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THE ANTIMUTAGENIC EFFECTS OF *ARCEUTHOBIUM AMERICANUM*

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THE ANTIMUTAGENIC EFFECTS OF *ARCEUTHOBIUM AMERICANUM*

by

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ABSTRACT

Extracts from European mistletoe (*Viscum album*) have been used in alternative cancer treatments for nearly a century, and the extracts have modest antimutagenic properties. The dwarf mistletoe (*Arceuthobium americanum*) and *V. album* are from the same family and share similar characteristics. Both are plant-plant parasites, taking water and minerals from their hosts. However, it remains unknown as to whether *A. americanum* extracts are also antimutagenic. The objective of this research is to determine if extracts of *A. americanum* have antimutagenic properties when tested against some standard mutagens. Methanol extraction of *A. americanum's* fruits and stem were conducted, and the extracts were tested against histidine dependent *Salmonella typhimurium* strains via the Ames test, a standard method of detecting mutagenicity/antimutagenicity. The *S. typhimurium* strains contain different mutations in assorted genes in the histidine operon and are more easily back mutated than others in the presence of mutagens. The increased *S. typhimurium* growth can then be an indicator of increased genetic mutations that resulted in histidine reversions. The same test was conducted with modifications to test for antimutagenic activity. This research revealed modest *A. americanum* antimutagenic properties similar to *V. album;* however, the results were not statistically significant and difficult to get replicates. This result is a first for the Arceuthobium genus. Future research into this field could find more concrete evidence into the potential for the plant.

Thesis Supervisor: Associate Professor Joanna Urban, PHD.

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INTRODUCTION

Cancer and mutagenesis

Cancer is a disease which occurs in eukaryotic cells when cell replication occurs at an uncontrolled rate (Stadler 2014). Many eukaryotes normally derogate cancer cells but in cases where the body cannot correct these cancer cells, treatments such as chemotherapy or surgery must be used (Kastan and Bartek 2004). Cancer can be the result of carcinogens that may occur in the diet, through smoke or other forms of exposure, which can cause mutations (Lou et al. 1998). The uncontrolled replication occurs when cellular phases (G_1, G_2, S and M) no longer are correctly regulated by the regulatory checkpoints (Kastan and Bartek 2004). The mutations in the genome often effect the proteins that regulate the cell cycle (Stadler 2014). Therefore, it appears that the underlying cause of cancers is due to extensive mutations in the genome (Casás-Selves and DeGregori 2011). Although these genetic mutations are essential evolutionarily, at excessive rates they show to cause damage to exposed cells (Casás-Selves and DeGregori 2011). There are several types of mutations which effect the genome which are caused by either an insertion, deletion, replacement or duplication of a base pair (Habibi Najafi and Pezeshki 2013; Paulson 2018). The results of these can be a single base pair substitution, a frameshift mutation or a repeat expansion (Habibi Najafi and Pezeshki 2013). A frameshift mutation shifts the genomic DNA generally as a result of an insertion or deletion of a base pair – this may ultimately result in the genetic codons being completely different, as shown in Figure 1 (Habibi Najafi and Pezeshki 2013). A single-base pair substitution is caused by a replacement of a single base pair which may result in the amino acid for the subsequent codon being changed, this is also shown in Figure 1 (Habibi Najafi and Pezeshki 2013). Repeat expansion on the other hand is when a small section of DNA is repeated several times (Paulson 2018); however, this is not relevant to this study.

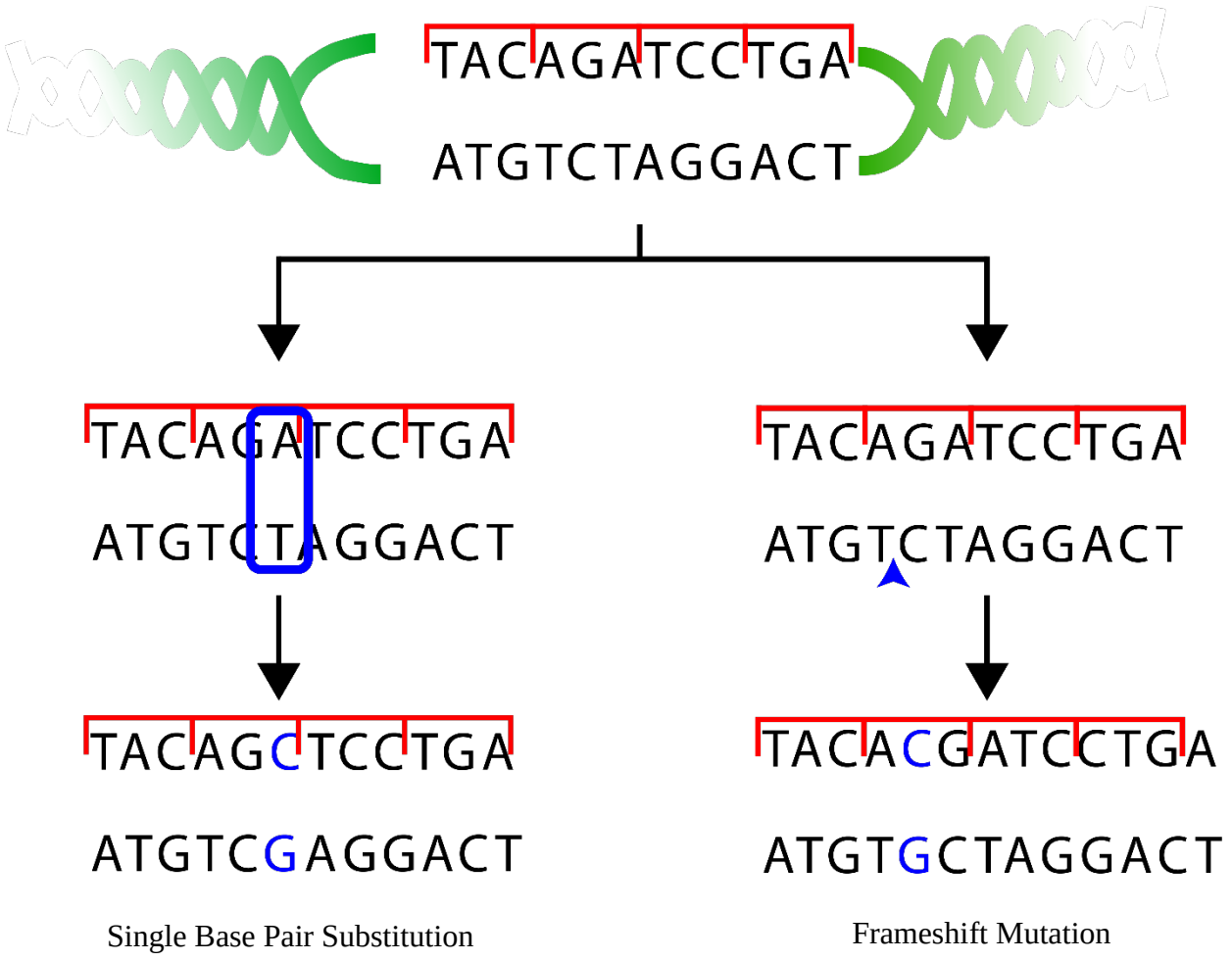

Figure 1. Two kinds of mutations shown in blue, the codons are indicated within the red lines, Single base pair substitution shows the replacement of a A=T base pair with a C≡G base pair (Habibi Najafi and Pezeshki 2013. The Frameshift mutation shows an insertion of the highlighted C≡G base pair which shifts the codons out of their original state (Habibi Najafi and Pezeshki 2013).

Antimutagens & Mutagens

Different chemical carcinogens can cause different mutations – often through a unique mechanism (Słoczyńska et al. 2014). They may act directly or indirectly on the genome through oxidation, generation of free radicals or other mechanisms (Słoczyńska et al. 2014). Some substances can inhibit the activity of mutagens – these are termed antimutagens (Kusamran et al. 1998). While antimutagens prevent the occurrence of mutagens, anticarcinogens more broadly impede cancer directly (Kusamran et al. 1998). Therefore, some anticarcinogens work by killing the host cell containing the cancer via apoptosis (Stewart et al. 2003); but, this does not work on the actual mutation itself (Kusamran et al. 1998). Antimutagens can be thought as a category of anticarcinogen (Lou et al. 1998). There are a variety of antimutagens, bio-antimutagenics describe antimutagens that effect the cellular process, while desumutagen inactivates the mutagen itself (Lou et al. 1998). There are several compounds which contain antimutagenic properties (Słoczyńska et al. 2014). For example, substances such as magnesium salts assist in prevention of oxidative damage. (Bronzetti et al. 2000). A commonly used antimutagen which assists in preventing exposure to the genetic mutations is sunscreen which prevents the penetration of UV light which causes mutations within the epithelial cells (Lucas 2011). Other antimutagens may help prevent the formation of free radicals or also act as an anti-oxidant (Słoczyńska et al. 2014). The use of practical antimutagenics can help with prevention and treatment of cancer (Zhang et al. 2014). This is very important due to the lethality of cancer (Stadler 2014). Therefore, the topic of antimutagenics may be a relatively unexplored resource for cancer treatment and prevention (Słoczyńska et al. 2014).

The Principle of the Ames Test

The Ames test is a tool which can assess the rate and type of mutations (Ames 1973; Kilbey et al. 2012). When done correctly the Ames test can be done in quick and effective manner (Kilbey et al. 2012). The type of mutations that are assessed are single base pair substitutions and frameshift mutations achieved with two particular strains of *Salmonella typhimurium* (Ames 1973; Kilbey et al. 2012). Strain TA98 is used for frameshift mutation assessment and TA100 is used for single base pare substitution assessment (Kilbey et al. 2012). This is achieved by a modification to the genome of the *S. typhimurium'*s gene which produces histidine – an essential protein for growth (Kilbey et al. 2012). Normal *S. typhimurium* cells produce histidine when needed; however, the TA98 and TA100 have the histidine genes knocked out (Kilbey et al. 2012). Thus, the cells no longer produce their own histidine and it needs to be added for the cells to grow (Kilbey et al. 2012). Therefore, the cells are incubated with limited histidine and a potential mutagen. If this mutagen causes a reversion to the histidine producing genes then the cells should be able to grow when the provided histidine runs out by producing their own histidine (Kilbey et al. 2012). This process is however entirely by chance; therefore, some cells will grow in a negative control due to spontaneous mutations resulting in reversion (Hebert et al. 2015). Therefore, the Ames Test has shown success in indicating substances which are mutagenic to cells and has been used often as a tool to assess the toxicity of substances (Ames 1973; Kilbey et al. 2012). Tests have also been done to test the opposite effect of a substance – which is if it prevents the mutagenic effects of a substance when included with the mutagen (Hong and Lyu 2012). This allows for testing of antimutagens (Hong and Lyu 2012).

European Mistletoe & cancer treatment

In Germany and other European countries, chemotherapy is occasionally combined with mistletoe injections (Ernst 2006). Some studies show that the use of mistletoe as a combined treatment in cancer does show clinical benefits (Kienle et al. 2006). In contrast, there are several clinical trials showing no benefit of using mistletoe treatment (Kleijnen and Knipschild 1994). Regardless, past research has shown antimutagenics appearing in the *Viscum album* primarily by studying the lectins extracted from the plants utilizing the Ames Test (Hong and Lyu 2012). The reason for the exact mechanism for this reported antimutagenic effects is unknown (Hong and Lyu 2012). Other research has shown anti oxidant activity within the mistletoe (Vicas et al. 2012). Thus, there is potential that this anti oxidant activity is blocking the oxidizing effects of the mutagen. It may be these antioxidant properties which allows for the antimutagenic behaviour of the extracts (Hong and Lyu 2012; Vicas et al. 2012).

Dwarf Mistletoe

A. americanum also known as Lodgepole Pine Dwarf Mistletoe, which is parasitic to the *Pinus contorta* (Fort Collins et al. 2002). This mistletoe shares the same family as the *Viscum album* – the European mistletoe. Due to this close phylogeny – the two share similar characteristics as regarding their parasitic nature; however, there are some distinct differences. The *A. americanum* can retrieve a significant amount of carbohydrates from its parasitic host (Hawksworth and Wiens 1998); whereas, the *Viscum album* primarily obtains water and minerals from its host (Kahle-Zuber 2008). Therefore the additional carbohydrates within the *A.americanum* may provide new substances to be investigated. Research in 2012 showed that *Viscum album* appears to produce antimutagenic activity from its lectins (Hong and Lyu 2012). With the different nutrient requirements (Hawksworth and Wiens 1998) – there is potential for other antimutagenic products

and potentially more potent products in the *A. americanum*. The research into the existence of lectins within *A. americanum* has yet to be done – therefore analyzing the plant's potentially antimutagenic activity would provide a novel starting place.

MATERIALS AND METHODS

Site Description Sample Collection

Samples were collected two times from the same location to have enough material for the project. The site of collection was near Stake lake at (50.573659, -120.434551). The host trees appeared to be primarily of the *Pinus* genus and had no notable swelling that is associated with the parasitism. Host trees were not chosen in a systematic approach as it was desired to collect a high raw biomass. Male and female plants were identified and collected into separate bags for each sex. Sex was identified based on notable differences between the male and female morphologies shown in Figure 8 in Appendix A. Bags were driven back to TRU campus and stored in the Science building in room S365 for roughly 2 days in a -20 ℃ freezer for the initial collection. This was done for preservation. No plant samples were stored in -80℃.

Methanol Extraction and Filtration

The samples that had been collected underwent methanol extraction. This technique involved weighing out the mass of the plant and crushing it in a mortar and pestle. It should be noted that the plant is not frozen to -80℃; thus, the plant didn't shatter. The entire plant was used in the extraction. The plant was then transferred into a 500 mL Erlenmeyer flask which was filled with 450 mL of methanol. Afterwards, the flask was sonicated utilizing a probe sonicator which was set to roughly 20V. Sonication was done from roughly 2-3 hours and afterwards the methanol was poured off into a storage container and 450 mL of fresh methanol was added. Sonication was repeated 2-3 times, each time removing and re-adding the same volume of fresh methanol. The

color of the extract changed throughout the process, from a dark green to a near clear. This indicated that the pigments were extracted and likely other organic solvents as well; therefore, the extracts were combined and filtered. There were 3 conditions: male extracts, female extracts and male and female extracts planned to be tested separately. The extracts were stored at room temperature until evaporation of the methanol was conducted. An overview of the extraction procedure is shown in Figure 2. The retrieved extracts still contained a considerable amount of solid plant debris. This was removed using simple gravity filtration. The filter used was cheese cloth to allow for a quicker extraction with high volumes; however, the same can be achieved utilizing paper filters. The cheese cloth was packed into a funnel and a beaker was placed beneath for extraction. Afterwards, the filtration was repeated several times to achieve a homogeneous mixture. Originally, the extract had much more opacity from the solid debris which were removed, and the mixture was green and clear. This was stored at room temperature in a closed container until evaporation was conducted.

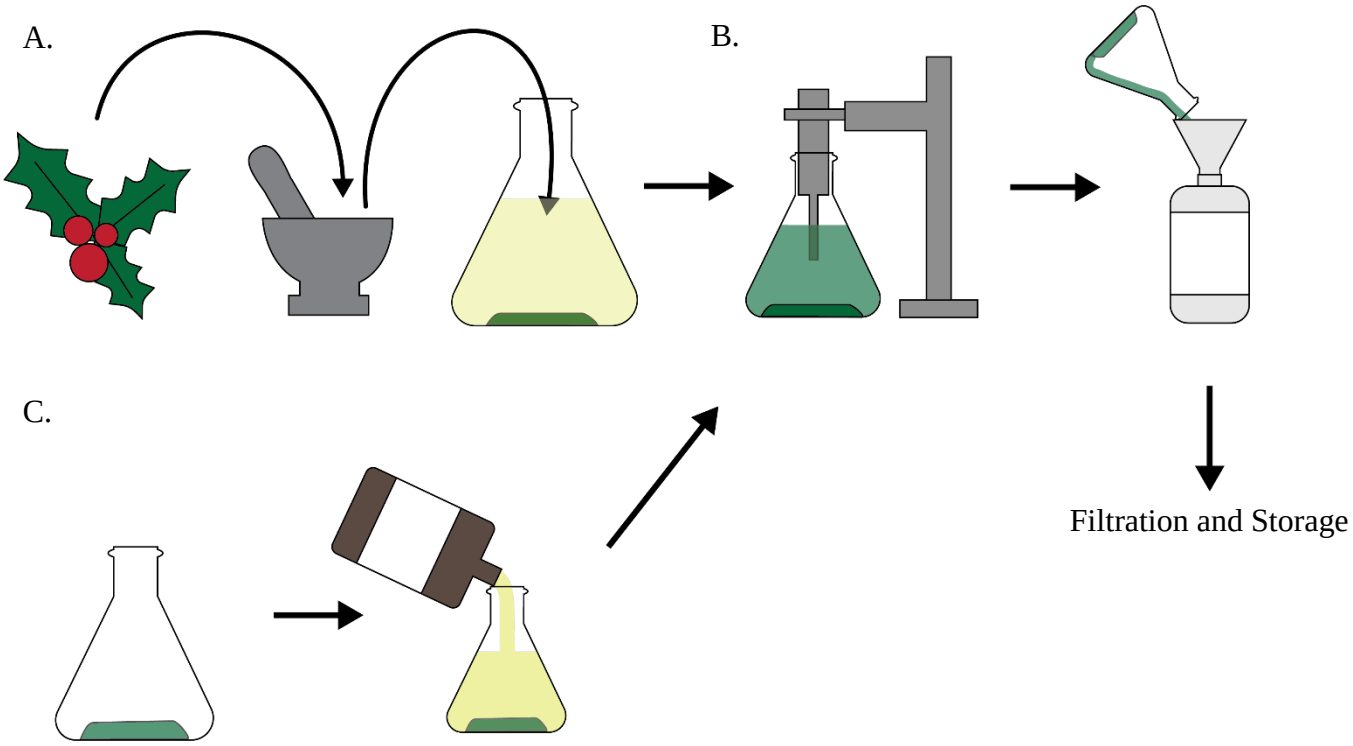

Figure 2. Extraction methods used for *A.americanum.* A. shows the initial steps of collecting and crushing the whole plant in a mortar and pestle and then placing the plant in a 500 mL flask with 450 mL of methanol. B shows sonication via probe sonicator which is done for roughly 2-3 hours at a time; afterwards, the methanol extract is poured into a container which is stored at roughly room temp in a dark room. C is the solid remnants of the extract after the methanol is poured off, this solid extract have 450 mL of methanol added and step B is repeated. Step C is repeated at most 2 times.

Rotary Evaporation and Nitrogen Evaporation

After methanol extraction was conducted, rotary evaporation and nitrogen evaporation was conducted to remove all the methanol to have pure extracts. Originally rotary evaporation was going to be utilized solely; however, it was difficult to evaporate all the methanol and as a result nitrogen evaporation was utilized as well. An overview for both methanol and nitrogen evaporation is shown in Figure 3.

Rotary Evaporation

The filtered methanol extracts were collected from storage. They were placed within a 500 mL round bottom flask. The flask was cleaned thoroughly with ethanol, and methanol to remove any residue. The flask was filled until roughly half full, thus not all extracts could be evaporated off at a single time. Rotary evaporation was conducted at approximately 40℃ which is below the boiling point of methanol. This is done because the vacuum created by the rotary evaporator depresses the boiling point of methanol. While evaporating, a noticeable amount of bumping occurred. Bumping refers to sudden volatile boiling of a substance resulting a large air bubble to be released. This is an issue as bumping often shoots substance into the bump suppressor of the rotary evaporator which may be contaminated. Thus, any substance in the bump suppressor was discarded. Strangely, bumping occurred the most at low methanol volumes at approximately under 100 mL - while a smooth boil occurred more often at higher volumes of around 250 mL. Once the majority of the methanol was boiled off, the remaining fluid did not appear to boil off. Thus, this was stored in a small container and transferred for low flow nitrogen evaporation.

Nitrogen Evaporation

Nitrogen evaporation was conducted on the remaining extracts which remained after rotary evaporation. The extracts were distributed among approximately 10 test tubes. These tubes contained a low amount of the extract in each tube of approximately 1-2 mL. The nitrogen evaporation apparatus was cleaned with ethanol and the tips were placed near the middle of the test tubes. Flow was attempted to be kept high enough that the evaporation would be efficient but not too high as to cause splashing of the fluids. Evaporation was conducted for roughly an hour and the vials were assessed to confirm that they lacked methanol. This was tested by increasing the flow of nitrogen slightly and checking for any splashing. When there appeared to be no liquid left the test tubes were covered with Parafilm and stored in a fume hood at room temperature for future use.

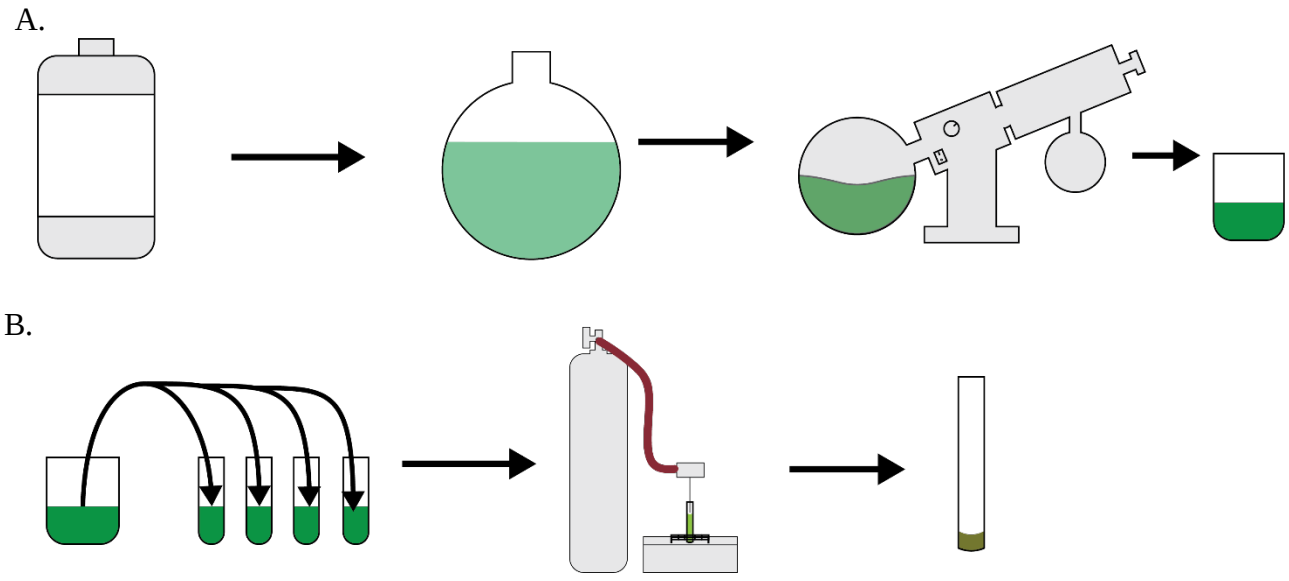

Figure 3. Flowchart of evaporation techniques used to remove all methanol. A shows transfer of the extracts to a round bottom flask which is then used to evaporate the fluids off; afterwards, the remaining fluids are collected and in B are transferred to several small test tubes. Nitrogen evaporation was used to evaporate the remaining extracts to get a solid brown mass.

Ames Test for Antimutagenicity

There were two variations of the Ames test which were run over the course of the study. The first test described as the "Traditional Ames Test" utilized a protocol similar to the one described by Ames in 1973 (Ames 1973). The Xenometrix Ames Assay was utilized later in the research due to difficulty with results from the traditional Ames Test. In both cases, the strains of bacteria used were TA98 and TA100.

Growth Procedure

Both TA100 and TA98 were grown under the same conditions and stored in the same freezer. A stock TA98 and TA100 was obtained from a -80°C freezer. The stock retrieved from the suppliers was stored in DMSO, the self-created stock was stored in glycerol. These stocks were retrieved from the freezer and left to thaw at room temperature. Afterwards the stock was incubated. The media was nutrient broth with 5 g/L NaCl. This media was 10 mL either in a test tube or Erlenmeyer flask. Growth was always done at 37°C and the rotations per minute (RPM) were adjusted between 150 RPM and 250 RPM to achieve the best growth. After overnight growth, the optical density (OD) was measured, and tests were conducted depending on if the OD was sufficient. Typically, an OD of greater than 2.0 is needed but 1.5 was still utilized typically at higher bacterial volumes. Negative controls of pure growth media were implemented after contamination was found in early tests.

Flash Freezing

Cells retrieved from the manufacturer stock were flash frozen in glycerol. This was done in three separate trials. The third trial achieved the best results. Trial 1, 2 and 3 are shown in Table 1. However only trial 3 will be discussed. This trial used 9 mL of the overnight culture and centrifuged it at 2000 rpm for 10 minutes. This resulted in a cell pellet. Most of the supernatant

was removed, and the pellet was resuspended in roughly 1 mL of media. 200 µL of the resuspended cells were transferred into microcentrifuge tubes and 200 µL of glycerol was added. The two components were mixed, and the cells were flash frozen with liquid nitrogen. Afterwards, these cells were stored in a -80°C freezer for future use.

Traditional Ames Test

The traditional Ames test was conducted as per chapter 6 in the Handbook of Mutagenicity Test Procedures (Kilbey et al. 2012). This utilized a top agar and a low glucose concentration plates. The top agar contained 0.6% Difco agar and 0.5% NaCl. The top agar used was stored from previous a research project. Top agar was melted in a microwave and kept a liquid in a water bath of approximately 50°C. Biotin and histidine are added to the top agar along with the positive and negative controls. The top agar is left to cool slightly prior to the addition of the bacteria. Afterwards the top agar is quickly mixed and poured over top of the low concentration glucose plate. The plate is swirled to allow for even distribution of top agar and left to cool for approximately 10 minutes. Afterwards, the plates are incubated at 37°C for 48 hours. The number of colonies were counted after the 48 hours have passed.

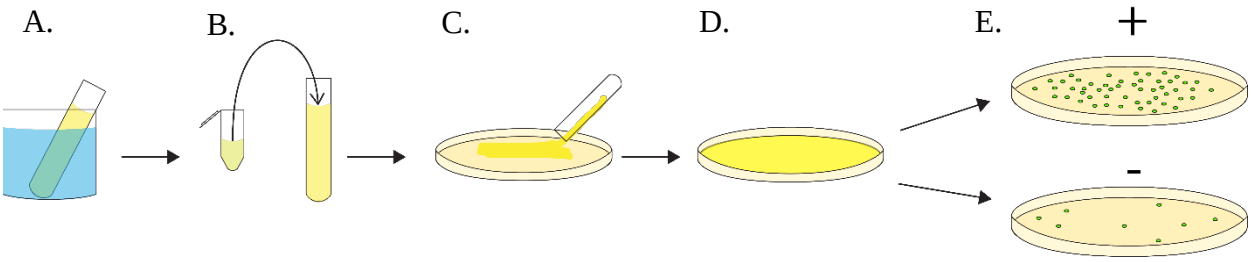

Figure 4. Flowchart for the Traditional Ames Test. A shows the top agar in a hot water bath (~50°C). B shows incubation of the appropriate bacteria into the top agar after it has cooled slightly. C and D show the pouring and spreading of the top agar on the low glucose concentration plate. E shows expected growth results for positive control (+) and negative control (-), with more growth on the positive control than the negative.

Modified Xenometrix™ Ames Assay:

The protocol for the Xenometrix™ Ames Assay was modified to utilize 96 well plates entirely versus the 384 well plates. This used a 24 well plate which was used to dilute the antimutagen with the mutagen. This is shown below in Figure 5**.** After the dilution is concluded, a positive control of 4-nitroquinoline N-oxide (2-NQO) and 2 nitrofluorene (2-NF) is used. The positive control stock contained a 100 ug/mL of 2-NF and 5 ug/mL of 4-NQO. The positive control was solely the mutagens with no antimutagen. The test and controls use an exposure media included with the kit and the negative control is exposure medium solely. The ingredients of the exposure media are not specified in the kit. 240 μL of the mistletoe sample was mixed with the 40 μL of the mutagen. This underwent serial dilution shown in Figure 5 B by taking 140 μL and adding it to another well. This receives 140 μL of exposure media and is further diluted utilizing the same steps. For TA98 – 6.3 mL of exposure media were added to 0.7 mL of bacteria culture. For TA100 – 6.65 mL of exposure media were added to 0.35 mL bacteria culture. 240 μL of these stocks were added to each of the corresponding wells. Afterwards, 1 mL of indicator media was added to each of the wells. Afterwards, 50 μL of each well was transferred to a 98 well plate as shown in Figure 5 D. The plates were inoculated at 37℃ for 48 hours and wells were counted. Yellow indicated growth and purple indicated no growth. If the well was in between colours it was marked as growth. Statistical analysis via SINGLE factor Anova was conducted to measure the significance between the data sets.

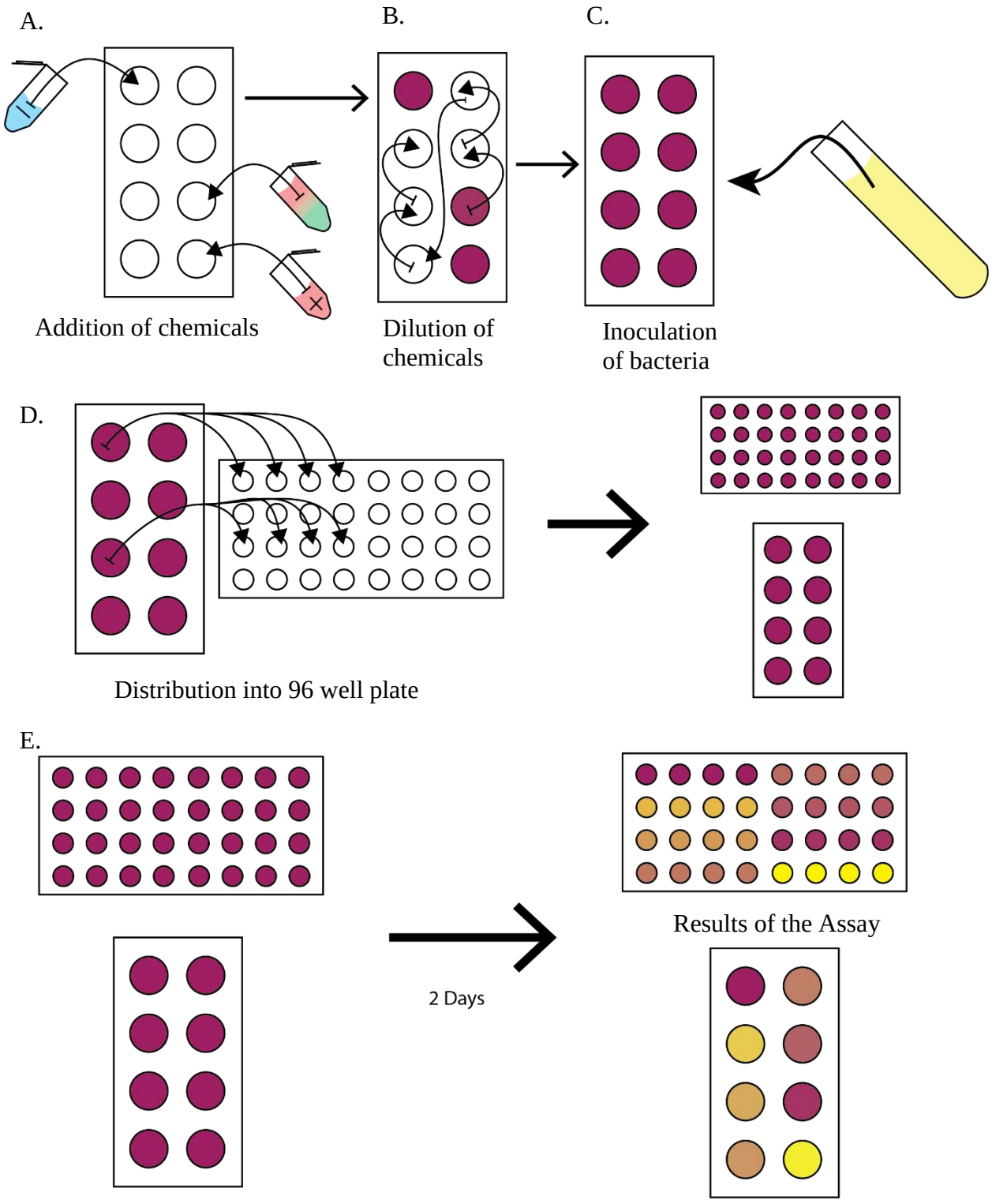

Figure 5. Flowchart of the modified Xenometrix™ Ames Test. A shows the addition of the antimutagen with the mutagen and the addition of the positive and negative control. B shows the dilution of the mutagen and antimutagen from the stock, downwards. C shows the incubation of the bacteria into each well, exposure media is added during this step as well. D shows the transfer

from the 24 well plate to the 96 well plate. E shows the 48 hour incubation and the expected colour changes. Yellow indicates reversion and purple indicates no reversion.

RESULTS

Methanol Extraction

Soaking the crushed plant in methanol with sonication showed the release of pigments as the methanol changed to a dark green fluid. As more extraction runs were completed, the methanol became less green and the solid plant also lost much of its pigments. This is demonstrated in Figure 6, which shows the difference between three extraction runs versus one extraction run. The filtration of the extraction material still left a green tinted fluid, this indicates that the pigments were solubilized within the methanol. Therefore, an assumption was made that the organic compounds were also solubilized.

Rotary & Nitrogen Evaporation

Rotary evaporation resulted in incomplete evaporation of the substance. There was residue left on the inside of the round bottom flask which may have slightly decreased the yield. The remaining methanol was transported to test tubes tubes and was slightly cloudy and dark green. When this methanol had undergone nitrogen evaporation, it had turned to a brown waxy solid. When water was added for Ames testing – it appeared to be a brown liquid.

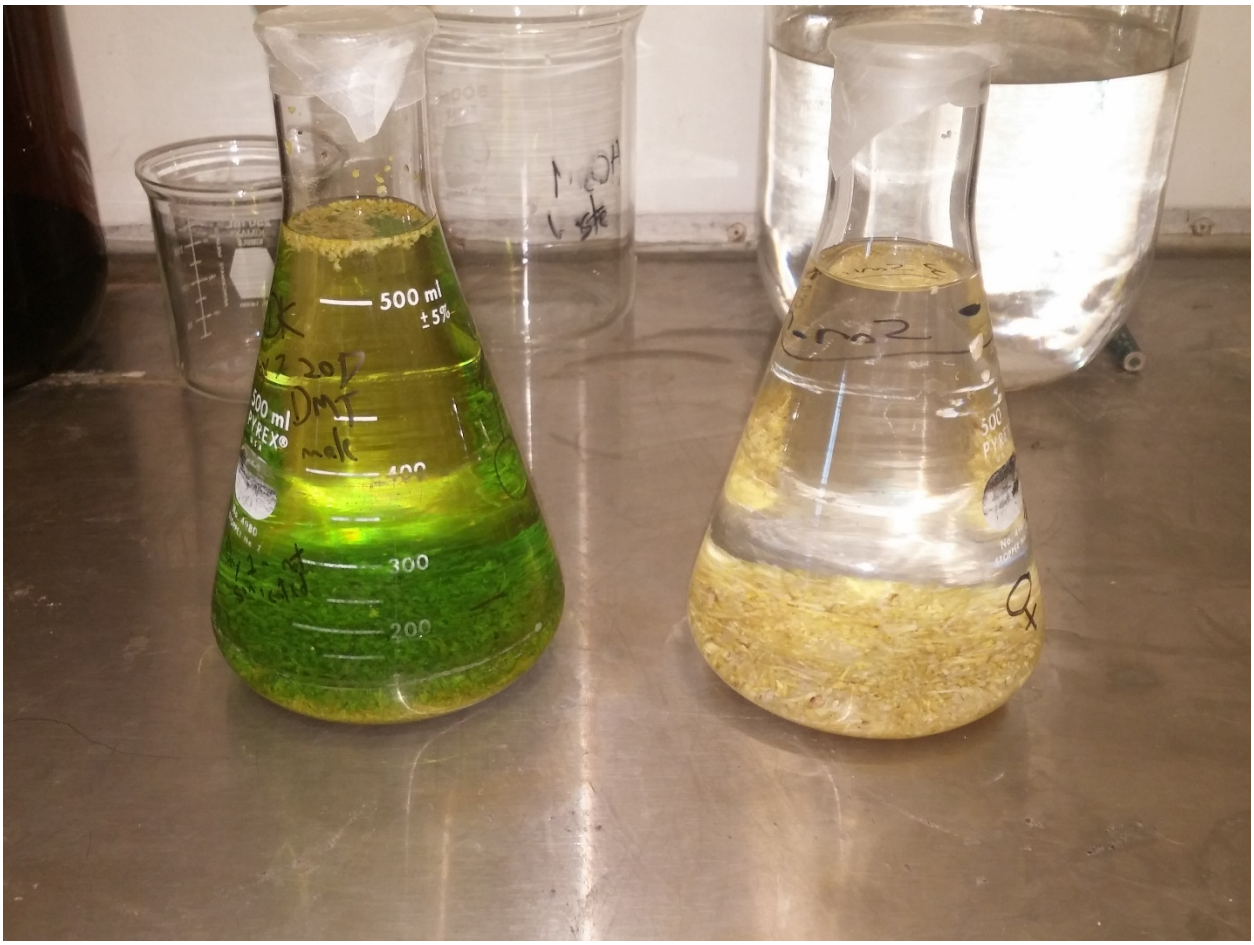

Figure 6. Dwarf Mistletoe extraction in 500 mL of methanol. The left shows a new extraction with the first addition of 450 mL methanol, the right shows an exhausted extract with its third addition of 450 mL of methanol. Both have been sonicated for two hours.

Ames Test for Antimutagenicity

Growth and Competency

When growing cells overnight, generally an OD of 2.0 or greater is desired for the bacteria; however, this was difficult to achieve with the bacteria used. Generally, the count would get close to 2.0 but never surpassed it. The exact reason for this is uncertain but may have affected the results of the Ames test. Typical values were seen between 1.5 – 2.0. Testing for standard Ames Test did not use a negative control in the growth conditions. There was one notable example were *S.auereus* contamination appeared to be present in gram staining. Therefore, negative controls were used for the later Ames tests to ensure easy recognition of contamination. In most cases, the negative control had a O.D. of less than 0.1 indicating no bacterial growth; however, there was a notable exception and the growth media was believed to be contaminated; therefore, these cells were discarded.

Flash Freezing

Flash freezing of the cells were attempted three separate times. Each occurrence of flash freezing utilized glycerol to prevent the formation of ice crystals. The methodology was similar for each process; however, slight modifications were made to get the best cell growth results. The three trial conditions are listed in Table 1. Each trial utilized a 50% mixture of glycerol. Trial 1 and 2 did not pellet the cells before freezing, as a result it appeared that the growth may have been less optimal compared to trial 3 which had the bacteria pelleted before glycerol was added.

Traditional Ames Test

The traditional Ames Test was attempted only with controls to attempt to get correct positive and negative results. This appeared to not be the case most of the time. Two of the trials are shown in Table 2 which demonstrate the incorrect growth values. The O.D. for these cells used had an O.D

of greater than 1.5. Trials that had a low O.D of below 1.5 were not tested and were discarded. Some contamination was also seen during these trials on a TA98 plate. Issues with the traditional Ames Test lead to the utilization of the Xenometrix™ Ames Testing plate.

Xenometrix™ Ames Test

The Xenometrix™ Ames test showed the most substantial results of any antimutagenic activity. Due to the 96 well trial assay, each group has a maximum of 4 revertants for each trial. Therefore, the average values are taken among the three trials used. The results are shown in Figure 7 and show that when the mistletoe and antimutagen are diluted to 1:8, this results in least amount of revertants with an average of 1.6 out of 4 wells containing revertant among the trials. The 1:16 dilution showed an average of 2.6 revertants. Single factor ANOVA was run for the 1:8 dilution to determine if the results within the three trials were significantly close to one another. This appeared to not be so with a P-value of 0.32.

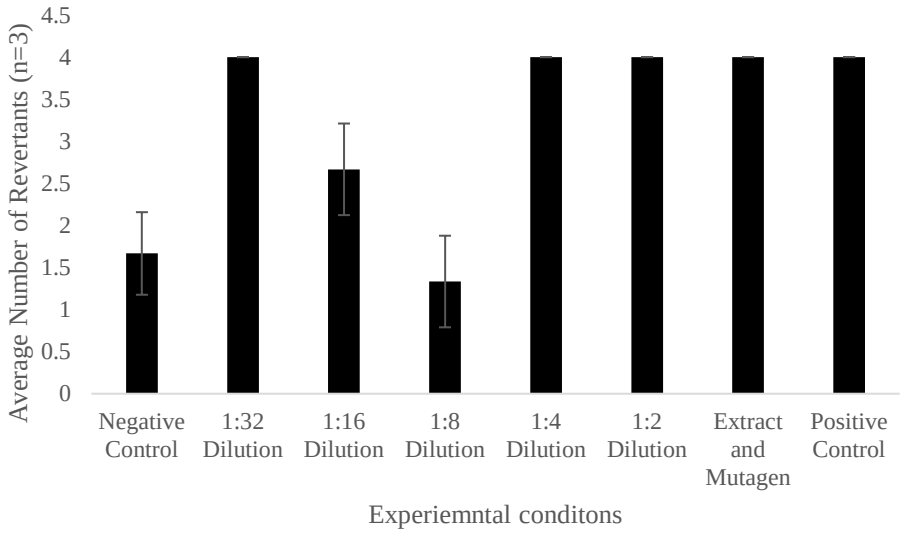

Figure 7. The mean results for the Xenometrix™ Ames Test with the average number of revertants shown and standard error plotting on the error bars, lower indicates less mutagenicity/

DISCUSSION

Extraction & Evaporation

The extraction process appeared to be successful based on the transfer of pigment into the methanol. The pigment extracted was likely chlorophyll A and chlorophyll B. This indicates that the cells appeared to be successful extraction of organic solvents. Based off this information it was deduced that lectins may have also been extracted along with other organic compounds. However, no analytical testing was done to confirm this. Storage of the extraction was at room temperature – this may have lead to break down of the organic compounds over time. The extracts were stored for approximately 6 months prior to being utilized in the Ames testing – this likely also influence the results. In future work it would likely be good to store the extracts in a freezer for stability, or to test the extracts much sooner after the extraction process has been completed.

Traditional Ames Tests

The results of the traditional Ames tests appeared to have a variety of issues with the controls incorrectly working. There may be several reasons for this. Some of the materials were re-used from previous testing. This includes the top Agar; therefore, there may have been issues not noticed with this trial. There may have also been issues with the cell viability – as the cells were grown from a frozen stock and then attempted to be reflash frozen using a small volume of nitrogen which may not have been sufficient. The results indicate that the cell viability may be a big issue as negative control values sometimes had more growth than the positive control plates. This may be the result of these cells being selected against slowly during the freezing and melting processes.

Xenometrix™ Ames Tests

The Xenometrix™ Ames Assay showed to give a difference in growth between the mistletoe trials particularly at 1:8 and 1:16 dilutions. The reason for this is uncertain and it should be dually noted

that the antimutagen and mutagen were both diluted with one another. However, the dilution of the mutagen is unlikely a cause of any alteration of results since lower concentrations did not see the same low revertant rates that would be expected if this were the case. It must also be noted that among the three trials for these dilutions – the results were significantly different from one another based on the ANOVA tests; thus, it cannot be said with certainty that this is not an anomaly. These results differ much from past research by (Hong and Lyu 2012), research on the *Viscum album* and indicate that there is an overall less antimutagenic effect at higher dilutions. Therefore, it is uncertain as to why the increased dilution caused greater antimutagenicity.

The *A. americanum* demonstrated modest antimutagenic properties; however, more vigorous testing would have to be done to assess any significant findings. A major issue was getting controls to work correctly with the antimutagenic assays. Future testing with a calibrated Ames Test procedure would allow for a greater understanding of any underlying antimutagenicity.

APPENDIX

Appendix A. Plant collections figures.

Figure 8. Dwarf mistletoe plants, female is on the right and male is on the left.

Appendix B. Flash Freezing Conditions.

Table 1. Flash freezing results of cells for three different trials with variations in reagents and cell culture conditions. The O.D. was taken after a sample was grown for 14 hours.

Trial Number:	Reagents:	Cell culture conditions:	O.D. After growing for 14 hours
1	• 500 µL dH2O • 500 µL glycerol • 200 µL of cells in media	200 µL of cells incubated into mixture	1.4
2	• 500 µL media • 500 µL glycerol	cells in growth media and glycerol added	1.2
3	• 200 µL media • 200 µL glycerol	Cells pelleted and frozen with glycerol	1.6

Appendix C. Results of Traditional Ames Test.

Table 2. Colony counts from the traditional Ames Test plates. The * marks contamination.

Trial	Strain	Positive Control (colony count)	Negative Control (colony count)
1	TA100	28	150
	TA98	8	33
2	TA100	173	141
	TA98*	77	66

Appendix E. Results of Xenometrix™ Ames Test.

Table 3. Results of three Xenometrix™ Ames Test trials labeled A, B and C. The values show the average number of revertants with a maximum of 4 and minimum of 0 for trials A, B and C.

Category	A	B	C	Sum:	Avg:	Revertants:
Negative Control	4	0	3	7	2.3	1.7
1:32 Dilution	0	0	0	0	0	4
1:16 Dilution	4	0	0	4	1.3	2.7
1:8 Dilution	4	4	0	8	2.7	1.3
1:4 Dilution	0	0	0	0	0	4
1:2 Dilution	0	0	0	0	0	4
Extract and Mutagen	0	0	0	0	0	4
Positive Control	0	0	0	0	0	4

Table 4. Anova single factor test between trials of three of the Xenometrix™ Ames Test trials.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
A	8	12	1.5	4.3
B	8	4	0.5	2
C	8	3	0.38	1.1

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