Leaves in Traditional Pickle Brine, Tannins, and Their Role in Pickle Texture

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Research funded by Mount Mary University.

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APPROVAL

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ABSTRACT

Pickle texture is important for consumer acceptance. It is theorized that tannins from leaves may play a role in the firmness and crispness of the cucumbers. The purpose of this work herein is to understand the effect of native leaves used in vinegar-brined cucumber pickle recipes on pickle texture.

Aim number 1 of this thesis was to measure the total phenol content (TPC) found in a 3% NaCl, 0.05M acetic acid brine before and after the addition of currant, raspberry, black tea, and oak leaves compared to a control. TPC was measured in mg of gallic acid equivalents (GAE) at 20 minutes, 5 Days, 10 Days, 15 Days, and 30 Days using the Folin-Ciocalteu method, and pH and temperature were recorded.

Aim number 2 was to investigate the effect of ellagic acid on cucumber pickle firmness and crispness as compared to a negative control (nothing added), a positive control (modern standard of $CaCl_2$ [5]), and raspberry leaves. Brine in jars were prepared the same as in Aim number 1 and samples taken at 5 Days and 15 Days. Alcohol insoluble solids (AIS) content was used as an indicator of firmness, and crispness was measured by texture analyzer.

Literature is scant on the effect of tannins on pickled cucumbers. Literature was found on the tannin content of the leaves and on causes of pickle softening.

TPC was measured in significant quantities in all leaf types and at all time points. GAE content varied across the different leaf types; raspberry leaf treatment produced the greatest TPC of 176.8 mg/mL GAE on Day 5 and oak leaf treatment had the lowest of 33.3 mg/mL GAE on Day 5 with varying levels for all treatments and time periods ranging between these. AIS for pickles treated with raspberry leaf brine was not significantly different from the positive control

on both days, and the texture was found to be similarly crisp to the control on Day 15. Ellagic acid did not show any effects on firmness or crispness.

This thesis concludes that historic recipes' inclusion of leaves in brine to improve crispness were valid. Further studies should mitigate bacterial and fungal contamination as experienced while completing Aim number 2. It should also attempt to isolate the tannin(s) responsible for the phenomenon, and the mechanism by which this occurs.

DEDICATION

I dedicate this thesis to my family. Their support is much appreciated. My husband and younger son often joined me in the joy of eating pickles with blini, and my older son at age 3 declared on Thanksgiving he was thankful for pickles. It was only natural I choose to research pickles.

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Cucumber pickles are made by using long fermentations in a salt brine, or using an acidified brine over a short time period.[1] Cucumber pickles are an acidified food, and in the United States, regulations require that their pH must be below 4.6, and the water activity above 0.85.[2,3] Cucumber pickle recipes use many techniques to preserve or enhance crispness such as the modern vinegar-based quick method, often with additives or the longer salt brine fermentation method with leaves added.[4,5] Crispness is defined as the intensity of sound, expected to be sharp, clean, fast, and high pitched, made after the first bite with the incisors.[6] The use of leaves to maintain crispness during fermentation was a common historic process, but modern quick pickle methods usually use calcium chloride and vinegar.[4,5] During an interview with a septuagenarian in Moscow, Russia (L. Savitskaya, phone communication July 28, 2020) about pickled cucumbers, it was realized that many common recipes used in this region include currant leaves or leaves of regional plants, like raspberry, as part of quick pickling (non-fermented, vinegar) brine, a hybrid of old and new recipes. When asked why they added leaves, it was stated that it was for crispness. This thesis sets out to understand the reason leaves from native plants were included in vinegar brined cucumber pickle recipes.

Agricultural extension and food science university websites mention leaves, often grape, were added to cucumber pickle recipes due to the tannins from those leaves inhibiting a pectinase enzyme, but where that knowledge came from was not referenced.[4,5] Pectins (pectic polysaccharides) are found in cucumber cell walls as well as in the middle lamella where they help bind cells together.[7] Certain indigenous and exogenous enzymes can degrade pectin, which cause cell walls to become separated, resulting in softer pickles and undesirable textural attributes to consumers.[8,9,10] Tannins, polyphenols found in the leaves of various plants, can complex with enzymes such as polygalacturonase, inhibit their action, and disrupt the softening process.[8] Alternatively, there is some research that indicates ellagitannins and proanthocyanidins (condensed tannins) can form complexes directly with the pectin polysaccharides.[7] Either by inhibiting pectin degradation enzymes or by directly complexing with pectin, tannins could have an effect on cucumber pickle crispness, but not if those tannins are not released in pickling brine in enough quantities to make an impact. Conversely, they may provide a novel source of cucumber pickling/crisping agent.

Aim number 1 of this thesis objective was to first measure the total phenol content found in a quick pickling brine similar to hybrid quick pickle recipes before and after the addition of currant, raspberry, black tea, and oak leaves. Currant and raspberry leaves were chosen because they were originally suggested by L. Savitskaya and used historically. Black tea and oak leaves were included because they have been mentioned as readily available substitutes in cookbooks.[1,11] The brine recipe, base on the formulation proposed by the interviewee, consisted of an aqueous solution of 3% sodium chloride and 0.05 M acetic acid solution with added leaves (2g, dried). Samples of the brine were taken after steeping for 20 minutes, and then again at 5, 10, 15, and 30 days. Brine pH was measured, and the total phenol content was measured on a UV-VIS spectrophotometer using the Folin-Ciocalteu method. Statistical significance was calculated. Once it was established that phenols are present, a literature research was used to estimate tannin levels and identify some of those tannin types.

Aim number 2 of this thesis was to investigate the effect of the equivalent exogenous tannins found in Aim 1 on pickle crispness as compared to a negative control (brine with no leaves), a positive control (brine with a concentration modern standard of $CaCl_2$ [12]), and a leaf variety successful from Aim number 1, and then to theorize as to whether the action of tannins on polygalacturonase or pectin plays a role. The cucumber crispness would be measured using a texture analyzer, and the cucumber firmness would be measured by alcohol insoluble solids. Statistical significance was calculated.

There is a lot of research about different types of tannins present in the leaves of plants, and limited research into tannin interactions with pectin and pectin enzymes. Most of this research concerns the juice, jelly, and wine industries.

The research proposed here is novel. The hypothesis is that a significant quantity of polyphenols will be found from leaves in brine, and that they can enhance brined cucumber crispness. This research could lead to new preservatives/crisping agents that could be labeled as natural, which is popular among consumers.

Literature Review

Phenols include flavonoids (including flavan-3-ols and anthocyanidins) and non-flavanols, which include phenolic acids, or tannins, as well as hydroxycinnamoyls and stilbenes.[13] They can be further broken down into hydrolysable and condensed tannins.[13] Some publications include anthocyanidins as tannins, others do not, and it becomes difficult to pin down how exactly how to classify a tannin.[7] Tannins are a broad class of substances generally defined as special phenolic compounds that have an ability to combine with proteins and other polymers.[7] Since enzymes are proteins, it would be a reasonable assumption that a tannin could combine with an enzyme responsible for pectin degradation and deactivate it. Further literature research to support this statement was undertaken. This idea that tannins prevent cucumber softening from tannin interaction with a pectinase appears to have come from an article published in 1958 in which they concluded that tannins extracted from myrobalan tannins do inhibit pectolysis of low-methoxy pectin but tannins extracted from uncaria gambir did not.[8]

More recent research confirms that pickled cucumbers can soften from polygalacturonic acid hydrolysis with both exo- and endo- sources of polygalacturonase (a pectinase often found in fungus associated with vegetables) acting on the pectin.[9,10] Polygalacturonase catalyzes the hydrolytic cleavage of the α-1,4 glyosidic bonds of pectic acid.[14] Calcium chloride is used currently as a preservative of texture. Not only can it inactivate polygalacturonase, it interacts with demethylesterfied pectin to for a pectin-Ca^{2+} network.[14] Tannins can perform similar roles chemically. There are of course many other reasons for pickled cucumber softening besides this, including salt type and level, the temperature of the brine, the pH, the type and quality of cucumber, and fungal cellulases.[10,15] There are also mechanisms outside of pectin softening that can contribute to a fruit softening.[14] There is very little literature out there directly related to tannins and cucumber softening, but there is literature out there that investigates the effect of tannins on pectin in fruits and in isolation. One article suggests that ellagitannins and proanthocyanidins (condensed tannins), but not gallotannins impede the enzymes of pectin.[16] Another indicates that complexes occur between tannins, specifically from grape skins and seeds, and both insoluble and soluble cell wall material directly.[17] This would indicate that tannins could improve pickled cucumber crispness through either the inactivation of the enzyme polygalacturonase preventing the degradation of pectin, or through the direct complex of several types of tannins on pectin in cell walls directly. It is unknown whether the leaves in these acid-brined pickle cucumber recipes leach enough of the right tannins into the brine to have an effect, and if they do, whether it is related to the enzymes present in the brine or directly related to cell wall interactions.

Black tea total tannins, measured after hot water extraction, are reported in literature to range between 6.20 – 8.33 percent (w/w) using UV-VIS spectrophotometer against a tannic acid standard.[18] Tannin types found in black teas include theaflavins, thearubigans, catechins, and ellagitannins.[19,20] Black currant leaves were found by HPLC to contain epigallocatechin, catechin, and epicatechin among many others as displayed in Figure 1.[21] However, this was discovered by ethanol/phosphoric acid extraction and may not represent that released in the brine.

Table 1. Within-groups correlations between discriminating variables and standardized canonical discriminant functions for black currant populations according to their phenolic compounds in leaves.

	Function			
	1	2	3	4
Eigenvalue	8.424[a]	1.818[a]	1.000[a]	0.377[a]
% of variance	72.5	15.7	8.6	3.2
Cumulative %	72.5	88.2	96.8	100
Canonical correlation	.945	.803	.707	.523
Functional coefficients				
EGC	**−0.879**	0.224	0.388	−0.283
CAT	0.368	0.272	0.198	0.148
ECAT	−0.282	**0.667**	**−0.801**	−0.087
NCA	0.016	0.219	0.375	−0.308
CGA	0.261	0.388	0.299	−0.094
MMGLU	0.454	0.006	−0.229	−0.257
QRUT	0.053	0.057	0.19	−0.552
QGAL	−0.058	0.214	**0.557**	0.579
QGLU	**0.574**	**−0.815**	0.191	**−0.667**
QMGLU	−0.309	0.108	**−0.617**	**1.194**
KRUT	−0.06	−0.029	−0.049	0.381
KGLU	−0.295	**0.665**	0.302	**0.651**
IRUT	0.146	0.312	−0.009	0.247
IRGLU	0.186	−0.198	0.014	0.196
KMGLU	0.332	−0.22	−0.052	**−1.008**

Note: Values in bold indicate phenolic compounds that are the most important for each function. EGC, epigallocatechin; CAT, catechin; ECAT, epicatechin; NCA, neochlorogenic acid; CGA, chlorogenic acid; MMGLU, myricetin-malonylglucoside; QGAL, quercetin-3-O-galactoside; QGLU, quercetin-3-O-glucoside; QRUT, quercetin-3-O-rutinoside; QMGLU, quercetin-3-6-malonylglucoside; KRUT, kampferol-3-O-rutinoside; KGLU, kaempferol-3-O-glucoside; IRUT, isorhamnetin-3-O-rutinoside; IRGLU, isorhamnetin-3-O-glucoside; KMGLU, kaempferol-malonylglucoside.
[a]First four canonical discriminant functions were used in the analysis.

Figure 1: Tannin quantities by HPLC in Black currant leaves.[21]

Raspberry leaves total phenolic content measured by Folin-Ciocalteu method coupled with UV-VIS spectrophotometry ranges from 49.7% - 78.0%, and had several tannins identified by HPLC as seen in Figure 2.[22] These samples were extracted in hot water for only 20 minutes.[22]

Table 2. Concentration of antioxidants in leaves water extracts and their participation (% AC) in total antioxidant capacities of the extracts

Medicinal plant/compound	c (mg/l)	% AC	c (mg/l)	% AC	c (mg/l)	% AC
Strawberry	sample I (2202 ± 332)*		sample II (3295 ± 357)*		sample III (2241 ± 225)*	
Haklic acid	1.2	< 1	5.9	< 1	2.0	< 1
Ellagic acid	21.9 ± 0.1	9.9	34.5 ± 3.6	10.5	21.2 ± 1.3	9.7
(+)-Catechin	45.6 ± 2.4	10.1	29.8 ± 12.2	4.4	98.8 ± 9.9	21.6
Epigallocatechin	3.1	< 1	4.1	< 1	8.0 ± 1.3	1.6
Procyanidin B1	3.0	< 1	11.8 ± 0.2	1.8	5.4 ± 0.8	1.4
Total		> 20.0		> 16.7		> 34.3
Blackberry	sample I (2504 ± 58)*		sample II (3112 ± 110)*		sample III (3091 ± 255)*	
Gallic acid	1.4	< 1	1.1	< 1	1.6	< 1
Ellagic acid	22.7 ± 2.1	8.6	15.7 ± 1.6	5.0	14.0 ± 1.5	4.7
Quercetin-3-D-glucoside	6.2	< 1	23.7	< 1	21.8	< 1
(+)-Catechine	-	-	2.6	< 1	1.2	< 1
(−)-Epicatechine	6.8 ± 1.9	1.4	70.9 ± 4.5	11.6	4.6	< 1
Epicatechingallate	0.8	< 1	3.6	< 1	1.8	< 1
Procyanidin B1	8.5 ± 0.0	1.8	2.8	< 1	3.6	< 1
Total		> 11.8		> 15.6		> 4.7
Raspberry	sample I (2104 ± 258)*		sample II (2181 ± 463)*		sample III (1344 ± 176)*	
Gallic acid	1.2	< 1	1.9	< 1	1.5	< 1
Ellagic acid	16.4 ± 0.9	7.8	19.7 ± 4.6	11.0	10.8 ± 0.1	7.7
(+)-Catechine	0.6	< 1	0.3	< 1	0.5	< 1
(−)-Epicatechine	3.6	< 1	1.9	< 1	1.5	< 1
Procyanidin B1	3.8	< 1	3.7	< 1	-	-
Total		> 7.8		> 11.0		> 7.7

The data are expressed as mean ± SD ($n = 3$ for compounds with % AC > 1); *Antioxidant capacities (DPPH) of leaves water extracts are expressed as means ± SD ($n = 3$), mg ascorbic acid/l

Figure 2: Tannin quantities by HPLC in raspberry leaf.[22]

Finally, oak leaf methanol extract showed measurable quantities of a wide variety of tannins by HPLC as seen in Figure 3.[23] Another study using methanol extraction found evidence of ellagitannins and proanthocyanidins by UPLC-Q-TOF-MS/MS.[24]

It was found some herbs used in traditional pickling recipes had high antioxidant levels and could be used as a preservative, but that levels could vary widely between plants.[25] This

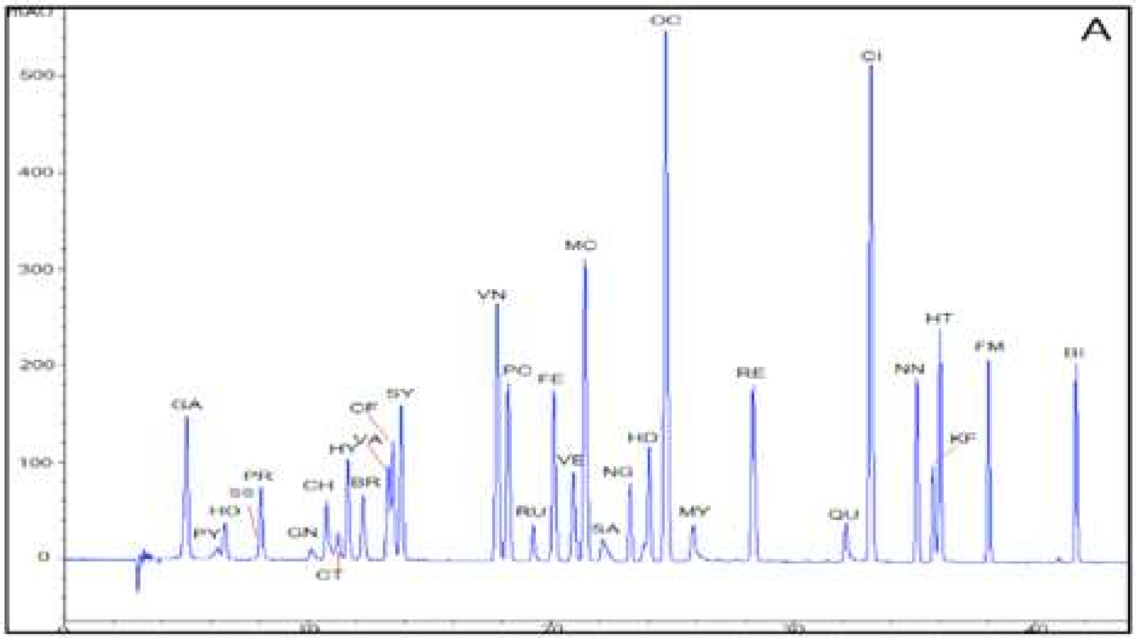

Figure 3. HPLC chromatogram of phenolic compounds on *Quercus* species. A, 32 Standard phenolic compounds; B, *Quercus salicina* Blume; C, *Quercus acuta* Thunberg. GA, Gallic acid; PY, Pyrogallol; HO, Homogentisic acid; SS, 5-sulfosalicylic acid; PR, Protocatechuic acid; GN, Gentisic acid; HY, *p*-Hydroxybenzoic acid; BR, *β*-Resorcylic acid; CT, (+) Catechin; CH, Chlorogenic acid; VA, Vanillic acid; CF, Caffeic acid; SY, Syringic acid; VN, Vanillin; PC, *p*-Coumaric acid; RU, Rutin; FE, Ferulic acid; VE, Veratric acid; MC, *m*-Coumaric acid; SA, Salicylic acid; BZ; Benzoic acid, NR; Naringin, OC; *o*-Coumaric acid, MY; Myricetin, RE; Resveratrol, QU; Quercetin, CI; *t*-Cinnamic acid, NN; Naringenin, KF; Kaempferol, HT; Hesperetin, FM; Formononetin, BI; Biochanin A.

Figure 3: HPLC ID of various phenolic compounds in oak leaves.[23]

suggests that finding an antioxidant/tannin that could be added in controlled exogenous amounts could be used as a pickle preservative/crisping agent. It would appeal to consumers looking for "natural" preservatives.

Some tannins have a reputation as being "antinutritive" by binding iron in the gut, although more recent studies are more positive.[26] Scientists used to think of tannins as anti-nutritive due to older animal studies, but have since been shown to have great anti-oxidant and anti-microbial activity.[27] The safety and consumer sensory appeal of exogenous tannins would not be included in this study, but would be an important consideration. There is not literature research evidence to conclude that, without methanol, oak or current leaves would leach tannins in significant levels, and no research on how brine would affect phenol or tannins levels.

Methods

All research was conducted in the Mount Mary University science department unless otherwise noted.

Aim number 1

Green leaves of Ribes rubrum (Red currant) and Ribes Nigrum (black currant) were

Figure 7: Dried & chopped currant leaves

Figure 6: Dried & chopped oak leaves

Figure 4: Dried and chopped raspberry leaves

Figure 5 Black Tea leaves from bag

gathered in the 53097 zip code of the USA on September 5, 2021. They were placed in a Warring Pro DHR30 dehydrator on the low setting (43.3 °C) for 18 hours and then immediately placed in vacuum sealed plastic using a Food Saver. This process was repeated on September 6, 2021 for green leaves of Rubus idaeus ‘Nova’ (Summer Bearing Raspberry) in the 53029 zip code, and again for the green leaves of Quesrcus palustris (Pin Oak) gathered on September 7, 2021 in the 53029 zip code. Kroger brand Black Tea bags with a best by of JUN18 23B were purchased. On October 22, 2021 the vacuum sealed leaves were removed from their packaging and ground in a food processor to the same approximate size and weighed (see Figures 4-6). Select leaves were weighed before and after drying. Two grams represents 3.3 fresh raspberry leaves, 7.6 fresh currant leaves, and 2.5 fresh oak leaves. Those numbers are in line with the ranges of fresh leaves used in historic recipes.

The purchased leaves were removed from their bags and weighed (see Figure 7). DI water was boiled and cooled. Jars and lids were sanitized in a dishwasher on a sanitary cycle. Into 3 glass quart jars per treatment type, the samples were prepared according to Table 1 resulting in a 3% NaCl 0.05M acetic acid brine. Temperature and pH were recorded after the dried leaves were added.

Table 1: Formulation of treatment types for Aim 1

Ingredient	Currant	Raspberry	Oak	Black Tea	Control
DI water (mL)	789	789	789	789	789
Glacial Acetic Acid (mL) (Sigma 320099 lot # SHBG2118V)	2.2	2.2	2.2	2.2	2.2
NaCl (g) (Sigma S9888 lot # SLCC4856)	24.0	24.0	24.0	24.0	24.0
Ground leaf (g)	2.0	2.0	2.0	2.0	0

Jars were then shaken at 0, 10, and 20 minutes. At 20 minutes, 3 samples were taken per jar (a total of 3 jars per sample type with 3 samples taken per jar) and temperature and pH of brine recorded.

The Folin-Ciocalteu method for TPC using UV-VIS spectrophotometry was used measure total phenolics released under these conditions. All Folin-Ciocalteu tests were run according to the suggested modifications.[28,29] This method is slightly variable but is comparable to the AOAC method and has more literature to compare.[30] A 250 µl sample was mixed with 1 mL of 1:10 v/v diluted Folins & Ciocalteu's phenol reagent (Sigma F9252 lot # SLCJ8281) and left to sit at ambient for 5 minutes. Then 1 mL of a 10% Na_2CO_3 (Sigma 223530 lot # SLCK3834) solution was added and the mixture was left at ambient temperature for 30 minutes. Gallic acid standards were created by dissolving 0.1000g gallic acid (Sigma 67384 lot # SLCJ8281) into 10mL of 95% ethanol, and then diluted to 100 mL with DI water. Dilutions were then made to create 25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, 125 mg/mL, and 150 mg/mL standards. They were first scanned for absorbance across the visible spectrum using disposable cuvettes and a Jenway 7205 scanning UV-VIS spectrophotometer, and it was verified a peak occurred at 765nm as literature suggested.[30] A standard curve at 765nm was then developed, and repeated on multiple days.

Then on Days 5, 10, 15 and 30 the temperature and pH of the jars were taken and the Folin-Ciocalteu method described above was repeated. These numbers were converted to gallic acid equivalents (GAE) using the standard curve and Beer's law to calculate the total phenolic content (TPC). ANOVAs with Tukey multiple comparisons (SPSS software) and a MANOVA (JMP software) were then run to determine significance at the 0.05 level. Through literature research, it was determined that ellagic acid was most like to be present in at least the raspberry leaf treatment. This discussion can be found in the Discussion section of the paper. The dihydrate form of ellagic acid (EMD Millipore 324683 lot # 3574647) was chosen based on cost, solubility in water, and availability. The amount, 150mg, to be added to a sample jar in Aim 2 was chosen.

Aim number 2

Mucci Farms "Cute Cumbers" cucumbers (sell by dates of 22JAN17 and 22JAN20, imported from Canada) were purchased from a local Costco, sorted by size, and rinsed. Only unblemished cucumbers between 9cm – 11cm were selected. Raspberry leaf tea was purchased (Traditional Methods Organic Raspberry Leaf Tea lot # MAY2024LB0009825), bags were opened and leaves weighed. Brine was prepared exactly as in Aim number 1 and the test ingredients were added according to Table 2. Three jars of each sample type were prepared.

Table 2: Formulations of treatment types for Aim 2

Ingredient	Ellagic Acid	Positive Control	Negative Control	Raspberry Leaf
DI water (mL)	789	789	789	789
Glacial Acetic Acid (mL)	2.2	2.2	2.2	2.2
NaCl (g)	24.0	24.0	24.0	24.0
Cucumbers	8	8	8	8
Sample variable	150 mg Ellagic Acid	2.0 g Calcium Chloride (Ball Pick Crisp granules lot#B212213-2952)	N/A	2.0 g

The brine temperature and pH were measured on Days 0, 5, and 15. Observations on brine clarity and cucumber color were noted.

Texture Analysis

On Day 5, two cucumbers were selected from the jar and each one had the middle 4 cm cut out and then sliced in half. The pickle crispness was analyzed with a Brookfield CT-3 Texture Analyzer on these pieces, cut side down, using a 6mm cylinder probe (see Figure 8). On Day 15 three cucumbers were selected from the jar and each one had the middle 4 cm cut out and were left whole. The pickle crispness was analyzed with a Brookfield CT-3 Texture Analyzer on these pieces using a TA7 Knife Edge 60mm W probe (see Figure 9). On both days a TPA test with a trigger of 5.0g, deformation of 10.0mm, a speed of 5 mm/sec, and a distance of 17mm was used. Hardness 1 measurement was recorded.

Alcohol Insoluble Solids

The alcohol insoluble solids (AIS) of pickle flesh was performed as described by Yoo et

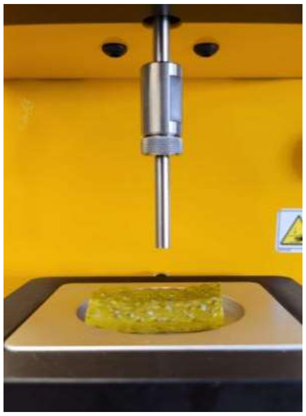

Figure 8: 6mm probe sample set up

Figure 9: Knife edge probe setup

al with slight changes.[15] The pieces from a single jar from the texture test were recombined and roughly chopped. Three 10-g samples were weighed and combined with 95% undenatured ethanol (Lab Alley EAP200 lot 282885), and an immersion blender was used until samples were pulverized, approximately 1 minute per sample. The samples were then left undisturbed for 40 minutes at 60°C. A Buchner funnel system was set up and a paper filter (No 100 7cm JHMUNKTELLS Swedish filter) was used. Samples were rinsed twice with 80% ethanol and once with diethyl ether. They were then dried overnight at 40°C and weighed. The weight loss before and after drying was used to calculate the AIS.

Both texture hardness and AIS were averaged and compared across different brine treatments using ANOVA with multiple comparison Tukey (SPSS software).

Results

Aim number 1

Total Phenolic Content

The total phenolic content (TPC) of the brine for each treatment type and time period is reported in gallic acid equivalents (GAE), which was calculated by the standard curve found in Appendix A. Note that for each treatment type and time period 3 jars of brine were prepared and each jar was sampled 3 times for a total of 9 data points per jar and time point. The control, which had no leaves added, displayed zero phenolic content. As can be seen in Figure 10, raspberry leaves measured the highest TPC, followed by black tea leaves, currant leaves, and

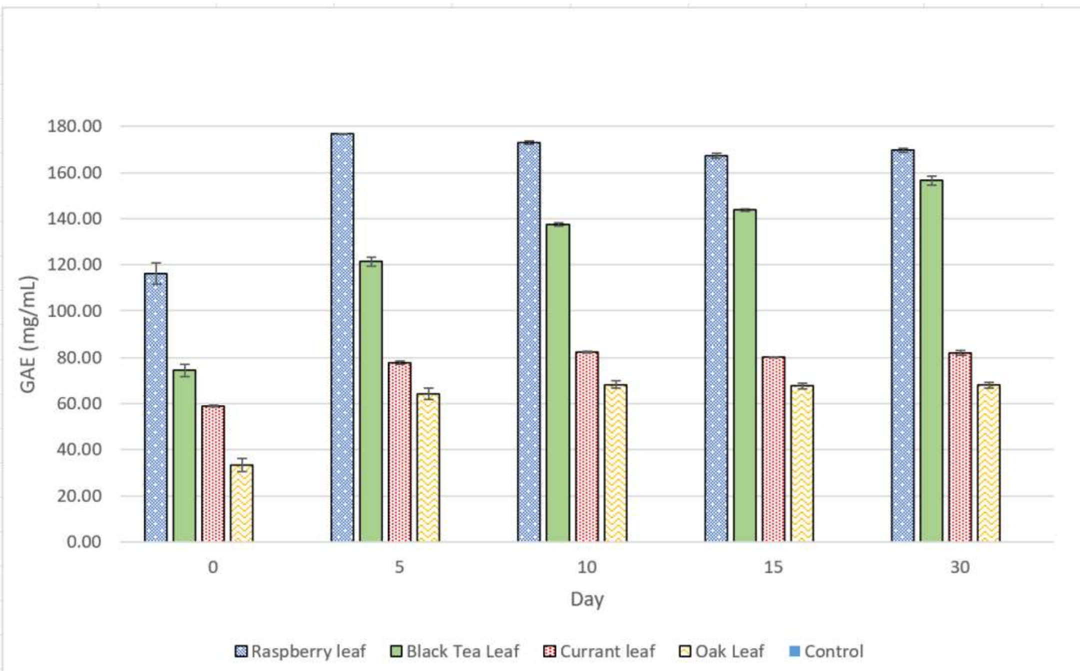

Figure 10: TPC in mg/mL GAE displayed by day and treatment type. Note that the control stayed 0.

then oak leaves.

Significant differences at the $p=0.05$ level were calculated by ANOVA Multiple Comparisons Tukey HSD and found to be $p<0.001$ for all treatment types as compared to the control and each other for each day sampled. Appendix A contains the descriptive statistics for each treatment type and day, and the Multiple Comparison tables by day.

Figure 11 displays this same data over time. MANOVA F test calculated a p<0.0001, displaying significance differences over time within all interactions. Further, it can be noted that regression analysis for each treatment type in brine over time shows that the elution pattern follows a logarithmic curve with a good coefficient of determination for currant leaves, black tea leaves, and oak leaves and a moderate coefficient of determination for raspberry leaves.

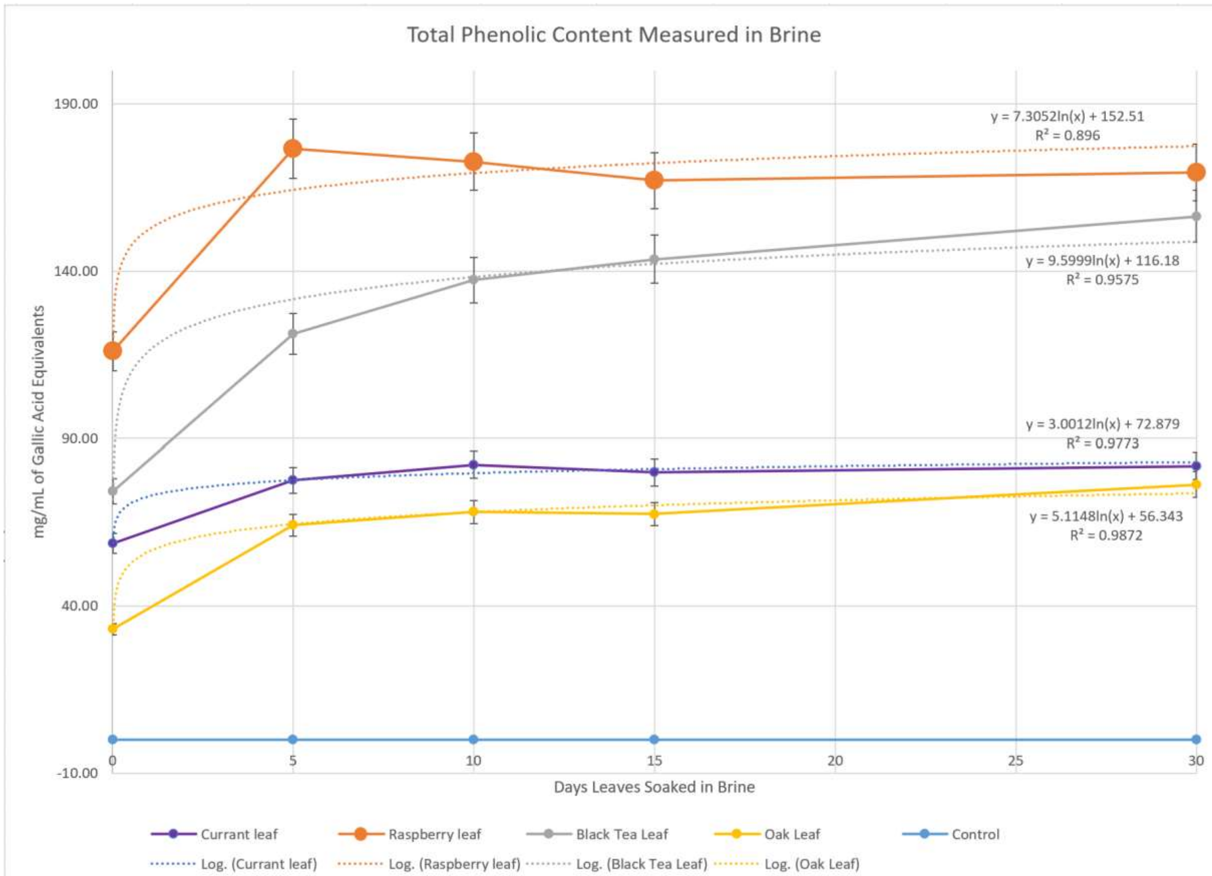

Figure 11: TPC in GAE (mg/mL) in brine over time with regression analysis

pH and Temperature

The starting pH for all treatments including the control was 3.09. The pH of the control brine showed good stability over the 30 days of the experiment as seen in Figure 12. Unlike the

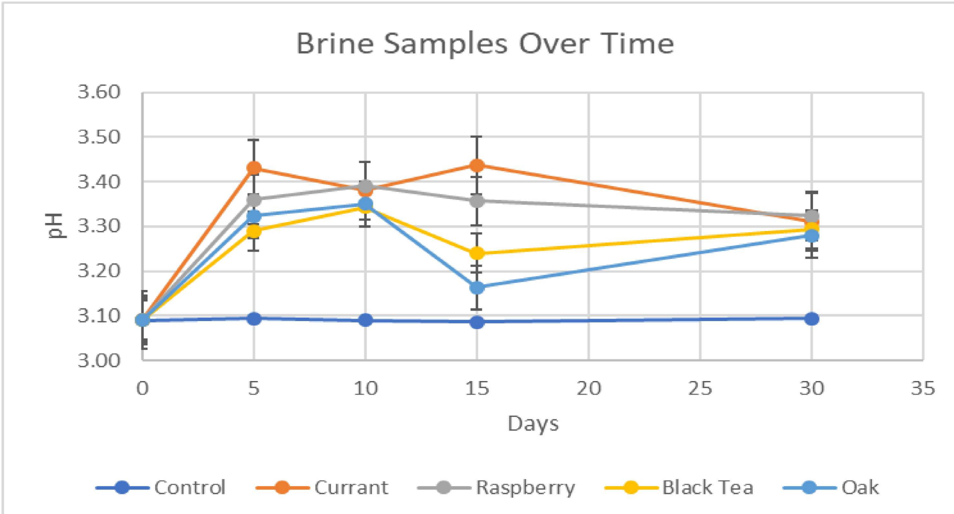

Figure 12: pH of brine over time by treatment type

control, the other treatment types showed a change in pH after 5 days which increased in pH to around 3.30 – 3.45, which was treatment dependent. Over the remaining time points, pH varied slightly within ±0.5 for each treatment. Day 0 temperatures of the brine was 31.1 °C, Days 5 - 30 temperatures were 21.6 °C to 22 °C.

Observations

Several observations were made. Figure 13 shows the jars at Day 30. Throughout the 30 days the majority of raspberry leaves stayed floating on the surface or in the top third, whereas

Figure 13: Treatment jars on day 30. From left to right: Currant, Raspberry, Black Tea, Oak, Control

the other three leaf types settled to the bottom of the jars. The green leaves of the raspberry, currant, and oak produced a green-beige brine color, and the black tea leaves produced an amber brine color. The currant and raspberry had a pleasant green, herbal aroma, the oak had a forest aroma, and the black tea had a typical earthy, malty aroma; all had the vinegar aroma as well. At Day 30 some raspberry leaves showed some white on the leaves (see Figure 14), but it is unknown if it was salts crystalizing due to surface exposure or fungal contamination. One tea leaf jar started to show some contamination in the form of a floating, foamy mass reminiscent of a scoby as pictured in Figure 15.

Figure 14: White residue on raspberry leaves

Figure 15: Foamy, floating mass in black tea brine

Aim number 2

Alcohol Insoluble Solids

Alcohol insoluble solids (AIS) data was collected for Days 5 and Days 15. The results are displayed in Figure 16. Day 0 represents data from fresh cucumbers. Significant

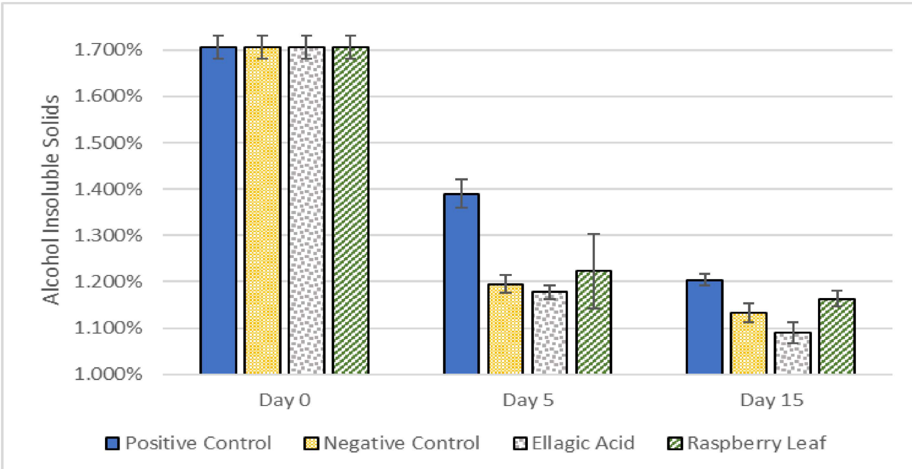

Figure 16: AIS (%) by treatment type and day

differences (p<.001) were calculated between fresh cucumbers and all treatment types on both days. On Day 5 the positive and negative controls were found to be significantly different at p=0.021, confirming the validity of the test. Table 3 for Day 5 shows the homogenous subset

AIS

Tukey HSD[a,b]

Treatment	N	Subset 1	Subset 2
Ellagic Acid	9	.0117778765	
Negative Control	9	.0119514048	
Raspberry Leaf	9	.0122253894	.0122253894
Positive Control	9		.0138935704
Sig.		.892	.057

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 1.783E-6.

a. Uses Harmonic Mean Sample Size = 9.000.

b. Alpha = 0.05.

Table 3: Day 5 AIS homogenous subsets

groupings that SPSS performs and as can be seen, raspberry leaves perform similar, although significantly different, to the positive control (p=0.057) and ellagic acid is not significantly different to the negative control (p=0.993). Raspberry leaf's performance to the positive control is tempered by the fact that it also is not significantly different compared to the negative control (p=0.972).

On Day 15 the positive and negative controls were also found to be significantly different at p=0.048, confirming the validity of the test. Table 4 for Day 15 shows the homogenous subset

AIS

Tukey HSD[a,b]

Treatment	N	Subset 1	2	3
Ellagic Acid	9	.0109054293		
Negative Control	9	.0113224626	.0113224626	
Raspberry Leaf	9		.0116343610	.0116343610
Positive Control	9			.0120413204
Sig.		.401	.640	.423

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 3.116E-7.

a. Uses Harmonic Mean Sample Size = 9.000.

b. Alpha = 0.05.

Table 4: Day 15 homogenous subsets

groupings changed. Raspberry leaf shows was not significantly different compared to the positive control (p=0.423), and ellagic acid shows similar performance the negative control (p=0.401). However, although raspberry leaf is still not significantly different from the negative control (p=0.640), it does show a stronger subset grouping compared to the positive control.

On both days, ellagic acid showed a significant difference from the positive control (Day 5, p=0.010 and Day 15, p<0.001), but not the negative control (Day 5, p=0.993 and Day 15 (p=0.401). A full tukey multiple comparisons and the descriptive statistics for both days can be found in Appendix A.

Texture Analysis

Texture data was collected on Day 5 using a 6mm cylinder probe as suggested in literature, but due to the softness and hollowness of the cucumbers some tests failed per the machine when the cucumber split instead of being punctured. Therefore, the group sizes were unequal. As a reference a commercial pickle, Great Gherkins Baby Whole Pickles, was measured to have an average hardness 1 of 3139.5 gf. The experimental treatments average hardness 1 readings are displayed in Figure 17. There was a significant difference between the positive

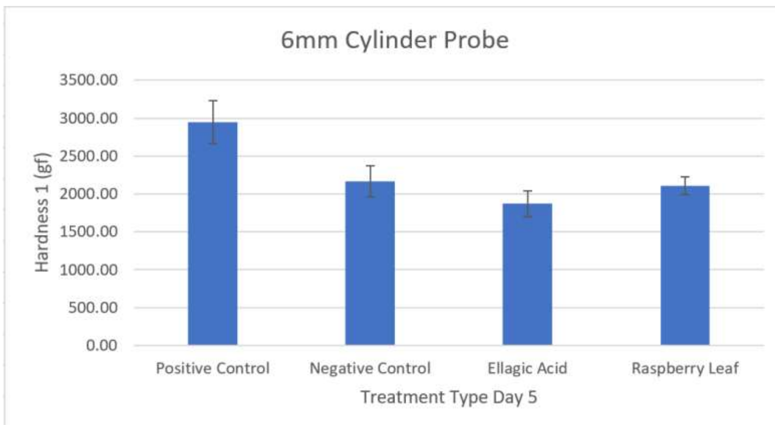

Figure 17: Day 5 hardness measurements by treatment type

control and the negative control (p=0.047) confirming the validity of the test. There were also significant differences between the positive control and ellagic acid (p=0.003) and the positive control and raspberry leaves (p=0.024).

There were no significant differences found between the negative control and ellagic acid (p=0.730) and raspberry leaf (p=0.996). The full Tukey and the descriptive statistics can be found in Appendix A.

Due to the difficulties with the probe type, for Day 15 the knife edge 60mm probe was used, and therefore statistical comparisons cannot be made between Days 5 and 15. The results can be found in Figure 18. As a reference a commercial pickle, Great Gherkins Baby Whole

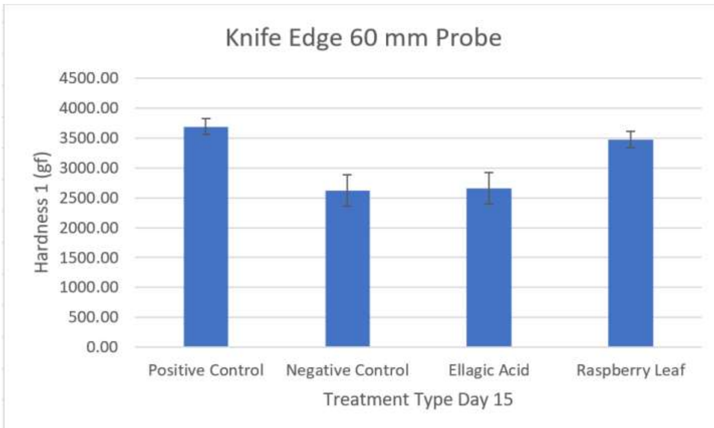

Figure 18: Day 15 hardness measurements by treatment type

Pickles, was measured to have an average hardness 1 of 4612 gf. Some commercial pickles were sliced all the way through on the initial compression, which did not happen with the experimental treatment types. There was a significant difference between the positive control and the negative control (p=0.005) confirming the validity of the test. There was also a significant difference the positive control and ellagic acid (p=0.007). There was no significant difference between the positive control and raspberry leaves (p=0.881). There were no significant differences found between ellagic acid and the negative control (p=0.999), but there were significant differences between the negative control and raspberry leaf (p=0.032). The full Tukey and descriptive statistics can be found in Appendix A.

pH and Temperature

The temperature for all 15 days stayed at 20.6 °C. The pH of the brine before the addition of cucumber or additives was 3.09. The graph of the pH changes can be found in Figure 19 and as can be seen all followed a similar pattern of rising pH over time.

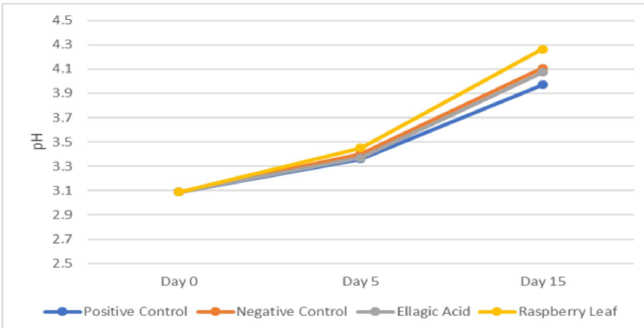

Figure 19: Aim 2 pH over time by treatment type

Observations

Some observations were noted. The ellagic acid did not dissolve completely in the brine, and the brine stayed an unpleasant, opaque, grey-beige color the entire experiment duration. See Figure 20 for a picture.

Figure 20: Aim 2 Day 0 cucumbers in brine. From left to right: Ellagic Acid, Negative Control, Positive Control, Raspberry Leaf

On Day 5, the cucumbers had equally lost their bright green color and had taken on the typical yellow-green color of a pickled cucumber. The brines of the positive control, negative control, and the raspberry leaf stayed clear, although the raspberry leaf brine did have a yellow-green hue similar to the cucumbers (see Figure 21).

Figure 21: Aim 2 Day 5 cucumbers in brine. From left to right: Raspberry Leaf, Positive Control, Negative Control, Ellagic Acid

On Day 15, the cucumbers and brines had similar hues to Day 5, but the positive and negative controls became cloudy (Figure 22). In all treatment types some individual jars displayed a

Figure 22: Aim 2 Day 15 cucumbers in brine. From left to right: Ellagic Acid, Negative Control, Positive Control, Raspberry Leaf

white slimy feeling, potential mold on the cucumbers (Figure 23), and in a few jars, some of the cucumbers exposed to the air developed a dry, blue-green mold (Figure 24). All jars had vinegar and cucumber aromas, and the raspberry jar had an additional, faint herbal aroma.

Finally, many cucumbers started to develop some hollowness internally as displayed in Figure 25. Due to the fungal and/or bacterial contamination, the experiment was terminated after

Figure 24: White, slimy substance Day 15

Figure 23: Blue-green surface mold Day 15

Figure 25: Hollow center of brined

Day 15. However, the jars were kept for 30 days, and it was noted that the brine treated with raspberry leaves never became cloudy, even the other samples were turbid likely due to contamination. For the raspberry leaf treated brine, it seemed that the cloudiness causing agent had settled to the bottom of the jars resulting in a clearer brine compared to the other treatments. All pickles in all treatments held at 30 days at room temperature in the dark disintegrated upon handling.

Discussion and Conclusions

Aim number 1

The purpose of Aim number 1 was to determine if leaves steeped in brine did leach phenolic compounds of measurable quantities. There were measurable, significant TPC levels leached into the brine that followed a logarithmic pattern. The Folin-Ciocalteu method for measuring TPC in mg/mL of GAE produced results with good precision. A minimum acceptable threshold was not established, but literature indicates herbs used in pickling that produced a TPC of 74 – 120 mg/mL (by tannic acid equivalents) in brine was found to be a good preservative for pickled vegetables.[25] The results from this study showed good agreement with TPC values falling within this range. All types of dried, finely chopped leaves produced significant phenol content in the pickling brine within 20 minutes of contact time, although some leaves resulted in greater TPC than others. This trend continued over the next 30 days. The results of anywhere between 33.2 mg/mL TPC and 176.8 mg/mL TPC fall within the range of various literature.[19,22,28] Of specific interest, Buricova et al steeped raspberry leaves in plain water at 98°C for 20 minutes and had an average TPC of 68.9 mg/mL.[28] In this experiment, raspberry leaves were steeped in brine at only 31.1°C for 20 minutes and had an average of 116.3 mg/mL, 168% more than in water. The lowered pH may facilitate the release of phenolic compounds into the brine, especially tannins, which tend to hydrolyze in acidic conditions since it has been observed by others.[13] The raspberry treatment's high TPC levels, with a high of 176.8 mg/mL on Day 5, could also be an artifact of the raspberry leaves staying suspended in the brine, instead of settling on the bottom like the other leaf types, thereby having more surface exposure to the brine.

Polyphenolic compounds include the groups of phenolic acids, flavonoids, and tannins.[29] Hydrolyzable tannins are polymers of gallic or ellagic acid.[29] As stated in the literature review, all leaf types were found by other researchers to have various types of tannins present in them when identified by HPLC.[18,19,20,21,22,23] Tannin identification and quantitifcation was beyond the scope of this research, but based on the part literature leaves, including raspberry leaf, were shown to contain tannins and anthocyanins with ellagic acid being the majority of that percentage.[22] Other leaves in the same study displayed varying quantities of tannins, but all types detected tannins at some level.[22] Thus it is likely that tannins, in particular ellagic acid, are likely present in the leaves used in this study.

Ellagic acid was chosen for the second Aim since it has been confirmed and previously identified in plant leaves. The author is aware that 150 mg of ellagic acid would be present in a quart jar of brined raspberry leaves and cucumbers, but this quantity was chosen to represent the upper limits of possibility and solubility. The high presence of ellagic acid in raspberry leaves, as noted in literature, was a determining factor to be used in Aim number 2.[22] Ellagic acid is sold as a supplement to consumers, so there is already some familiarity as a "helpful, natural" chemical, which is important to consider for commercialization. Several studies have shown it to have many potential benefits including reducing oxidative stress from acrylamides *in vitro*, and demonstrating anti-diabetic potential in mice.[31,32]

Aim number 2

Aim number 2 attempted to determine if ellagic acid and raspberry leaf performed similar to the industry standard of calcium chloride in maintaining a firmer, crisper pickled cucumber. In brining cucumbers with various treatments, calcium chloride, the positive control, was expected to have a significantly higher AIS and this was shown experimentally to be true. A higher AIS demonstrate more linkages between pectin molecules and therefore a firmer cucumber.[15] The 1.39% AIS of the Day 5 positive control was within the target range of 1.39 – 1.43 which represents brined cucumber with a desired firmness.[15] The raspberry leaf treatment at 1.22%, although not significant at the 0.05 level, was significant at the 0.10 level and falls within the range of acceptable, although not as desired pickle firmness.[15] By Day 15, the effects of possible contamination on AIS could be seen in the lowered values across all treatments, although both the positive control and raspberry leaf did show some effect in slowing down the process of texture degradation. It suggests that something in raspberry leaves, that is leached into the brine, participates in pectin linkage preservation in a similar, though slightly less effective, manner than calcium chloride. Ellagic acid, with its very similar performance to the negative control in its effects on AIS, does not participate in preserving pectin linkage.

The results from the texture analysis to quantify pickle crispness were variable. The positive control had a greater effect on hardness compared to other treatments, closer to a commercial pickle, as would be expected. On Day 5, using the 6mm cylinder probe, raspberry leaf and ellagic acid showed more similarities with the negative than the positive control, indicating that they just don't affect texture. However, on Day 15, using the knife edge probe, the raspberry leaf treatment showed a similar effect on texture to the positive control, but not the negative control. Ellagic acid continued to show no effect on texture. The effect from raspberry leaf was more pronounced towards the end of practical shelf life, when contamination was observed in the jars. The preservation of a firmer texture is possibly due to an unknown tannin coupling with the extra exogenous polygalacturonase enzymes produced by the contamination. Conversely, it may not have been polygalacturonase and pectin that was the enzyme responsible for the softening; other enzymes involved in other mechanisms, like the breakdown of the hemicellulose arabinoxylan may have been responsible.[14] This would need to be confirmed by experimentation.

It is disappointing that ellagic acid showed no significant activity. This may be due to its low solubility, leaving not enough available in solution to form complexes, or its inability to directly bind with pectin as calcium chloride does. A different tannin may have better success. It also possible that a phenolic that is not a tannin is involved.

The lack of cloudiness in the raspberry leaf treatment is an interesting observation that may indicate tannins of some sort are present in the brine. An aqueous extract or raspberry leaf should be further pursued for a commercial use as a potential clarifying agent. The cloudiness seen in the negative and positive controls is most likely due to a combination of dead and living yeast cells as well as the polygalacturonase that they produce.[9] Tannins complex with proteins, such as those found in yeast cells and enzymes, which may account for the clarifying effect seen.[7] It is interesting to note that even with the contamination, the legal definition of a pickled food requires the pH to be below 4.6, which this experiment met.[2,3]

Final Conclusions

Overall, this thesis can conclude that leaves of currants, raspberry, black tea, and pin oak do release phenols in significant quantities into pickling brine in a time frame in which a refrigerated vinegar brined pickle would be consumed (normally within 2 weeks). Raspberry leaf did show a positive effect on pickle firmness and pectin linkage, and a positive effect of crispness over time. Tannins could definitely contribute to the firmness of a pickle based on literature, but by experimentation it is not ellagic acid. The AIS numbers and texture analysis of the raspberry leaf and the positive control would indicate an effect directly on pectin linkage, but the clarity of the raspberry brine and the contamination issues could indicate there was simply less polygalacturonase acting on the cucumber flesh due to tannin-protein complexes binding the enzyme.

Combined with the appealing herbal aroma and the clarity of the brine, it is easy to see why recipes still include native leaves in quick pickling brines. This thesis concludes that historic recipes' inclusion of leaves for crispness were valid. Further studies should be done to try and isolate the tannin(s) responsible for the phenomenon, and the mechanism by which this occurs.

Improvements to Future Studies

This study would have benefited greatly if individual tannins could have been isolated and quantified in Aim number 1, instead of relying on literature reviews. HPLC and NMR both have some techniques that could be used, although the identity of tannins is still an evolving process.[24] Polygalacturonase activity can be determined by a series of involved assays as well as SEM to look at cell structure and other tests, and it is recommended that future studies include a test to help separate the cause of the positive effects seen.[7] The amount of leaves used should be investigated to find the optimal quantity, as well as using fresh vs dried leaves. A determination of the solubility limits of the subsequent tannins isolated should be performed to help determine the correct quantity to be used.

Aim number 2 had a lot of problems with contamination that could have masked the effects of the results, although the general trends are in line with expectations for the positive and negative controls. The unknown bacterial and fungi contaminates would contribute greatly to the softening of the cucumbers.[6,7,22] This experiment should be repeated to include a blanching step for the cucumbers at 98°C as indicated in the literature.[9] This would drastically reduce the likelihood of contamination. Alternatively, the brining cucumbers could be stored under refrigeration to discourage contamination. The study should also be repeated using the knife probe on all days studied, and the inclusion of a Day 10 might yield better trends over time.

Further studies should include water activity measurements and culturing of any contamination found for safety. A larger variety of tannins should be trialed in Aim 2, and the mechanisms of any successes should be explored. Once safety has been established, the inclusion of sensory taste studies would be important.

Finally, the cucumbers available this season, outside of factories, are not specific to pickling, and therefore do not hold up as well in brine. They seemed to have very thick skins, which may inhibit the ability of tannins to reach in the cucumber flesh. This seemed to have a huge effect on probe choice as the original probe worked well for fresh and commercial pickles, but not those produced by this study. The cucumbers also may have been wax coated, masking the effects. If repeating the experiment, cucumbers specifically grown for pickling should be used.

Appendix A: Additional Data

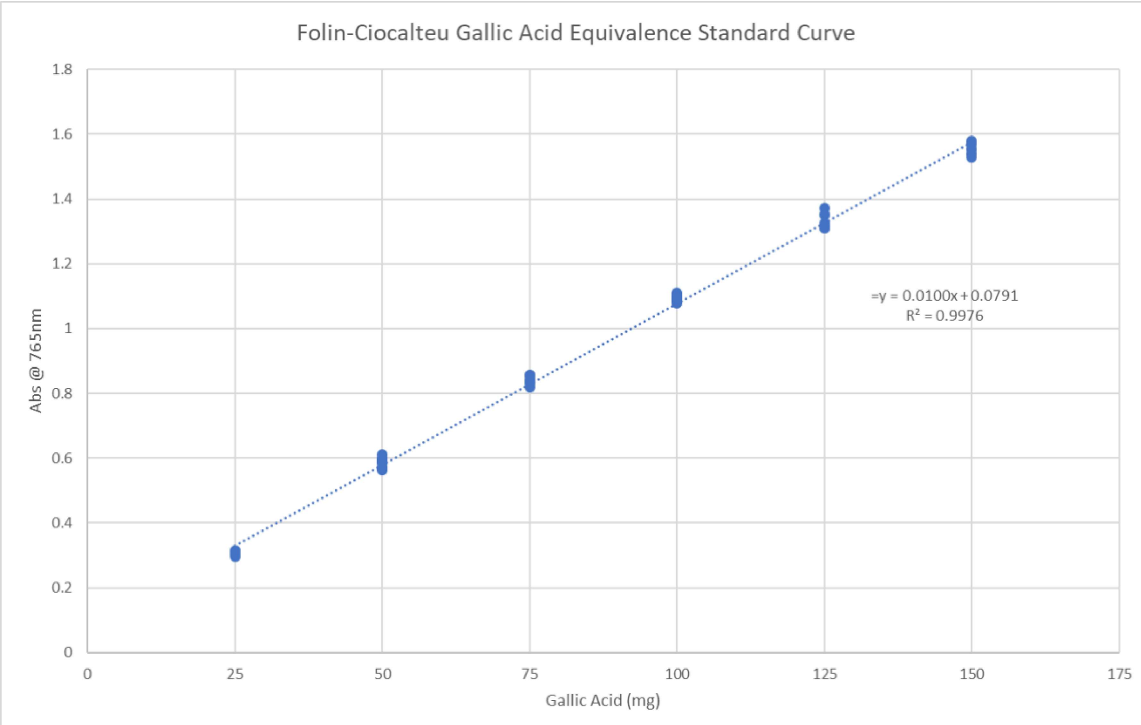

Figure 26: GAE standard curve used in Aim 1 TPC calculations

Table 5: Aim 1 Black Tea Leaf Treatment GAE descriptive data

	Day 0	*Day 5*	*Day 10*	*Day 15*	*Day 30*
Mean	74.48	121.47	137.50	143.74	156.57
Standard Error	2.66	1.93	0.87	0.48	1.94
Median	72.15	120.87	138.20	143.33	157.73
Standard Deviation	4.60	3.34	1.51	0.83	3.35
Sample Variance	21.16	11.15	2.28	0.69	11.24
Skewness	1.70	0.78	-1.64	1.68	-1.37
Range	8.26	6.60	2.77	1.50	6.40
Minimum	71.52	118.47	135.76	143.20	152.79
Maximum	79.78	125.07	138.53	144.70	159.19

Table 6: Aim 1 Currant Leaf Treatment GAE descriptive data

Currant GAE	*Day 0*	*Day 5*	*Day 10*	*Day 15*	*Day 30*
Mean	59.02	77.79	82.50	80.19	82.10
Standard Error	0.39	0.57	0.33	0.19	1.21
Median	59.15	77.55	82.41	80.35	80.98
Standard Deviation	0.68	0.99	0.57	0.33	2.09
Sample Variance	0.46	0.98	0.33	0.11	4.36
Skewness	-0.85	1.04	0.68	-1.65	1.72
Range	1.33	1.93	1.13	0.60	3.70
Minimum	58.29	76.95	81.98	79.81	80.81
Maximum	59.62	78.88	83.11	80.41	84.51

Table 7: Aim 1 Oak Leaf Treatment GAE descriptive data

oak	*Day 0*	*Day 5*	*Day 10*	*Day 15*	*Day 30*
Mean	33.29	64.43	68.34	67.74	68.19
Standard Error	2.86	2.53	1.49	1.17	1.22
Median	35.06	64.62	67.82	68.45	68.25
Standard Deviation	4.96	4.39	2.59	2.03	2.12
Sample Variance	24.57	19.23	6.70	4.11	4.48
Skewness	-1.40	-0.19	0.87	-1.38	-0.12
Range	9.43	8.76	5.10	3.87	4.23
Minimum	27.69	59.95	66.05	65.45	66.05
Maximum	37.12	68.72	71.15	69.32	70.28

Table 8: Aim 1 Raspberry Leaf Treatment GAE descriptive data

Raspberry	*Day 0*	*Day 5*	*Day 10*	*Day 15*	*Day 30*
Mean	116.259	176.8317	172.8994	167.2676	169.7114
Standard Error	4.628951	0.221328	0.735238	1.01559	0.918154
Median	116.0035	176.8206	173.0549	167.0565	169.2226
Standard Deviation	8.017578	0.383352	1.27347	1.759054	1.590289
Sample Variance	64.28156	0.146958	1.621726	3.094272	2.529018
Skewness	0.143251	0.130284	-0.54134	0.532146	1.25239
Range	16.02905	0.766462	2.532656	3.499065	3.065847
Minimum	108.3722	176.454	171.5553	165.6236	168.4228
Maximum	124.4012	177.2205	174.088	169.1226	171.4887

Table 9: Aim 1 Control Treatment GAE descriptive data

	Day 0	*Day 5*	*Day 10*	*Day 15*	*Day 30*
Mean	0	0	0	0	0
Standard Error	0	0	0	0	0
Median	0	0	0	0	0
Standard Deviation	0	0	0	0	0
Sample Variance	0	0	0	0	0
Skewness	0	0	0	0	0
Range	0	0	0	0	0
Minimum	0	0	0	0	0
Maximum	0	0	0	0	0

Table 10: Aim 1 TPC in GAE Day 0 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: GAE (setting control to 0)

Tukey HSD

(I) Treatment Type	(J) Treatment Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Currant Leaves	Raspberry Leaves	-57.2402569*	2.507282660	<.001	-64.4012851	-50.0792288
	Black Teat Leaves	-15.4625340*	2.507282660	<.001	-22.6235621	-8.30150583
	Oak Leaves	25.7264574*	2.507282660	<.001	18.56542925	32.88748556
	Control	59.0187233*	2.507282660	<.001	51.85769517	66.17975148
Raspberry Leaves	Currant Leaves	57.2402569*	2.507282660	<.001	50.07922876	64.40128507
	Black Teat Leaves	41.7777229*	2.507282660	<.001	34.61669477	48.93875108
	Oak Leaves	82.9667143*	2.507282660	<.001	75.80568616	90.12774247
	Control	116.258980*	2.507282660	<.001	109.0979521	123.4200084
Black Teat Leaves	Currant Leaves	15.4625340*	2.507282660	<.001	8.301505830	22.62356214
	Raspberry Leaves	-41.7777229*	2.507282660	<.001	-48.9387511	-34.6166948
	Oak Leaves	41.1889914*	2.507282660	<.001	34.02796324	48.35001954
	Control	74.4812573*	2.507282660	<.001	67.32022915	81.64228546
Oak Leaves	Currant Leaves	-25.7264574*	2.507282660	<.001	-32.8874856	-18.5654293
	Raspberry Leaves	-82.9667143*	2.507282660	<.001	-90.1277425	-75.8056862
	Black Teat Leaves	-41.1889914*	2.507282660	<.001	-48.3500195	-34.0279632
	Control	33.2922659*	2.507282660	<.001	26.13123776	40.45329407
Control	Currant Leaves	-59.0187233*	2.507282660	<.001	-66.1797515	-51.8576952
	Raspberry Leaves	-116.258980*	2.507282660	<.001	-123.420008	-109.097952
	Black Teat Leaves	-74.4812573*	2.507282660	<.001	-81.6422855	-67.3202292
	Oak Leaves	-33.2922659*	2.507282660	<.001	-40.4532941	-26.1312378

Based on observed means.
The error term is Mean Square(Error) = 28.289.

*. The mean difference is significant at the 0.05 level.

Table 11: Aim 1 TPC in GAE Day 5 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: GAE (setting control to 0)
Tukey HSD

(I) Treatment Type	(J) Treatment Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Currant Leaves	Raspberry Leaves	-99.0401961*	1.307291959	<.001	-102.773941	-95.3064510
	Black Teat Leaves	-43.6772152*	1.307291959	<.001	-47.4109604	-39.9434701
	Oak Leaves	13.3630951*	1.307291959	<.001	9.629349931	17.09684027
	Control	77.7914837*	1.307291959	<.001	74.05773852	81.52522886
Raspberry Leaves	Currant Leaves	99.0401961*	1.307291959	<.001	95.30645095	102.7739413
	Black Teat Leaves	55.3629809*	1.307291959	<.001	51.62923570	59.09672605
	Oak Leaves	112.403291*	1.307291959	<.001	108.6695461	116.1370364
	Control	176.831680*	1.307291959	<.001	173.0979346	180.5654250
Black Teat Leaves	Currant Leaves	43.6772152*	1.307291959	<.001	39.94347008	47.41096042
	Raspberry Leaves	-55.3629809*	1.307291959	<.001	-59.0967260	-51.6292357
	Oak Leaves	57.0403104*	1.307291959	<.001	53.30656518	60.77405552
	Control	121.468699*	1.307291959	<.001	117.7349538	125.2024441
Oak Leaves	Currant Leaves	-13.3630951*	1.307291959	<.001	-17.0968403	-9.62934993
	Raspberry Leaves	-112.403291*	1.307291959	<.001	-116.137036	-108.669546
	Black Teat Leaves	-57.0403104*	1.307291959	<.001	-60.7740555	-53.3065652
	Control	64.4283886*	1.307291959	<.001	60.69464342	68.16213376
Control	Currant Leaves	-77.7914837*	1.307291959	<.001	-81.5252289	-74.0577385
	Raspberry Leaves	-176.831680*	1.307291959	<.001	-180.565425	-173.097935
	Black Teat Leaves	-121.468699*	1.307291959	<.001	-125.202444	-117.734954
	Oak Leaves	-64.4283886*	1.307291959	<.001	-68.1621338	-60.6946434

Based on observed means.
The error term is Mean Square(Error) = 7.691.

*. The mean difference is significant at the 0.05 level.

Table 12: Aim 1 TPC in GAE Day 10 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: GAE (setting control to 0)

Tukey HSD

(I) Treatment Type	(J) Treatment Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Currant Leaves	Raspberry Leaves	-90.3980615*	.9253155820	<.001	-93.0408472	-87.7552757
	Black Teat Leaves	-54.9964122*	.9253155820	<.001	-57.6391980	-52.3536264
	Oak Leaves	14.1628813*	.9253155820	<.001	11.52009557	16.80566711
	Control	82.5013360*	.9253155820	<.001	79.85855023	85.14412177
Raspberry Leaves	Currant Leaves	90.3980615*	.9253155820	<.001	87.75527570	93.04084724
	Black Teat Leaves	35.4016493*	.9253155820	<.001	32.75886352	38.04443506
	Oak Leaves	104.560943*	.9253155820	<.001	101.9181570	107.2037286
	Control	172.899397*	.9253155820	<.001	170.2566117	175.5421832
Black Teat Leaves	Currant Leaves	54.9964122*	.9253155820	<.001	52.35362641	57.63919795
	Raspberry Leaves	-35.4016493*	.9253155820	<.001	-38.0444351	-32.7588635
	Oak Leaves	69.1592935*	.9253155820	<.001	66.51650775	71.80207929
	Control	137.497748*	.9253155820	<.001	134.8549624	140.1405339
Oak Leaves	Currant Leaves	-14.1628813*	.9253155820	<.001	-16.8056671	-11.5200956
	Raspberry Leaves	-104.560943*	.9253155820	<.001	-107.203729	-101.918157
	Black Teat Leaves	-69.1592935*	.9253155820	<.001	-71.8020793	-66.5165078
	Control	68.3384547*	.9253155820	<.001	65.69566888	70.98124042
Control	Currant Leaves	-82.5013360*	.9253155820	<.001	-85.1441218	-79.8585502
	Raspberry Leaves	-172.899397*	.9253155820	<.001	-175.542183	-170.256612
	Black Teat Leaves	-137.497748*	.9253155820	<.001	-140.140534	-134.854962
	Oak Leaves	-68.3384547*	.9253155820	<.001	-70.9812404	-65.6956689

Based on observed means.

The error term is Mean Square(Error) = 3.853.

*. The mean difference is significant at the 0.05 level.

Table 13: Aim 1 TPC in GAE Day 15 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: GAE (setting control to 0)

Tukey HSD

(I) Treatment Type	(J) Treatment Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Currant Leaves	Raspberry Leaves	-87.0767269*	.7470008852	<.001	-89.2102297	-84.9432242
	Black Teat Leaves	-63.5496817*	.7470008852	<.001	-65.6831844	-61.4161790
	Oak Leaves	12.4522274*	.7470008852	<.001	10.31872472	14.58573016
	Control	80.1908424*	.7470008852	<.001	78.05733969	82.32434513
Raspberry Leaves	Currant Leaves	87.0767269*	.7470008852	<.001	84.94322422	89.21022966
	Black Teat Leaves	23.5270452*	.7470008852	<.001	21.39354252	25.66054796
	Oak Leaves	99.5289544*	.7470008852	<.001	97.39545166	101.6624571
	Control	167.267569*	.7470008852	<.001	165.1340666	169.4010721
Black Teat Leaves	Currant Leaves	63.5496817*	.7470008852	<.001	61.41617898	65.68318442
	Raspberry Leaves	-23.5270452*	.7470008852	<.001	-25.6605480	-21.3935425
	Oak Leaves	76.0019091*	.7470008852	<.001	73.86840642	78.13541186
	Control	143.740524*	.7470008852	<.001	141.6070214	145.8740268
Oak Leaves	Currant Leaves	-12.4522274*	.7470008852	<.001	-14.5857302	-10.3187247
	Raspberry Leaves	-99.5289544*	.7470008852	<.001	-101.662457	-97.3954517
	Black Teat Leaves	-76.0019091*	.7470008852	<.001	-78.1354119	-73.8684064
	Control	67.7386150*	.7470008852	<.001	65.60511225	69.87211769
Control	Currant Leaves	-80.1908424*	.7470008852	<.001	-82.3243451	-78.0573397
	Raspberry Leaves	-167.267569*	.7470008852	<.001	-169.401072	-165.134067
	Black Teat Leaves	-143.740524*	.7470008852	<.001	-145.874027	-141.607021
	Oak Leaves	-67.7386150*	.7470008852	<.001	-69.8721177	-65.6051123

Based on observed means.

The error term is Mean Square(Error) = 2.511.

*. The mean difference is significant at the 0.05 level.

Table 14: Aim 1 TPC in GAE Day 30 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: GAE (setting control to 0)

Tukey HSD

(I) Treatment Type	(J) Treatment Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Currant Leaves	Raspberry Leaves	-87.6099178*	1.041929530	<.001	-90.5857636	-84.6340719
	Black Teat Leaves	-74.4689855*	1.041929530	<.001	-77.4448314	-71.4931397
	Oak Leaves	13.9073941*	1.041929530	<.001	10.93154822	16.88323992
	Control	82.1014429*	1.041929530	<.001	79.12559702	85.07728873
Raspberry Leaves	Currant Leaves	87.6099178*	1.041929530	<.001	84.63407192	90.58576362
	Black Teat Leaves	13.1409323*	1.041929530	<.001	10.16508641	16.11677811
	Oak Leaves	101.517312*	1.041929530	<.001	98.54146599	104.4931577
	Control	169.711361*	1.041929530	<.001	166.7355148	172.6872065
Black Teat Leaves	Currant Leaves	74.4689855*	1.041929530	<.001	71.49313966	77.44483136
	Raspberry Leaves	-13.1409323*	1.041929530	<.001	-16.1167781	-10.1650864
	Oak Leaves	88.3763796*	1.041929530	<.001	85.40053373	91.35222543
	Control	156.570428*	1.041929530	<.001	153.5945825	159.5462742
Oak Leaves	Currant Leaves	-13.9073941*	1.041929530	<.001	-16.8832399	-10.9315482
	Raspberry Leaves	-101.517312*	1.041929530	<.001	-104.493158	-98.5414660
	Black Teat Leaves	-88.3763796*	1.041929530	<.001	-91.3522254	-85.4005337
	Control	68.1940488*	1.041929530	<.001	65.21820295	71.16989466
Control	Currant Leaves	-82.1014429*	1.041929530	<.001	-85.0772887	-79.1255970
	Raspberry Leaves	-169.711361*	1.041929530	<.001	-172.687206	-166.735515
	Black Teat Leaves	-156.570428*	1.041929530	<.001	-159.546274	-153.594583
	Oak Leaves	-68.1940488*	1.041929530	<.001	-71.1698947	-65.2182030

Based on observed means.
The error term is Mean Square(Error) = 4.885.

*. The mean difference is significant at the 0.05 level.

Table 15:Aim 2 Day 5 AIS Descriptive Statistics

Day 5 AIS	*Raspberry Leaf*	*Positive Control*	*Negative Control*	*Ellagic Acid*
Mean	0.012225389	0.01389357	0.011951405	0.011777877
Standard Error	0.000799316	0.000305377	0.000193339	0.000151476
Median	0.012527583	0.013567134	0.011770524	0.011851852
Standard Deviation	0.002397947	0.000916132	0.000580018	0.000454428
Sample Variance	5.75015E-06	8.39298E-07	3.36421E-07	2.06505E-07
Kurtosis	4.168693	-0.327205159	-1.004265984	-1.626408326
Skewness	-1.371569436	1.014742593	-0.003337517	-0.01961838
Range	0.009073571	0.002522593	0.001762793	0.00125515
Minimum	0.00673	0.013099298	0.011034826	0.01117
Maximum	0.015803571	0.015621891	0.012797619	0.01242515
Sum	0.110028505	0.125042134	0.107562644	0.106000889
Count	9	9	9	9

Table 16: Aim 2 AIS Day 5 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: AIS
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Raspberry Leaf	Positive Control	-.001668181	.0006294784	.057	-.003373666	.0000373037
	Negative Control	.0002739846	.0006294784	.972	-.001431500	.0019794693
	Ellagic Acid	.0004475129	.0006294784	.892	-.001257972	.0021529976
Positive Control	Raspberry Leaf	.0016681810	.0006294784	.057	-.000037304	.0033736657
	Negative Control	.001942166*	.0006294784	.021	.0002366809	.0036476503
	Ellagic Acid	.002115694*	.0006294784	.010	.0004102092	.0038211786
Negative Control	Raspberry Leaf	-.000273985	.0006294784	.972	-.001979469	.0014315001
	Positive Control	-.001942166*	.0006294784	.021	-.003647650	-.000236681
	Ellagic Acid	.0001735283	.0006294784	.993	-.001531956	.0018790130
Ellagic Acid	Raspberry Leaf	-.000447513	.0006294784	.892	-.002152998	.0012579719
	Positive Control	-.002115694*	.0006294784	.010	-.003821179	-.000410209
	Negative Control	-.000173528	.0006294784	.993	-.001879013	.0015319564

Based on observed means.
The error term is Mean Square(Error) = 1.783E-6.
*. The mean difference is significant at the 0.05 level.

Table 17: Aim 2 AIS Day 15 Descriptive Statistics

Day 15 AIS	Raspberry Leaf	Positive Control	Negative Control	Ellagic Acid
Mean	0.011634361	0.01204132	0.011322463	0.010905429
Standard Error	0.000165569	0.000129966	0.00020372	0.00022949
Median	0.011787149	0.012065868	0.011441441	0.010862588
Standard Deviation	0.000496708	0.000389897	0.000611159	0.000688469
Sample Variance	2.46718E-07	1.5202E-07	3.73515E-07	4.73989E-07
Kurtosis	4.673134091	0.839365734	-0.049967488	0.533073517
Skewness	-2.025019844	0.071642621	0.14448694	0.280294931
Range	0.001632072	0.001354821	0.002032032	0.002372152
Minimum	0.01044	0.011394183	0.01035035	0.00978
Maximum	0.012072072	0.012749004	0.012382382	0.012152152
Sum	0.104709249	0.108371884	0.101902163	0.098148864
Count	9	9	9	9

Table 18: Aim 2 AIS Day 15 Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: AIS

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Raspberry Leaf	Positive Control	-.000406959	.0002631268	.423	-.001119865	.0003059463
	Negative Control	.0003118984	.0002631268	.640	-.000401007	.0010248041
	Ellagic Acid	.000728932*	.0002631268	.044	.0000160260	.0014418373
Positive Control	Raspberry Leaf	.0004069594	.0002631268	.423	-.000305946	.0011198651
	Negative Control	.000718858*	.0002631268	.048	.0000059521	.0014317635
	Ellagic Acid	.001135891*	.0002631268	<.001	.0004229854	.0018487968
Negative Control	Raspberry Leaf	-.000311898	.0002631268	.640	-.001024804	.0004010073
	Positive Control	-.000718858*	.0002631268	.048	-.001431764	-.000005952
	Ellagic Acid	.0004170333	.0002631268	.401	-.000295872	.0011299390
Ellagic Acid	Raspberry Leaf	-.000728932*	.0002631268	.044	-.001441837	-.000016026
	Positive Control	-.001135891*	.0002631268	<.001	-.001848797	-.000422985
	Negative Control	-.000417033	.0002631268	.401	-.001129939	.0002958724

Based on observed means.
The error term is Mean Square(Error) = 3.116E-7.

*. The mean difference is significant at the 0.05 level.

Table 19: Aim 2 Day 5 Hardness measurements (gf) Descriptive Statistics

Day 5	Positive Control	Negative Control	Ellagic Acid	Raspberry Leaf
Mean	2945.9	2167.1	1871.3	2106.2
Standard Error	281.9	207.2	170.1	115.5
Median	2603.0	2089.0	1882.5	2152.8
Standard Deviation	845.8	621.6	510.4	365.2
Sample Variance	715399.7	386418.7	260536.6	133358.4
Kurtosis	5.5	3.3	0.8	-1.9
Skewness	2.2	1.0	-0.9	-0.2
Range	2826.0	2345.5	1662.0	946.5
Minimum	2199.0	1182.0	845.0	1604.0
Maximum	5025.0	3527.5	2507.0	2550.5
Sum	26513.5	19503.5	16841.5	21061.5
Count	9	9	9	10

Table 20: Aim 2 Day 5 Hardness measurements Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: Hardness 1
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Raspberry Leaf	Positive Control	-839.794*	278.2111	.024	-1592.341	-87.248
	Negative Control	-60.906	278.2111	.996	-813.452	691.641
	Ellagic Acid	234.872	278.2111	.833	-517.674	987.419
Positive Control	Raspberry Leaf	839.794*	278.2111	.024	87.248	1592.341
	Negative Control	778.889*	285.4386	.047	6.792	1550.985
	Ellagic Acid	1074.667*	285.4386	.003	302.570	1846.763
Negative Control	Raspberry Leaf	60.906	278.2111	.996	-691.641	813.452
	Positive Control	-778.889*	285.4386	.047	-1550.985	-6.792
	Ellagic Acid	295.778	285.4386	.730	-476.319	1067.874
Ellagic Acid	Raspberry Leaf	-234.872	278.2111	.833	-987.419	517.674
	Positive Control	-1074.667*	285.4386	.003	-1846.763	-302.570
	Negative Control	-295.778	285.4386	.730	-1067.874	476.319

Based on observed means.
The error term is Mean Square(Error) = 366638.349.

*. The mean difference is significant at the 0.05 level.

Table 22: Aim 2 Day 15 Hardness measurements (gf) Descriptive Statistics

Mean	3691.3	2622.6	2665.2	3474.7
Standard Error	130.5	258.1	263.0	137.1
Median	3800.0	2861.0	2761.5	3554.0
Standard Deviation	391.4	774.4	789.1	411.2
Sample Variance	153220.8	599713.9	622675.1	169044.6
Kurtosis	-0.3	1.2	-0.5	-1.0
Skewness	-0.5	-1.1	-0.6	-0.7
Range	1243.0	2545.5	2315.0	1074.0
Minimum	2983.5	1026.0	1400.5	2842.0
Maximum	4226.5	3571.5	3715.5	3916.0
Sum	33221.5	23603.0	23987.0	31272.5
Count	9	9	9	9

Table 21: Aim 2 Day 15 Hardness measurements Tukey Multiple Comparisons

Multiple Comparisons

Dependent Variable: Hardness 1

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Raspberry Leaf	Positive Control	-216.556	292.9405	.881	-1010.237	577.126
	Negative Control	852.167*	292.9405	.032	58.485	1645.848
	Ellagic Acid	809.500*	292.9405	.044	15.818	1603.182
Positive Control	Raspberry Leaf	216.556	292.9405	.881	-577.126	1010.237
	Negative Control	1068.722*	292.9405	.005	275.040	1862.404
	Ellagic Acid	1026.056*	292.9405	.007	232.374	1819.737
Negative Control	Raspberry Leaf	-852.167*	292.9405	.032	-1645.848	-58.485
	Positive Control	-1068.722*	292.9405	.005	-1862.404	-275.040
	Ellagic Acid	-42.667	292.9405	.999	-836.348	751.015
Ellagic Acid	Raspberry Leaf	-809.500*	292.9405	.044	-1603.182	-15.818
	Positive Control	-1026.056*	292.9405	.007	-1819.737	-232.374
	Negative Control	42.667	292.9405	.999	-751.015	836.348

Based on observed means.
The error term is Mean Square(Error) = 386163.622.
*. The mean difference is significant at the .05 level.

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