteM200 spectrophotometer from Tecan. The wavelength used to measure the absorbance of oleuropein is 330 nm, which is unique to oleuropein and is not interfered by BSA.

2.6 Fractionation of D-lenolate®

The extraction method used to fractionate desired products from d-lenolate® was carried out using the soxhlet apparatus (Barnstead/Electrothermal). D-lenolate® powder (200 g) was extracted for 8-10 hours with methanol to ensure that all alcohol soluble compounds are collected. The resulting extract is then dried under vacuum and ground with a pestle and mortar to ensure that there is no clumping that would interfere with uniform separation during column chromatography. The dried extract was then further purified by performing the dry column vacuum chromatography (DCVC) (30). The advantage of DCVC as opposed to a conventional column chromatography is the separation can be applied to a larger scale (up to 100 grams). Silica gel of 15-40 μm was loaded and vacuumed packed into a large sintered glass funnel attached to a side arm flask. The dried extract was then applied evenly to the top of the silica gel and with the use of a vacuum; the extract was sequentially extracted with solvents with increasing polarity to ensure that compounds will be fractionated according to their polarity. D-lenolate® methanol residue was thus sequentially extracted starting with 100% hexane and ending at 100% ethyl acetate increasing in 10% increments. This was followed by 10% MeOH/Ethyl acetate, ending at 100% MeOH, also increasing in 10% increments. One liter fractions were collected and analyzed by thin layer chromatography (TLC).

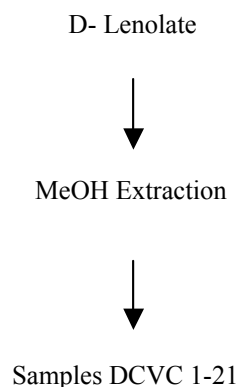


Figure 4. Flow-chart of the initial fractionation of d-lenolate with the soxhlet and the dry column vacuum chromatography.

2.7 Testing of D-lenolate® Fractions for Antimicrobial Activity

To test the 21 samples previously obtained for the presence of antimicrobial activity (DCVC1-DCVC21), the extracts were tested against a Gram positive bacterium, *Bacillus anthracis*, Sterne strain and a Gram negative bacterium, *Escherichia coli*, DH5 α strain. Determining antimicrobial properties were achieved by applying the disk diffusion assay and the growth inhibition test. Both procedures test for the growth of the bacteria, but the difference is disk diffusion is done on solid media as opposed to liquid media for growth inhibition.

Disk diffusion is a process where a disk shaped filter paper is saturated with the sample. The dried disk is then placed onto a lawn of bacteria on an agar plate. The plate is incubated for 24 hours at 37°C. A clear zone around the disk is will be observed and measured after a day of incubation if compounds on the disk have antimicrobial activity, inhibits growth.

Growth inhibition test differs from disk diffusion in that growth is determined in liquid media instead of solid media. Samples along with bacteria are diluted in liquid broth and incubated for four hours. Optical density (OD) is taken at 30 minute intervals to measure bacterial growth. Optical density is a measure of transmittance. Higher OD readings equal lower transmittance, which would indicate bacterial growth. Relative OD is the readings of all the ODs relative to the optical density at time 0 hr. Optical density readings were measured using the Labsystems iEMS Reader MF.

Bacteria treated with methanol were used as a negative control, which does not harm the bacteria at our tested concentration, for both antimicrobial testing procedures since all samples were resuspended in methanol and chloramphenicol was used as the positive control.

2.8 Purification of D-lenolate® Fractions

Further separation and purification of the DCVC10, DCVC11, and DCVC12 samples was achieved by column chromatography. Fractions DCVC10-12 were selected for further purification because results of the growth inhibition tested indicated that these 3 samples showed the greatest antimicrobial activity. This is procedure started out with dissolving 3.0g of the combination of samples DCVC10 and DCVC11 in about 25 ml of 50% MeOH/CHCl₃. The sample was loaded into a column that has been packed with a slurry (stationary phase) of 222.0 grams of 100-200 mesh silica gel in 50% MeOH/CHCl₃. A layer of glass wool was added to the top of the column to protect the column and sample from any disturbance when adding the solvents (mobile phase). The column was then flushed out with 100% CH₂Cl₂ to get rid of all the methanol and

chloroform creating a less polar column. This allowed the column to start at a nonpolar environment and work its way up to higher polarity conditions. The procedure was continued with 2 liters of 2.5% MeOH/CH₂Cl₂. 10 ml fractions were collected and each fraction was analyzed by TLC to determine if compounds have eluted and whether these compounds were pure. The fractions that contained the same compounds were combined. From these procedures, two sets of fractions with similar compound contents based on TLC analysis that exhibited antimicrobial properties were combined for further purification. This sample was labeled A1 for future references.

Sample A1 (1.5 grams) was dissolved in about 15 ml of 50% MeOH/CHCl₃. The sample was loaded into a column that was vacuumed packed with 270.0 grams of 100-200 mesh silica gel creating a dry column instead of a slurry (wet column). The column was then flushed out with 100% dichloromethane and continued with 2.5 liters of 5% MeOH/CH₂Cl₂. The fractions were collected and analyzed by TLC. Fractions A1.3, A1.4, and A1.5 presented antimicrobial activity and were combined (labeled A1.3 for future references) for further analysis by separation and purification.

Sample A1.3 (600 mg) was dissolved in about 4 ml of 2.5% ACN/CH₂Cl₂ and loaded into the column vacuumed packed with 110 g of 15-40 µm silica gel. Five hundred milliliters of 2.5% ACN/CH₂Cl₂ and 500 ml of 5% ACN/CH₂Cl₂ were used to run the column. Fractions of 3 ml were collected and analyzed by TLC. Analysis of these fractions presented no antimicrobial activity, so no further separation of these samples was necessary.

Sample DCVC10

Sample DCVC11



Sample A1



Sample A1.3

Sample A1.4

Sample A1.5



Sample A1.3.4

Sample A1.3.12

Figure 5. Flow-chart of the second set of purification.

A1 (from separation of DCVC10/DCVC11) was also separated by medium pressure liquid chromatography (MPLC). MPLC does not use gravity or a vacuum for the separation; instead, a liquid chromatography pump (Ace Glass) is used. This type of separation uses more pressure than gravity, but not as much pressure as the vacuum. 1.5 grams of the 2.5 grams have been previously separated during the second set of purification leaving 1.0 g for this procedure. The 1.0 g of sample was dissolved in 9 ml of 50% ACN/CH₂Cl₂ and loaded into a Michel-Miller (Ace Glass) column packed with about 110 grams of 5-40 μ m silica gel slurry. Dichloromethane (100%) was used to flush

out the 50% ACN/CH₂Cl₂. ACN/CH₂Cl₂ (300 ml of 8%) was used to run the column. 2 ml fractions were collected and analyzed by TLC. Analysis of these fractions presented no antimicrobial activity, so no further separation of these samples was necessary.

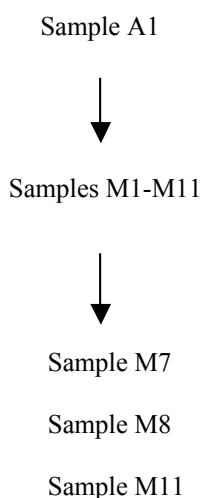


Figure 6. Flow-chart of the third set of purification.

A different set of column chromatography was also performed but with sample DCVC12 from the dry vacuum column chromatography. There were about 8.5 grams of fraction DCVC12 that could be separated and purified. This column was performed with the flash chromatography. Vacuumed is used as opposed to gravity as described during the previous separations. Four hundred grams of 100-200 mesh silica gel was used to vacuum pack the column very tightly. The 8.5 g of fraction DCVC12 was dissolved in about 11.4mL of 100% methanol and loaded into the column. Fractions of 300 ml were collected with the use of the vacuum and analyzed by TLC. Fraction B7 was selected for further purification due to the demonstration of antimicrobial characteristics.

B7 (50.0 mg) was dissolved in about 3 ml of 50% ACN/CH₂Cl₂ and loaded into a column that was vacuum packed with about 30.0 g of 15-40 µm silica gel. The column was first flushed with 100% dichloromethane and followed with about 300 ml of 12% ACN/CH₂Cl₂ and proceeded with 300 ml of 25% ACN/CH₂Cl₂. Fractions of 1 ml were collected and analyzed by thin layer chromatography. Of the 13 samples analyzed, 4 exhibited antimicrobial properties, but were not further separated due to the lack of quantity of the samples.

Sample DCVC12



Sample B7



Sample B7.5

Sample B7.6

Sample B7.10

Sample B7.11

Figure 7. Flow-chart of the fourth set of purification.

Figure 8 shows a complete flow-chart of the purification procedures. This provides an overview of the processes that took place from the first set to the last set of separations.

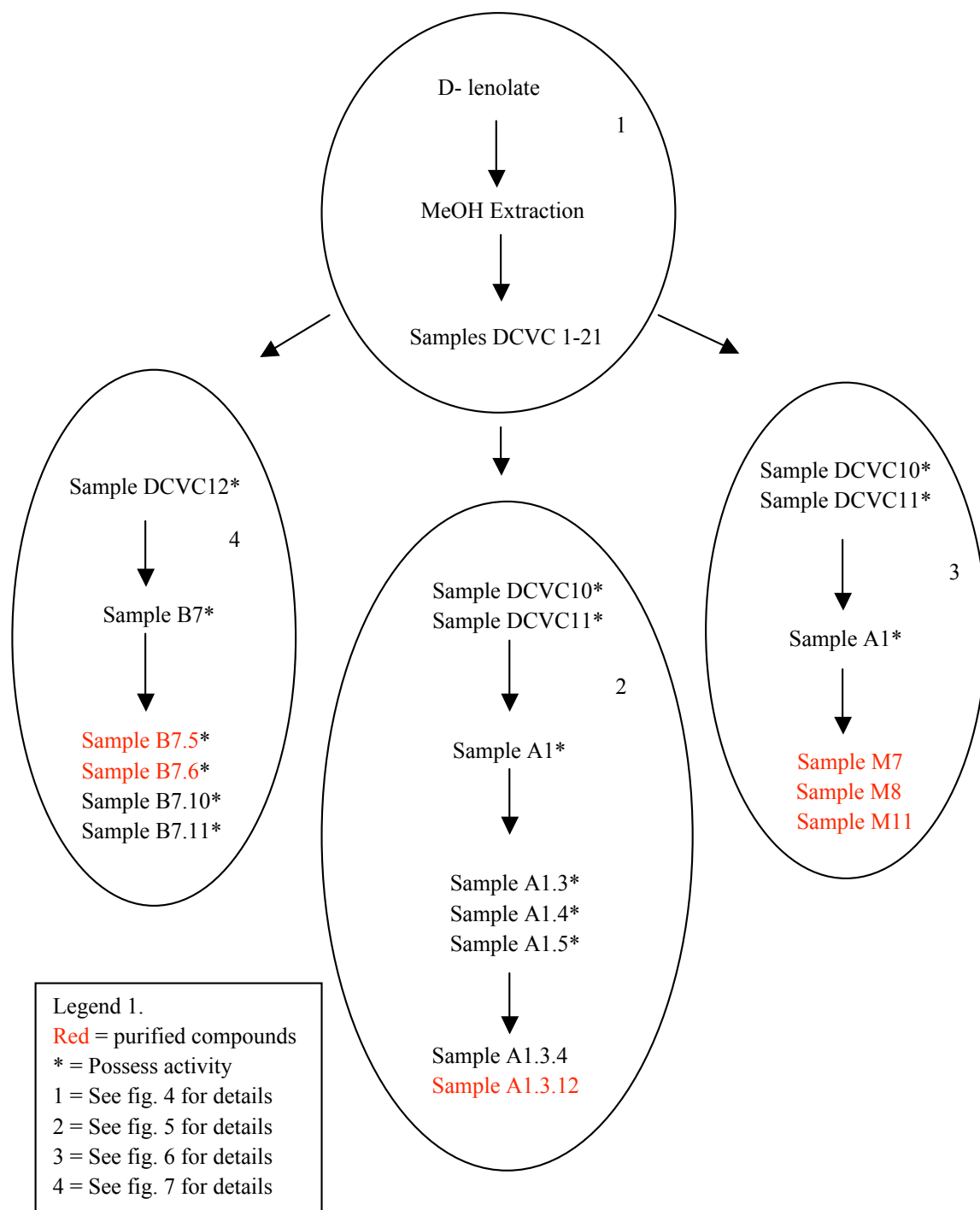


Figure 8. Flow-chart of the complete sequence of separations.

2.9 Testing of Fractions for Antimicrobial Activity

All of the samples obtained from the previous separations and purifications were tested for antimicrobial activity using the growth inhibition test at 1.0 mg/ml.

2.10 Comparing Antimicrobial Activity of D-lenolate® and Spanish Olive Leaf Extract

The antimicrobial activity of East Park Research's d-lenolate® and a comparable olive leaf extract from Spain were tested simultaneously against *Bacillus anthracis* and *Escherichia coli* using the growth inhibition assay at 1.0 mg/ml. The optical density readings were recorded and analyzed. The procedure was repeated to ensure the results were accurate.

2.11 Olive Leaf Extract Cytotoxicity against Macrophages

The mammalian cell line used for this part of the experiment is the J774a.1 murine macrophages. These cells were generously provided by Dr. Brojatsch at Albert Einstein College of Medicine, NY. There are certain conditions required for the medium and growth of these macrophages. One of the very important conditions needed is an incubator set at 37°C and humidified with 5% CO₂.

The culturing of the J774a.1 cells requires a medium of Dulbecco Modified Eagle's Medium (DMEM) without L-glutamine (Cellgro) that was supplemented with a few additions. These additions were 10% of dialyzed FBS (HyClone), 10% of GlutaMAX (Invitrogen), penicillin (100 IU/ml) from Cellgro and streptomycin (100 µg/ ml) also from Cellgro (31). The cells suspended in the complete medium were moved into a T-75 flask and placed in the CO₂ incubator to grow.

After 3 sub-culture procedures were performed (4-5 days between each sub-culture) and the cells have reached a confluence of about 80-90%, the cells were ready for testing. The macrophages were detached from the T-75 flask with phosphate buffered saline solution (PBS) without calcium and magnesium (Cellgro) that was supplemented with 5mM of EDTA. 80 μ l of macrophages (80,000 cells) were loaded into each well of the 96- well plate and incubated for 30mins to allow cells to attach. 10 μ l of propidium iodine (PI) is then added to each well and allowed to sit in the incubator for 10 minutes. PI is a fluorescent dye that stains the nucleus of the cell. PI leaks into the dead cells staining the DNA. PI will stain cells red only if the cells are dead. Living cells will not fluoresce. After the 10 minutes of incubation, a fluorescent reading (0 hr) was done using the InfiniteM200 apparatus (Tecan). Samples obtained from the separation procedures were loaded into their respective wells and the 96-well plate was placed back into the incubator. Fluorescent readings were taken at 0hr, .5hr, 1.0hr, 2.0hr, 3.0hr, and 4.0hr. Methanol was used as a negative control and a control with no treatment was also applied to the assay.

2.12 Artificial Gastric Juice Stability of Oleuropein

It is of interest to determine how stable oleuropein is in the gut of a human being to ensure that oleuropein can indeed be administered orally. Oleuropein was chosen for this experiment because pure oleuropein was commercially obtainable and has been shown to be an antimicrobial agent. This is a test that will help indicate if gastric juice from the stomach will or will not degrade the compound before it can reach the blood stream.

Oleuropein (Indofine Chemical Company, Inc.) was tested against artificial gastric juice (VWR) to determine its stability in this acidic environment. It was determined that each adult size capsule from East Park Research contains 500 mg of d-lenolate® . Because there is at most 20% of oleuropein in each capsule, 100 mg of commercial oleuropein was used for this test (East Park Research personal communication). The 100 mg of oleuropein was dissolved in 500 µl of MeOH. The 500 µl of solution was then added to a culture tube with 4.5 ml of artificial gastric juice, making the ratio of oleuropein to gastric juice 1:9. The tube was then incubated at 37°C. A TLC plate was spotted with the sample at 0hr, 1hr, 2hr, 3hr, 4hr, and overnight (18hr) of incubation. The plate was then developed with 15% MeOH/CH₂CL₂ and analyzed to determine if there were any changes to the oleuropein compound. Appearance of new compounds will show on the TLC plate as emerging spots. Changes to the compound would suggest oleuropein's instability in the presence of artificial gastric juice. If all the spots at different hours look the same, then the compound was not destroyed by the gastric juice.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Solubility of D-lenolate®

In order to determine which solvent was best to use for this project it was important to test the solubility of d-lenolate® in different solvents. This part of the procedure would help to determine which solvent would be used for the primary extraction process. Results showed that methanol was the best solvent to dissolve d-lenolate®, followed by DMSO, and double distilled water. Furthermore, it seems that d-lenolate® is composed mostly of polar, basic compounds. The compounds are assumed to be polar because of its high percentage of solubility in the polar solvents. The compounds are assumed to be basic due to its 75% solubility in 5% HCl, which dissolves basic compounds. Initial separation when testing for antimicrobial activity of crude extracts begin with alcohol extraction (10).

Table 1. Solubility of d-lenolate	
Solvent	Percentage soluble material
methanol	97.4%
DMSO	91.6%
water	80.9%
5% HCl	74.4%
tetrahydrofuran	70.1%
acetonitrile	26.8%
ethyl acetate	21.4%
dichloromethane	16.9%
hexane	4.6%
5% NaOH	0.0%

Table 1. Solubility of d-lenolate®. D-lenolate® was most soluble in MeOH, DMSO and water. D-lenolate was not very soluble in non-polar organic solvents or in basic solutions.

3.2 Qualitative Determination of Phenolic Compounds in D-lenolate®

It was determined that out of the 10 solvents tested, d-lenolate® was most soluble in methanol. According to Indofine Chemical Company, INC., commercial oleuropein is also soluble in methanol. To be sure that oleuropein is obtained in the sample extracted with methanol, a TLC plate was analyzed. When analyzing the developed plate with both the methanol extract and commercial oleuropein side by side, oleuropein was determined to be present. At 15% MeOH/CH₂Cl₂, pure oleuropein has a retention factor (R_f) of 0.33 ± 0.02 . R_f value is distance traveled by the compound divided by the distance traveled by the solvent.

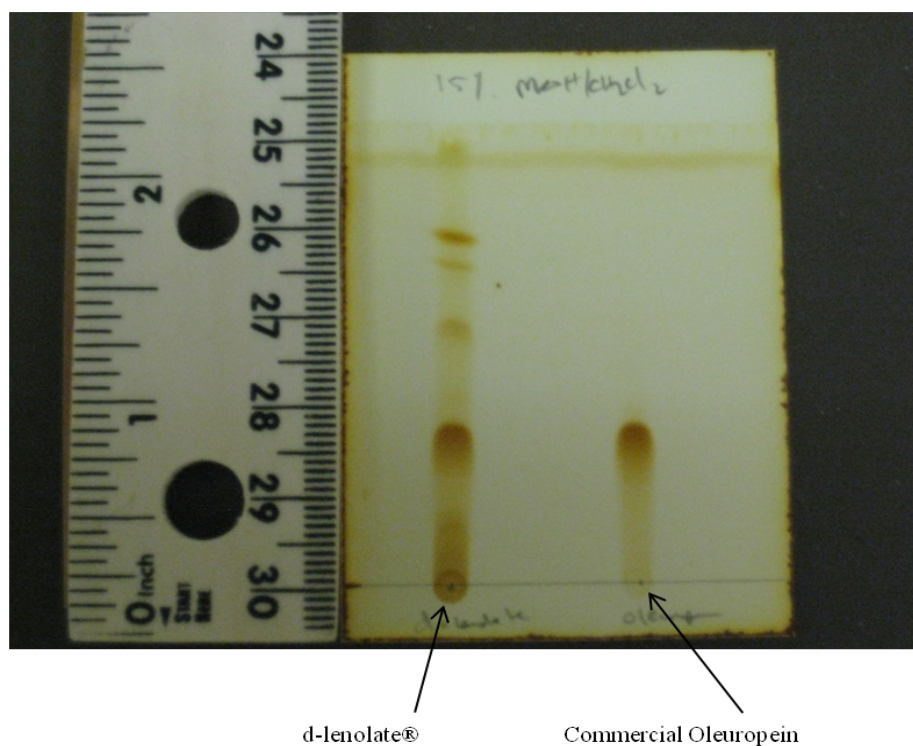


Figure 9. TLC plate of d-lenolate® & commercial oleuropein developed with 15% MeOH/CH₂Cl₂ solution.

To determine if the extract contains oleuropein, a compound with the R_f value of 0.33 ± 0.02 would be expected. Oleuropein was identified as the major active compound by comparison with a known commercial sample as seen in figure 9.

3.3 Purification of Oleuropein from D-lenolate®

As expected, pure oleuropein was eluted in the 15% MeOH/CH₂Cl₂ fraction by column chromatography. Oleuropein was extracted from both d-lenolate® and the olive leave extract from a competitor company (labeled “C”). Both extracted oleuropein were observed to be pure based on the analysis of TLC. A compound is considered pure when only a single spot on the plate is observed after development. ¹H NMR was also performed with both samples and compared to the ¹H NMR of the commercial oleuropein to ensure purity. Oleuropein was also attempted to be purified from olive leaf extract from a second competitor (labeled “B”). Despite the presence of oleuropein in the extract B, complete purification was not achieved.

3.4 Binding of Commercial Oleuropein to Serum

East Park Research claims that their patented extract, d-lenolate®, does not bind to blood serum. In order to test that, commercial oleuropein was used for preliminary testing to compile an appropriate protocol. Using commercial oleuropein allowed room for error because they can be repurchased as opposed to limited amount of extracted oleuropein. Oleuropein was tested for serum binding using the centrifuge filter assay. As seen in Fig. 10, oleuropein in the retentate shows a positive peak with a maximum of 1.2. Oleuropein

in the follow-through, on the other hand, shows a negative peak with a minimum of approximately 0.65. Thus, 20-35% of oleuropein seems to be bound to serum proteins.

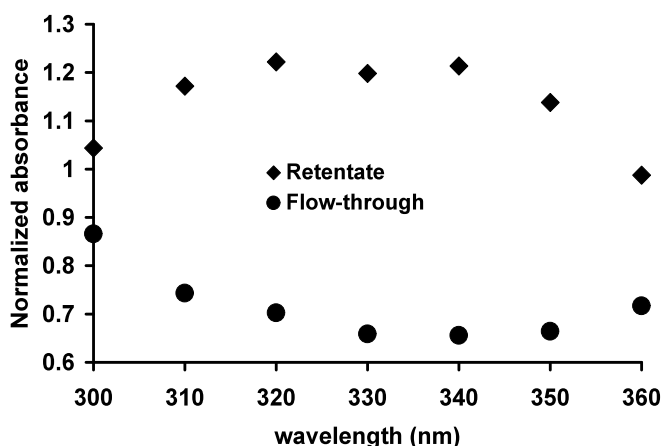


Figure 10. Analysis of oleuropein-serum binding. Retentate represents the normalized absorbance of serum-bound oleuropein. Flow-through represents the normalized absorbance of free oleuropein.

3.5 Testing of Oleuropein Binding to BSA

The testing for oleuropein binding to BSA was carried out with the Spectrum Equilibrium Dialyzer from SpectrumLabs instead of the centrifuge filter. The equilibrium dialyzer allowed for oleuropein to pass through the membrane in both directions as opposed to the centrifuge filter will only allow the crossing of oleuropein in one direction. Allowing the equilibrium dialyzer to run for 24 hours ensured that equilibrium is achieved.

During the 24 hours oleuropein diffuses back and forth until there is the same amount of free oleuropein on each side of the membrane. If oleuropein binds to BSA in one

chamber, then the free oleuropein from the second chamber will have to diffuse through the membrane until it reaches equilibrium again. So if binding occurs, one chamber will contain only free oleuropein, the other chamber would contain the same amount of free oleuropein in addition to bound oleuropein. Hence, the chamber with the BSA and oleuropein will now have a higher absorbance reading at 330 nm than the other chamber because it now contains more oleuropein than when it started. If binding does not occur, then both chamber readings should be the same.

According to the data obtained, it could be assumed that neither East Park Research extracted oleuropein or sample “C” extracted oleuropein bind to BSA in any of the conditions tested.

Binding of Bovine Serum Albumin to Extracted Oleuropein		
Concentration of Oleuropein	Absorbance at 330 nm	
	Oleuropein/BSA	Oleuropein
0.125 mM d-lenolate oleuropein	0.00640 ± 0.00297	0.00698 ± .00242
0.250 mM d-lenolate oleuropein	0.0114 ± 0.00312	0.0124 ± .00294
0.500 mM d-lenolate oleuropein	0.0202 ± 0.00396	0.0236 ± 0.00287
0.125 mM “C” oleuropein	0.00770 ± 0.00192	0.00760 ± 0.00271
0.250 mM “C” oleuropein	0.0167 ± 0.00112	0.0161 ± 0.00103
0.500 mM “C” oleuropein	0.0331 ± 0.00142	0.0370 ± 0.00118

Table 2. Binding of BSA to extracted oleuropein absorbance readings.

3.6 Fractionation of D-lenolate®

Twenty-one fractions from the dry column vacuum chromatography were collected and then dried. These 21 samples (labeled DCVC 1-21) were then tested for antimicrobial activity at saturated concentrations.

3.7 Testing of Lenolate Fractions for Antimicrobial Activity

When testing for antimicrobial activity using the disk diffusion test, none of the 21 samples (DCVC 1-21), d-lenolate® (starting product) nor methanol, exhibited antimicrobial activity after 24 hours of incubation. No clear zone was present, which would have indicated antimicrobial activity. A clear zone was observed around the disk applied with chloramphenicol (positive control) as seen in figure 11. The results did indicate that there is no antimicrobial activity, but this way of testing does not show the growth rate of the bacteria. These samples might have not inhibited bacterial growth, but it might have delayed the growth. The incubation of the plate for 24 hours will eventually give the bacterial lawn enough time to grow and cover the agar plate. Disk diffusion only gives a qualitative analysis of bacterial growth, whereas, growth inhibition test will provide more of a quantitative analysis.

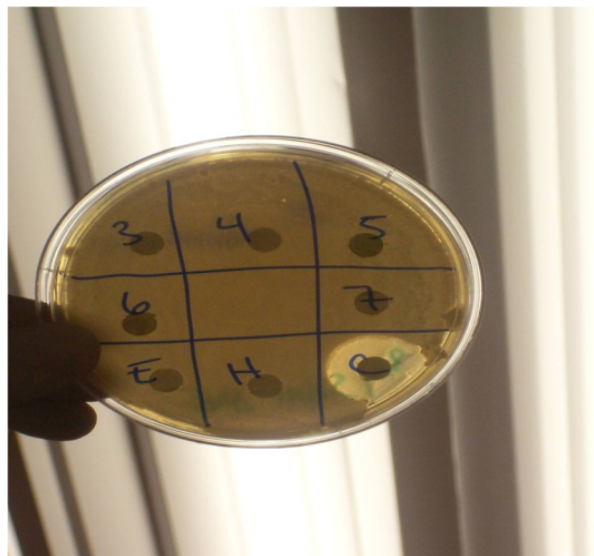


Figure 11. Disk Diffusion Assay. Only chloramphenicol exhibited antimicrobial activity.

However, growth inhibition tests at saturated concentrations of DCVC 1-21 determined some of the fractionated samples of d-lenolate® as well as d-lenolate® itself, displayed antimicrobial properties when tested against *B. anthracis* and *E. coli*. Results from the growth inhibition assay (Table 3) suggest that the fractionated d-lenolate® samples slowed the growth rate of both *B. anthracis* and *E. coli* but did not inhibit the growth completely.

When observing the results, interestingly d-lenolate® extracts and d-lenolate® itself were more potent in antimicrobial tests than purified oleuropein. This shows that oleuropein is not the only active compound in the d-lenolate® extract. Possible assumptions could be that there is another major compound that has greater inhibitory effect than oleuropein or that these compounds work synergistically to provide greater antimicrobial traits.

Bacteria growth in the presence of d-lenolate extracts after four hours of incubation		
Conditions	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	69 ± 5	57 ± 2
Chloramphenicol	7 ± 2	8 ± 1
d-lenolate®	32 ± 4	25 ± 4
40% EtOAc/Hex (DCVC5)	97 ± 6	83 ± 4
50% EtOAc/Hex (DCVC6)	88 ± 2	78 ± 4
60% EtOAc/Hex (DCVC7)	72 ± 3	92 ± 2
70% EtOAc/Hex (DCVC8)	81 ± 4	55 ± 3
80% EtOAc/Hex (DCVC9)	64 ± 2	53 ± 4
90% EtOAc/Hex (DCVC10)	38 ± 10	46 ± 2
100% EtOAc (DCVC11)	53 ± 4	43 ± 4
10% MeOH/EtOAc (DCVC12)	62 ± 3	37 ± 2
20% MeOH/EtOAc (DCVC13)	35 ± 4	26 ± 5
30% MeOH/EtOAc (DCVC14)	45 ± 4	25 ± 6
40% MeOH/EtOAc (DCVC15)	53 ± 4	33 ± 6
50% MeOH/EtOAc (DCVC16)	61 ± 4	35 ± 8
60% MeOH/EtOAc (DCVC17)	65 ± 2	42 ± 8
70% MeOH/EtOAc (DCVC18)	71 ± 2	45 ± 6
80% MeOH/EtOAc (DCVC19)	58 ± 4	36 ± 4
90% MeOH/EtOAc (DCVC20)	60 ± 3	41 ± 6
100% MeOH (DCVC21)	63 ± 3	48 ± 2

Table 3. Relative bacterial % growth in the presence of different d-lenolate fractions compared to control. Other samples from the separation were not included because there was no activity.

Samples DCVC13, DCVC14, and DCVC15 showed the greatest activity against both the Gram-positive and Gram-negative bacteria as seen from the table above. In any case, the focus of further purification would be on those three samples. However, TLC analysis

(figure 12) shows that each of these three samples contain oleuropein as the major component. Since oleuropein is known to possess antimicrobial properties, it is assumed that oleuropein played a role in the activity of fractions DCVC13, DCVC14, and DCVC 15. Hence, the focal point of future purification was shifted. Samples DCVC 13-21 all possess oleuropein based on TLC analysis and were not tested or purified further. Therefore, the focus is shifted to samples DCVC10, DCVC11, and DCVC12. These three samples have the greatest activity of all the samples without oleuropein. To assess compounds present in these three samples, thin layer chromatography of the 21 samples were again analyzed. The analysis of the TLC of the 21 samples showed the presence of three major compounds (amongst some minor compounds) that were at higher concentrations in DCVC10, DCVC11, and DCVC12 than in other samples. Because DCVC10, DCVC11, and DCVC12 showed greater activity than the other samples tested, it can be assumed that these three compounds might have a role with their ability to slow down bacterial growth.

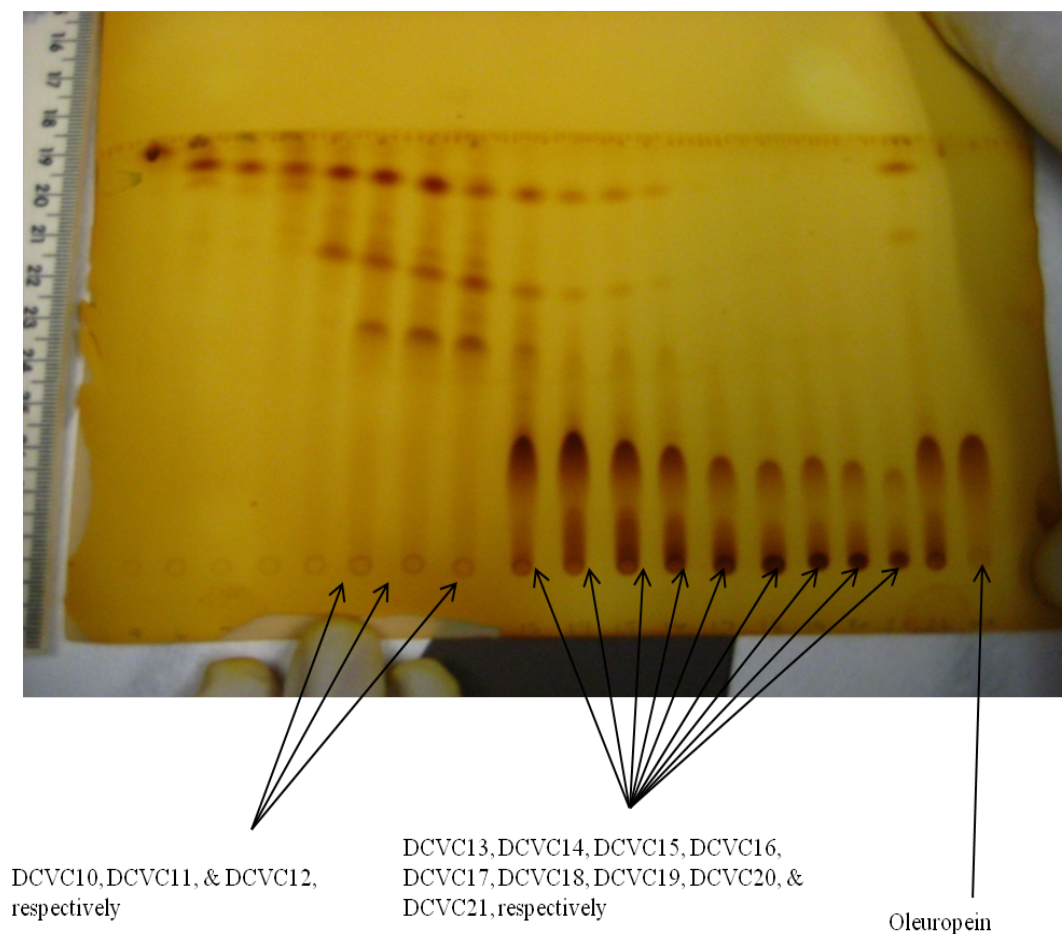


Figure 12. TLC plate of DCVC5-21, d-lenolate, and commercial oleuropein developed in 15% MeOH/CH₂Cl₂ solution.

The next step was to separate and purify the three compounds of samples DCVC10, DCVC11, and DCVC12. Since DCVC10, DCVC11 show similar compounds by TLC, these two extracts were combined for further purification. When the separation and purification process is done, purified compounds were tested again for antimicrobial activity.

3.8 Purification of D-lenolate® Fractions

The separation and purification of sample DCVC10 and DCVC11 (from separation of dry column vacuum chromatography (DCVC)) lead to the separation and purification of sample A1 (A6 + A7), which was followed by the separation and purification of samples A1.3 (A1.3, A1.4, and A1.5). The samples collected from these purifications were analyzed by thin layer chromatography. After looking at all the plates it was determined that only fraction A1.3.12 contained a pure compound. This compound corresponds to the 1st spot of DCVC10-12 from TLC plate observed above. Fraction A1.3.4 was partially purified and contained 3 compounds. These compounds correspond to the 3rd spot of DCVC10-12 with small amount of minor spots. Antimicrobial testing was done with all fractions obtained from this series of purification and analyzed for antimicrobial activity.

The separation and purification of DCVC12 lead to the separation and purification of sample B7. The samples collected from the purification of B7 were then analyzed by TLC. Samples B7.5 and B7.6 were the only samples determined to contain pure compounds. These two compounds correspond to the 2nd spot of DCVC10-12. Two other samples B7.10 and B7.11 were partially purified. These compounds correspond to the 1st and 2nd spot with a lot or minor compounds in between. The growth inhibition test was done with all of the samples collected and analyzed for antimicrobial activity.

The separation and purification DCVC10 and DCVC11 also lead to the separation and purification sample A1 by MPLC. The samples from the purification by MPLC were collected and analyzed by TLC. Samples 7M, 8M, and 11M show to contain the same pure compound. This compound corresponds to the 1st spot of DCVC10-12. All of the samples were tested for antimicrobial properties.

No structure analysis of the compounds was obtained due to the limited amount of samples obtained due to the many purification steps carried out. Nevertheless, purified samples have been submitted for mass spectrometry. Samples are being currently analyzed at the University of Arizona.

3.9 Testing of Fractions for Antimicrobial Activity

Growth inhibition test of samples obtained from the separation and purification of samples DCVC10 & 11 indicated that only samples A6 and A7 tested at 1.0 mg/ml possessed antimicrobial properties. These two samples were combined and labeled as A1 for future references. Table 4 below shows the growth rate in percentage of the two samples compared to the control (treated with only methanol) commercial oleuropein, and chloramphenicol.

Bacteria growth in the presence of DCVC10 & 11 extracts after four hours of incubation		
Samples	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	82 ± 3	90 ± 3
Chloramphenicol	10 ± 1	14 ± 2
A6	34 ± 6	59 ± 1
A7	58 ± 2	58 ± 2

Table 4. Relative % bacterial growth in the presence of different DCVC10 & 11 fractions compared to control. Samples A6 and A7 were combined and labeled as A1. Other samples from the separation were not included because there was no antimicrobial activity.

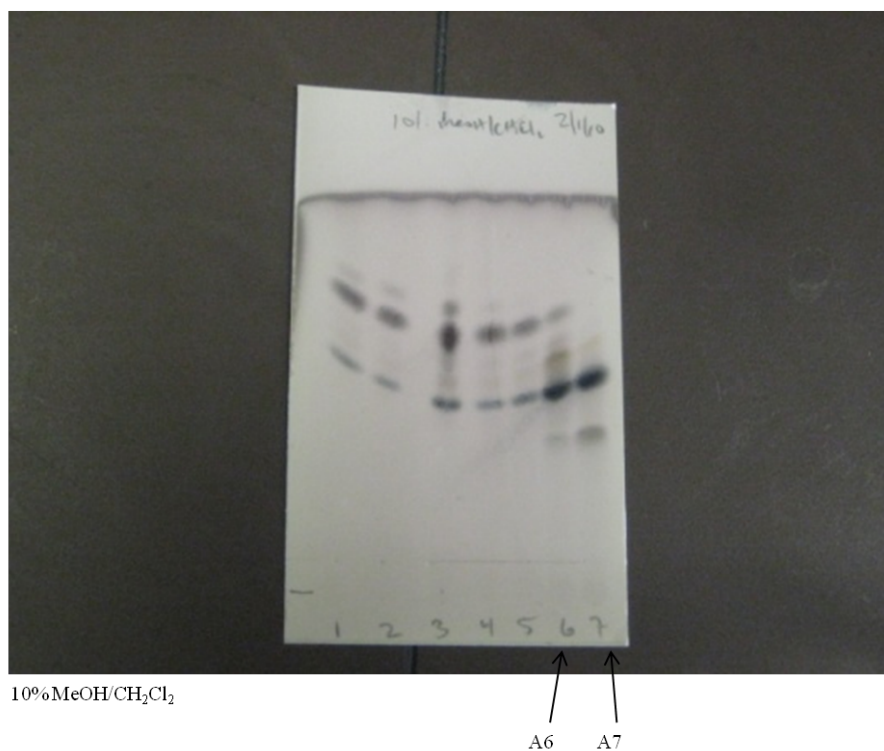


Figure 13. TLC plate of samples A6 & A7 developed in 10% MeOH/CH₂Cl₂ solution.

The next growth inhibition assay was performed with the samples obtained from the purification of sample A1. The results indicate that only samples A1.3, A1.4, and A1.5 tested at 1.0 mg/ml delayed the growth of *B. anthracis* and *E. coli*. Table 5 below shows the growth rate in percentage of the three samples compared to the control (treated with only methanol), commercial oleuropein, and chloramphenicol.

Bacteria growth in the presence of A1 extracts after four hours of incubation		
Samples	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	78 ± 4	87 ± 3
Chloramphenicol	10 ± 3	12 ± 2
A1.3	62 ± 4	73 ± 4
A1.4	67 ± 2	78 ± 5
A1.5	62 ± 4	74 ± 5

Table 5. Relative % bacterial growth in the presence of different A1 fractions compared to control. Other samples from the separation were not included because there was no antimicrobial activity.

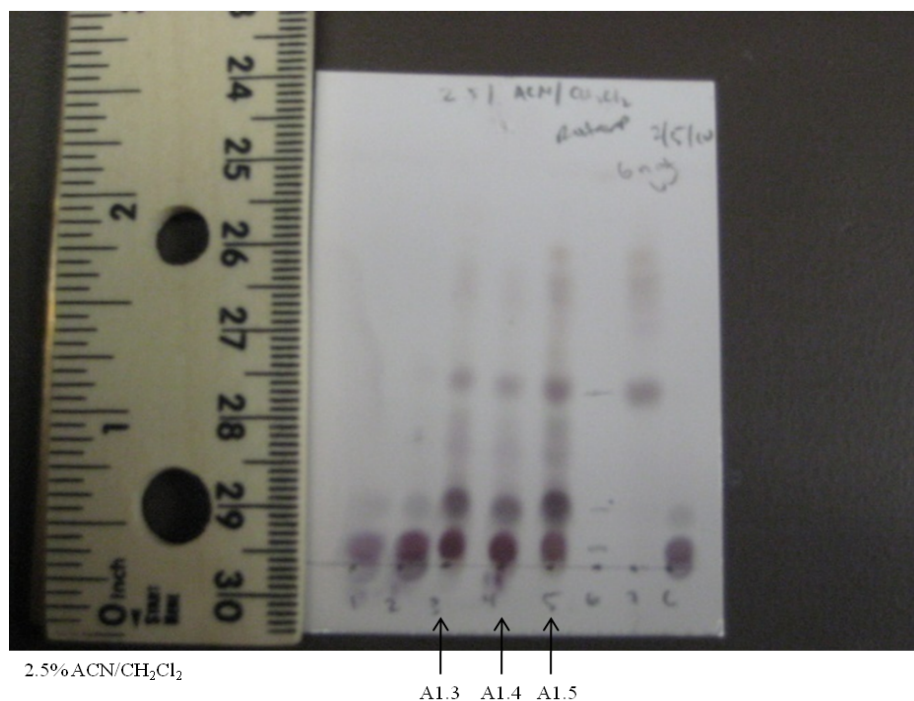


Figure 14. TLC plate of samples A1.3, A1.4, & A1.5 developed in 2.5% ACN/CH₂Cl₂ solution.

According to the data of the growth inhibition test performed at 1.0 mg/ml of samples obtained from the purification of A1.3, all samples lacked antimicrobial properties. Since A1.3.12 was completely purified according to TLC analysis (figure 15); we were hoping this compound exhibited antimicrobial activity. The procedure was repeated to ensure that the data was accurate.

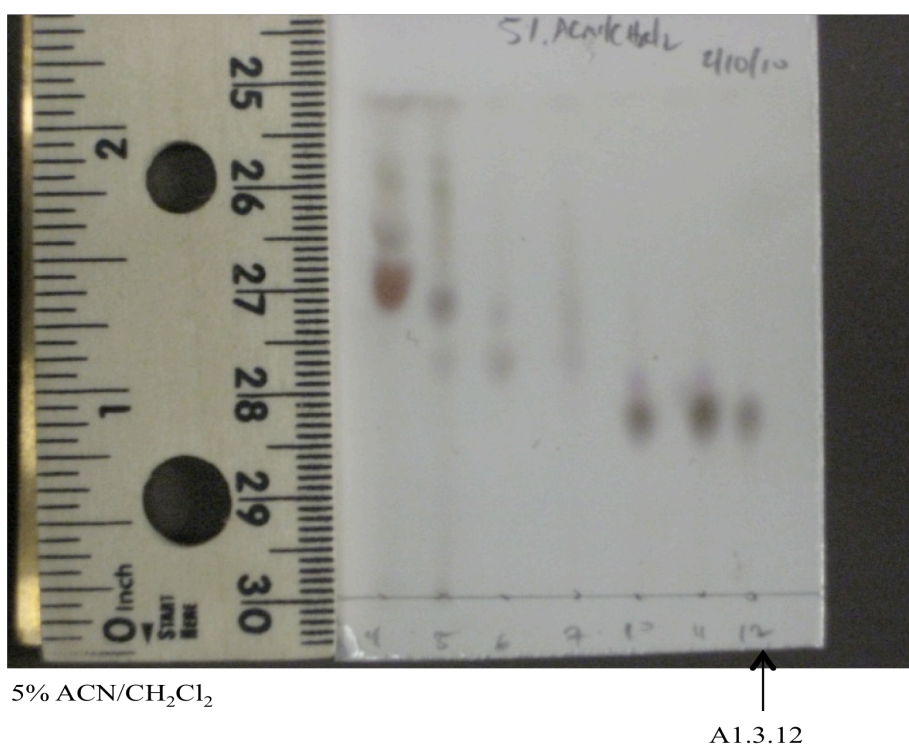


Figure 15. TLC plate with sample A1.3.12 developed in 5% ACN/CH₂Cl₂ solution.

The growth inhibition results for the samples obtained from the flash chromatography determined that samples B7, B8 and B10 at 1.0 mg/ml presented antimicrobial activity. Based on TLC analysis (figure 16), sample B10 contained oleuropein, which may have

played a big factor in its activity. This is strange because based on the TLC analysis of DCVC12 from chapter 3.7, DCVC12 did not contain oleuropein. DCVC12 was not further purified or tested due to this factor. Samples B7 and B8 exhibited similar antimicrobial activity, but there was more of B7 than B8, so B7 was further purified. Table 6 below shows the growth rate in percentage of the three samples compared to the control (treated with only methanol), commercial oleuropein, and chloramphenicol.

Bacteria growth in the presence of DCVC12 extracts after four hours of incubation		
Samples	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	76 ± 1	85 ± 2
Chloramphenicol	11 ± 2	10 ± 0
B7	14 ± 2	37 ± 9
B8	14 ± 4	20 ± 4
B10	18 ± 4	31 ± 6

Table 6. Relative % bacterial growth in the presence of different DCVC12 fractions compared to control. Other samples from the separation were not included because there was no antimicrobial activity.

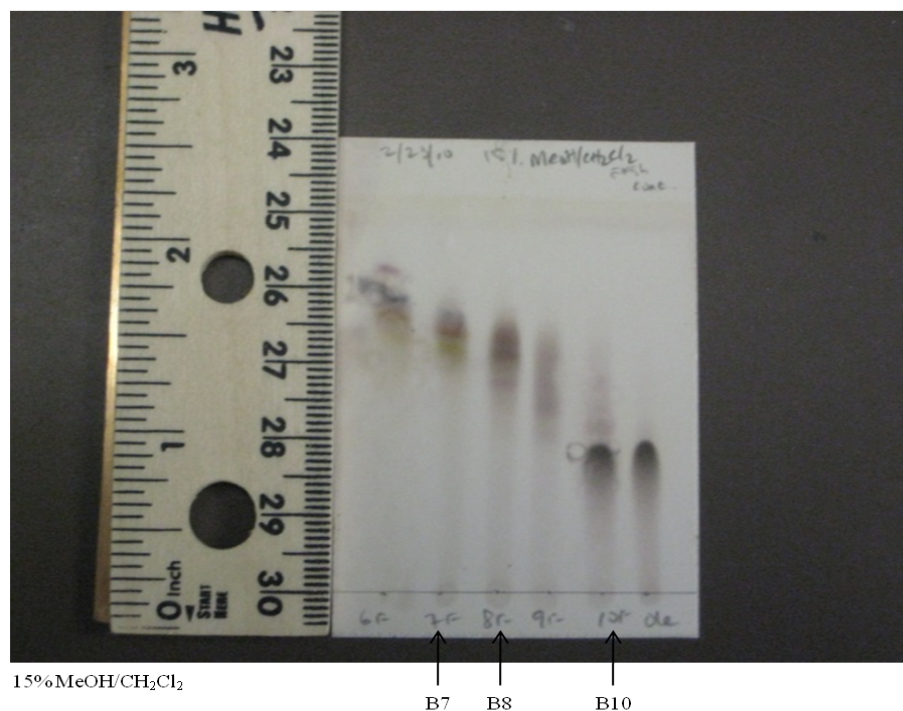


Figure 16. TLC plate of samples B7, B8 & B10 developed in 15% MeOH/CH₂Cl₂ solution.

Fractions from the separation and purification of B7 were obtained and tested for antimicrobial activity. Based on the results obtained from the growth inhibition test, samples B7.5, B7.6, B7.10 and B7.11 (from separation of B7) presented antimicrobial activity. According to TLC analysis (figure 17), samples B7.5 and B7.6 are the same compound, but the test shows that sample B7.5 does not exhibit antimicrobial properties as such of sample B7.6. Sample B7.6 was tested at 0.4 mg/ml because that was all that was obtained and sample B7.5 was tested at 1.0 mg/ml and yet B7.6 showed greater activity. The test was repeated and confirmed the results of the previous test. Sample B7.5 was then tested again at 0.4 mg/ml instead of 1.0 mg/ml to try to duplicate the results of sample B7.6. The results indicated that sample B7.5 does not possess any

antimicrobial activity at 0.4 mg/ml. The test was repeated and the same results were obtained.

Samples B7.10 and B7.11 also showed a great amount of activity. These two samples, which are not pure, exhibited greater activity than samples B7.5 and B7.6, which both seem to be pure. Table 7 below shows that growth rate in percentage of the four samples compared to the control (treated with only methanol), commercial oleuropein, and chloramphenicol.

Bacteria growth in the presence of B7 extracts after four hours of incubation		
Samples	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	75 ± 2	83 ± 2
Chloramphenicol	9 ± 5	12 ± 3
B7.5	63 ± 4	72 ± 4
B7.6	55 ± 5	75 ± 9
B7.10	48 ± 3	101 ± 3
B7.11	20 ± 2	64 ± 7

Table 7. Relative % bacterial growth in the presence of different B7 fractions compared to control. Other samples from the separation were not included because there was no antimicrobial activity.

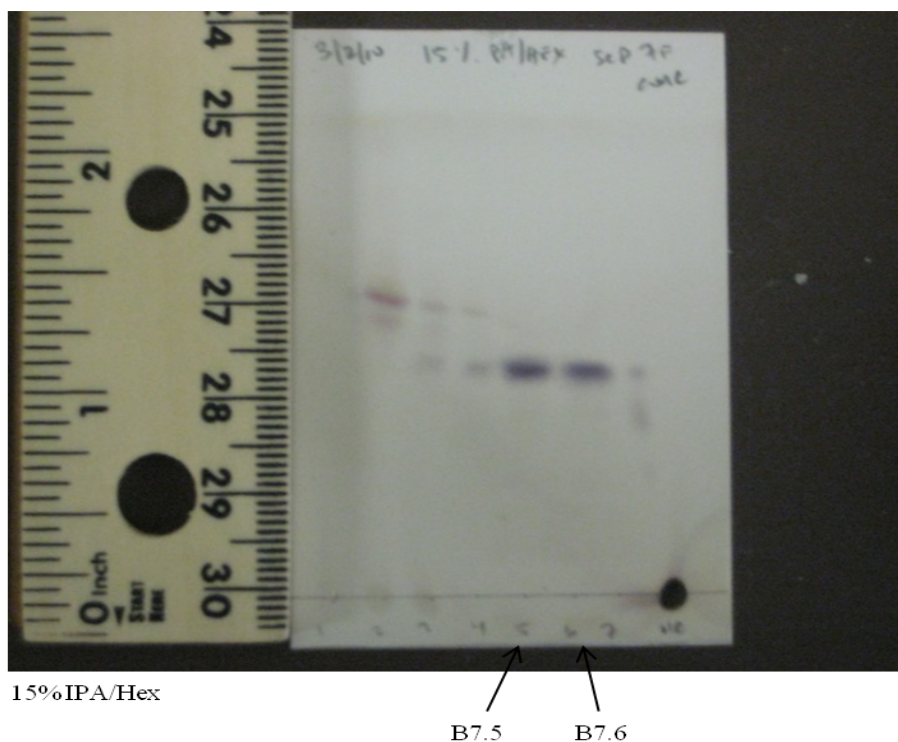


Figure 17. Samples B7.5 & B7.6 developed in 15%IPA/Hex solution.

Samples from the separation and purification of A1 with MPLC were tested against *B. anthracis* and *E. coli* for activity. The data collected indicated no antimicrobial properties of samples 1M through 11M. The test was repeated to confirm the previous results. Samples 7M, 8M, and 11M are pure and might even be the same compound based on TLC analysis (figure 18). Table 8 below shows the growth rate in percentage of the three samples compared to the control (treated with only methanol), commercial oleuropein, and chloramphenicol.

Bacteria growth in the presence of A1 (MPLC) extracts after four hours of incubation		
Samples	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	76 ± 1	84 ± 4
Chloramphenicol	14 ± 5	10 ± 0
M7	110 ± 2	93 ± 4
M8	110 ± 2	95 ± 2
M11	102 ± 2	105 ± 5

Table 8. Relative % bacterial growth in the presence of different A1 (MPLC) fractions compared to control. Other samples from the separation were not included because there was no antimicrobial activity.

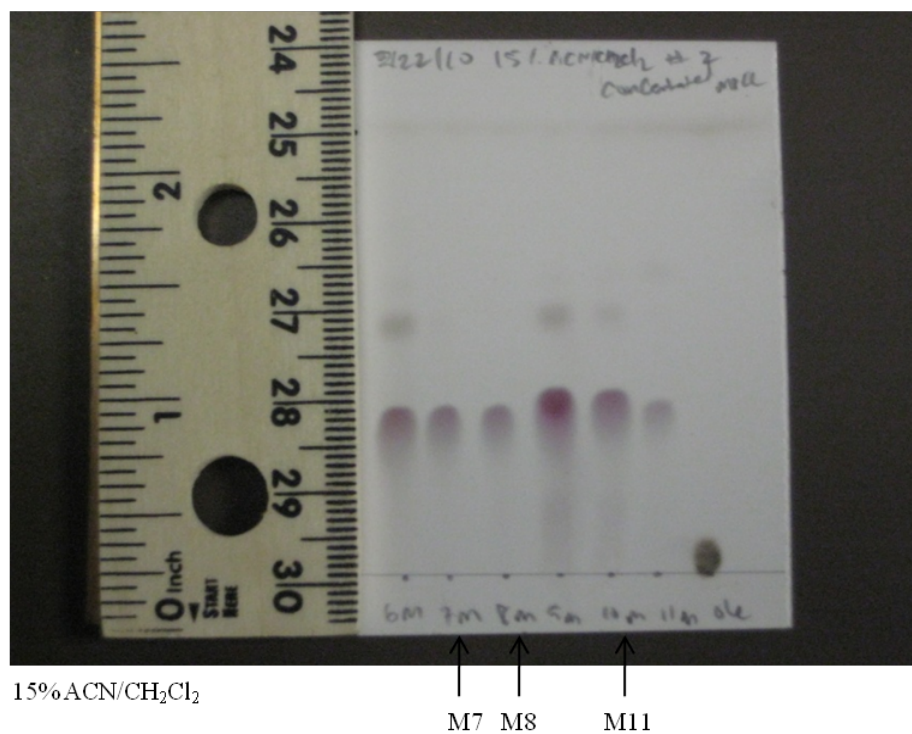


Figure 18. TLC plate of samples M7, M8, & M11 developed in 15% ACN/CH₂Cl₂ solution.

3.10 Comparing Antimicrobial Activity of D-lenolate® and Spanish Olive Leaf Extract

Both samples were extracted with methanol and tested at 1.0 mg/ml against *B. anthracis* and *E. coli*. The results indicated that both d-lenolate® and the Spanish olive leaf extract presented similar antimicrobial activity. TLC analysis also showed that the two extracts have similar composition (figure 19). Table 9 below shows the growth rate in percentage of the two samples compared to the control (treated with only methanol), commercial oleuropein, and chloramphenicol.

Bacteria growth in the presence of East Park Research and Spanish Olive Leaf Extract after four hours of incubation		
Samples	Relative % growth	
	<i>B. anthracis</i>	<i>E. coli</i>
Control (MeOH)	100 ± 0	100 ± 0
Oleuropein	85 ± 2	75 ± 2
Chloramphenicol	12 ± 1	12 ± 2
d-lenolate®	62 ± 7	53 ± 5
Spanish Olive Leaf Extract	56 ± 2	48 ± 3

Table 9. Relative % bacterial growth in the presence of East Park Research and Spanish Olive Leaf extracts compared to control.

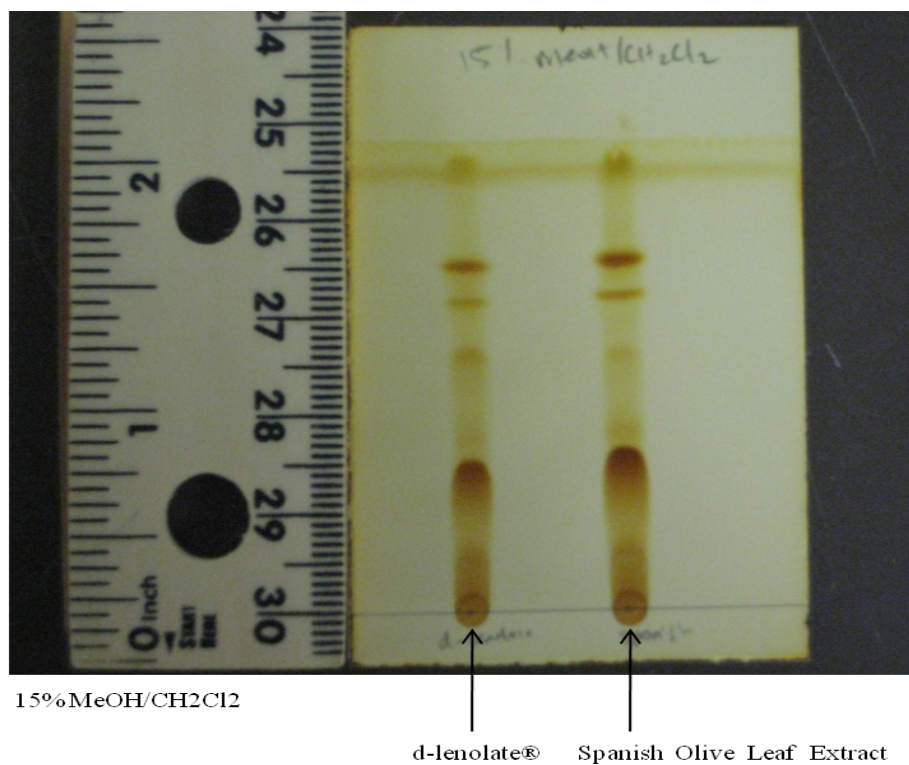


Figure 19. TLC plate of d-lenolate & Spanish Olive Leaf Extract developed in 15% MeOH/CH₂Cl₂ solution.

3.11 Olive leaf Extract Cytotoxicity against Macrophages

Macrophages were used in this experiment as opposed other human cells because these cells were readily available to us and our lab is familiar with the procedures of maintaining and growing these cells. All the samples obtained from the complete series of purifications, commercial oleuropein, d-lenolate®, and methanol extract of d-lenolate® were tested at 1.0 mg/ml to determine the cytotoxicity against macrophage cells. Increasing numbers during the fluorescent readings would indicate the killing of the cells. The numbers of the readings with the samples do not deviate too much from the readings of the wells with just methanol (control). Figure 20 shows the graph of relative fluorescence intensity compared to the control versus time (hr). If the samples were toxic to the macrophage cells, then a dramatic positive slope would be observed. According to the results, this was not the case and all samples extracted from d-lenolate® were assumed to be not cytotoxic against J774a.1 murine macrophages.

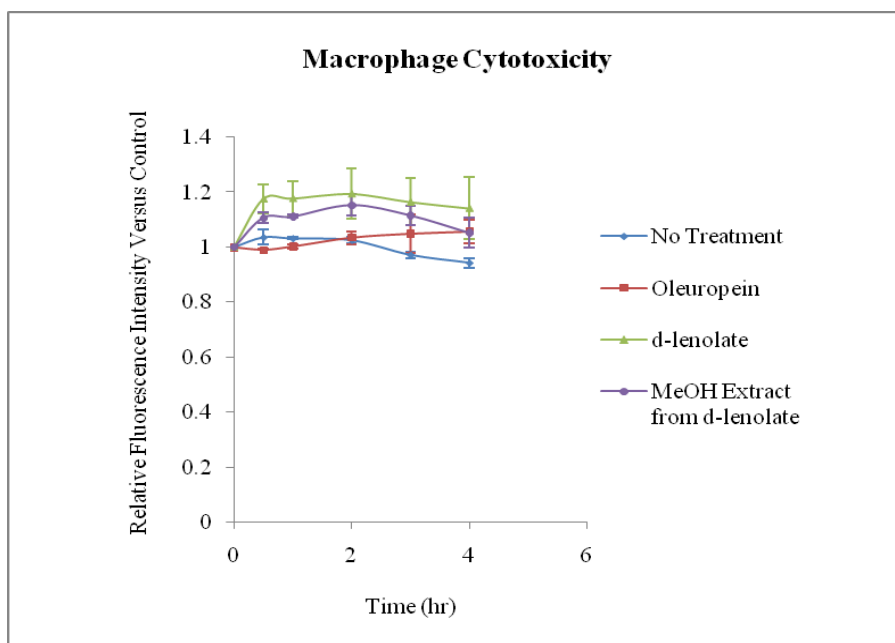


Figure 20. Macrophage cytotoxicity graph. Samples extracted from d-lenolate®, which provided similar results, were not included in the graph.

3.12 Artificial Gastric Juice Stability of Oleuropein

The most popular drugs are the most effective ones. The easiest way to administer medicine is through the mouth. When drugs are administered orally, they enter into the GI tract, an acidic environment. The gut of the stomach is design to degrade substances. When designing drugs of any sort, the stability of the drugs needs to be tested against gastric juice.

Oleuropein has been shown to possess antimicrobial activity. D-lenolate® contains oleuropein and is administered orally. The goal here is to determine how stable commercial oleuropein is in artificial gastric juice. As seen in figure 21, six points (0hr far left and overnight far right) were analyzed by the TLC. It could be seen at 0 hr; oleuropein seems to be pure and only contains one spot. After two hours of incubation, a

small amount of an additional compound along with oleuropein can be noticed. As the time of incubation increased, the addition compounds became more distinct and the intensity of the oleuropein decreased. After incubation overnight, three additional compounds along with oleuropein (total of four) can be seen clearly. It could be assumed that oleuropein is not very stable in artificial gastric juice. Based on the results of this test, gastric juice degrades oleuropein and in the process produces three unknown additional compounds.

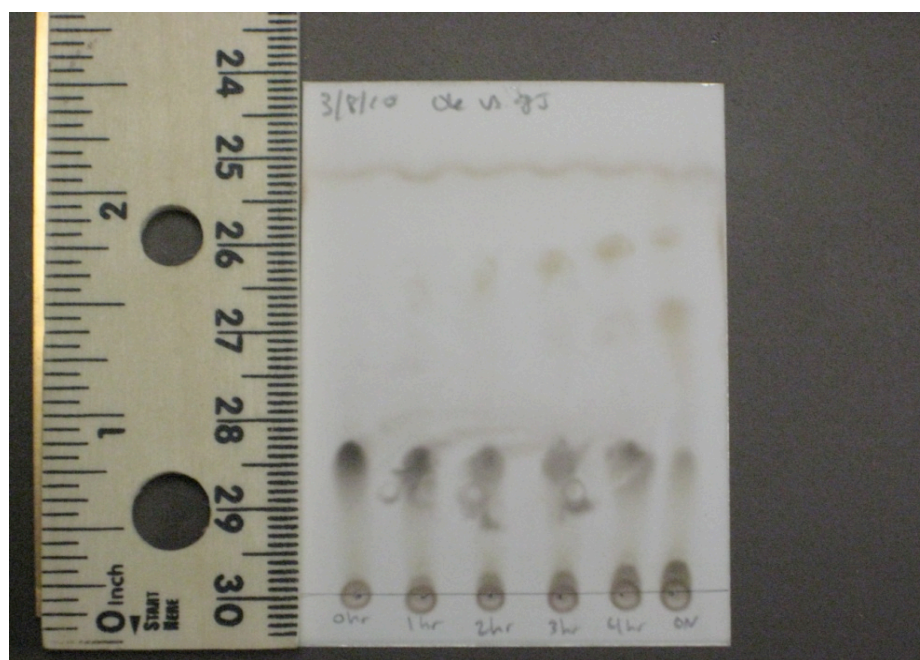


Figure 21. Stability of oleuropein to gastric juice. Oleuropein was incubated with artificial gastric juice and spotted for TLC 0, 1, 2, 3, 4, and 18 hours post-exposure. Degradation of oleuropein can be seen as new spots on the TLC plate.

CHAPTER 4

CONCLUSION

In the present study, preliminary results indicated that oleuropein binds to serum. This assumption was later concluded to be invalid. The centrifuge size-exclusion filter assay did not provide accurate results because it only allowed oleuropein to flow through the filter in one direction. If centrifuged long enough, all of the oleuropein could possibly end up on one side of the filter. This assay did not allow for equilibrium to occur, therefore, the results were not accepted. However, oleuropein was shown to not bind to BSA when performing the equilibrium dialysis assay. This assay allowed for equilibrium to be achieved. Oleuropein has been shown to possess antimicrobial activity and not binding to serum indicates that oleuropein is not only specific to serum and can be distributed to other cells in the body.

Our data shows that the acidic environment of the stomach can degrade oleuropein. It appears that oleuropein has the capability to flow through the intestine, but amount of oleuropein reaching the systematic circulation as the same compound first ingested is not very likely (32). The compound may be degraded as it passes through the digestive system. Whether these degradation products are harmful to humans, or are active against bacteria is yet to be determined. Thus, olive leaf extracts should be formulated to protect oleuropein from degradation by the GI tract.

Oleuropein has been shown to have antimicrobial activity. In our hands, olive leaf extracts and purified oleuropein can reduce bacterial growth but not eliminate it. In contrast, olive leaf components did not cause harm to J774a.1 macrophage cells tested. By retarding bacterial growth, olive leaf extracts could allow the immune system to clear up bacterial infections.

Interestingly, olive leaf extracts were more potent in antimicrobial testing than purified oleuropein. In fact, fractions of olive leaf extracts devoid of oleuropein were significantly more active than purified oleuropein. This shows that oleuropein is not the only active compound in olive leaf extract. However, when the oleuropein-less fractions were further purified, their antimicrobial activity was reduced or eliminated. This argues that the mixture of compounds in olive leaf extract is required for maximal anti-microbial activity and that no single component is the major antimicrobial compound. This could be a very clear indication that compounds within olive leaf extract do work synergistically and that separating the compounds will change their properties and reduce their activity.

Whether the compounds within the d-lenolate® extract provide greater antimicrobial properties as an extract or purified compounds is not conclusive, but the conclusive idea here is that d-lenolate® extracts at 1 mg/ml do possess 100% antimicrobial activity (bacteriostatic), but only delays the growth of *Bacillus anthracis* and *Escherichia coli*.

Further testing and future work of this project will include the purification of samples B7.10 and B7.11. The samples will then be tested for activity. The question of whether purified compounds or synergistic compounds provide greater activity will be answered. Samples B7.5 and B7.6 will again be tested for antimicrobial activity and their chemical structures will be determined.

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