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EXTRACTABLE AND NON-EXTRACTABLE POLYPHENOLS FROM APPLES:
POTENTIAL ANTI-INFLAMMATORY AGENTS

A Thesis presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2017

Food Science

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Approved as to style and content by:

_____
Hang Xiao, Chair

_____
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                              _____
                              Eric A. Decker, Member and Department
                              Head

DEDICATION

To my mother, for constantly supporting me and allowing the opportunity for me to receive a higher education.

ABSTRACT

EXTRACTABLE AND NON-EXTRACTABLE POLYPHENOLS FROM APPLES:

POTENTIAL ANTI-INFLAMMATORY AGENTS

SEPTEMBER 2017

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With diet being such a huge factor in the development of diseases, emerging research has supported that apple consumption is a promising candidate for disease prevention due to the high phenolic content it possesses. These polyphenols can be found in two forms: extractable polyphenols (EP) and non-extractable polyphenols (NEP). Polyphenols have been shown to have strong anti-inflammatory and anti-oxidant properties, but up until this point, most researchers focus on EP fractions, while NEP are neglected. After the EP extraction using acetone and acetic acid (99:1) from the Apple Boost powder, three additional extraction methods were conducted on the remaining powder residue to extract the NEP. These extractions put the residue in three different environments for hydrolysis to compare their extraction abilities: enzyme, alkaline, and acid. After analyzing the EP and NEP total phenolic content (TPC) levels, oxygen radical absorbance capacity (ORAC) assay was conducted to measure anti-oxidation capacity of each extraction, and in vitro anti-inflammatory assay was performed to evaluate the anti-inflammation

capacity of each extraction where inflammation was induced by LPS. The results showed that the NEP obtained from acid hydrolysis had the highest readings in both the TPC and ORAC assay, but did not show any anti-inflammatory effects in vitro. The EP extraction had the second highest readings in the TPC, ORAC and anti-inflammatory assays. The NEP enzyme extraction had the second lowest TPC and ORAC assay performance, but highest performance in the anti-inflammatory assay. The NEP alkaline extraction had the lowest TPC and performed poorly in both the anti-inflammatory assay and ORAC assay.

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CHAPTER 1

INTRODUCTION

Chronic diseases are the leading cause of death and disability for Americans, responsible for 7 of 10 deaths each year, and accounting for 86% of our nation's health care costs, according to the National Center for Chronic Disease Prevention and Health Promotion (CDC, 2017). Chronic diseases are among the most common, costly and preventable health problems Americans face, with some of the most common types being heart disease, stroke, type 2 diabetes, obesity, and cancer (CDC, 2017). About half of all adults, amounting to roughly 117 million people, have one or more chronic diseases while a quarter of adults have two or more chronic health conditions (CDC, 2017). Risk factors for developing chronic diseases include poor lifestyle choices such as low physical activity, smoking and tobacco use, excessive alcohol use, and dietary intake. Dietary intake has the strongest influence on obesity, diabetes, cardiovascular disease, and cancer development.

The apple has been discussed as a potential candidate in the fight against chronic diseases due to the polyphenols it possesses. Polyphenols are a naturally occurring compound in apples that have recently been found to be equally as effective as antioxidants in preventing free radicals from damaging cells in the body. This is primarily due to their ability to retard oxidation of low density lipoproteins and scavenge reactive oxygen species (Rana, 2016; Boyer and Liu, 2004). Secondary metabolites such as flavonoids, isoflavanoids, carotenoids, phenolic acids and dietary fiber are also helpful in

reducing oxidative stress in the body. Oxidative and inflammatory stress are two strong influences on the development of multiple chronic diseases.

When comparing apples to many other commonly consumed fruits in the United States, apples are seen to express the second highest levels of antioxidant activity and highest total concentration of phenolic compounds (Boyer and Liu, 2004). Additionally, apples have the highest portion of free phenolics when compared to other fruits (Boyer and Liu, 2004). This means that these compounds are not bound to other compounds in the fruits, and the phenolics may be more available for absorption into the bloodstream after consumption.

Whole foods, specifically fruits and vegetables, have been estimated to contain anywhere between 5,000 to 25,000 individual phytochemical species (Acosta-Estrada et al, 2014). Within varieties of apples, the structure of polyphenols is almost identical, but differ in concentration depending on the cultivar, maturity of fruit, conditions of cultivation, harvest, storage and infections (Kalinowska, 2014). Chlorogenic acid, the main phenolic acid present in the apple, can "scavenge" free radicals (Kalinowska, 2014; Wojdylo et al, 2008). Compared to eighteen other antioxidant compounds, including quercetin, gallic acid and α-tocopherol, chlorogenic acid was ranked second, immediately after the rutin, among antioxidants possessing the highest activity (Panzella et al, 2013).

The extractable polyphenol and non-extractable polyphenol have considerable differences in their bioavailability and movement through the digestive tract in the body. The extractable polyphenol, which is known as the most studied form of polyphenol, can be extracted by organic solvents such as acetone or methanol directly from a food matrix (Saura-Calixto et al, 2007). This is not the same with the non-extractable polyphenol. The

NEP must undergo a form of hydrolysis before being able to be extracted with an organic

solvent (Saura-Calixto et al, 2007). The most common examples of this hydrolysis are

enzyme, alkaline and acid hydrolysis. These differences lead to variances in digestion.

The EP will be subjected to absorption and metabolism in the stomach and small

intestine, while the NEP will remain almost fully intact until reaching the colon (Perez-

Jimenez et al, 2013). At this point, when the NEP is exposed to the colon and gut

microbiota, the NEP will be subjected to variations of fermentation, absorption and

metabolism (Perez-Jimenez et al, 2013). Noting these differences in how these

polyphenols are obtained and how they move through the body and become active is

crucial in understanding their ability to fight chronic diseases and their progression.

CHAPTER 2

MATERIALS AND METHODS

a. Materials and Chemicals

Apple Boost apple powder was used as the test product for all the experiments conducted. This apple peel powder is labelled as both an organic and non-GMO product, and comes from Leahy Orchards Inc., a family owned and operated apple orchard in Franklin Centre, Quebec. Leahy Orchards selected their highest quality apples from orchards in Quebec, Ontario and New York state. All Apple Boost powder containers were stored in a freezer at -20°C while the extractions were conducted.

b. Extractable Polyphenol (EP) Method

For the EP extraction, 350 mL of acetone/acetic acid (99:1) was used as the solvent to soak 50 grams of powder and extract the extractable polyphenols. This extraction was sonicated for 20 minutes and centrifuged for 10 minutes at 4,000 rpm and a temperature of 10°C. The solvent was removed and the residue was extracted two more times. After this, the supernatants were pooled. To remove the organic solvent from the final extraction, the extract was evaporated at 37°C. The remaining concentrated supernatant was left out over night to dry in the hood. The EP fraction was collected and stored in -20° for further studies.

c. Non-Extractable Polyphenol (NEP) Enzyme Hydrolysis Method

For the NEP enzyme hydrolysis, the remaining residue post-EP extraction was treated with viscozyme (2 U/mL) and pectinase (50 U/mL) for 12 hours at 50°C. The ratio of the sample to water was 1:20 g/mL. After this hydrolysis, the sample was freeze-

dried and extracted with ethyl acetate three times. After the third ethyl acetate wash, all three ethyl acetate washes were evaporated to reveal the NEP enzyme-fraction. The remaining concentrated supernatant was left out over night to dry in the hood. The NEP enzyme hydrolysis fraction was collected and stored in -20° for further studies.

d. Non-Extractable Polyphenol (NEP) Alkaline Hydrolysis Method

For the NEP alkaline hydrolysis, the residue post-EP extraction was treated with 2M NaOH (1:4 m/v) to induce an alkaline environment. Then, nitrogen gas was sprayed over the residue to remove oxygen and the residue was hydrolyzed at 37°C for 1 hour in a water bath. Next, the pH was adjusted to 2 with 6 M HCL. The residue was then washed three times with ethyl acetate. After the last ethyl acetate wash, all three ethyl acetate washes were evaporated to reveal the NEP alkaline-fraction. The remaining concentrated supernatant was left out over night to dry in the hood. The NEP alkaline hydrolysis fraction was collected and stored in -20° for further studies.

e. Non-Extractable Polyphenol (NEP) Acid Hydrolysis Method

For the NEP acid hydrolysis, the residue post-EP extraction was treated with 2 M HCL (1:8 m/v), flushed with nitrogen gas to remove oxygen and heated at 80°C for 1 hour. The pH was then adjusted to 2 with 6M NaOH. The residue was then washed three times with ethyl acetate. After the last ethyl acetate wash, all three ethyl acetate washes were evaporated to reveal the NEP acid-fraction. The remaining concentrated supernatant was left out over night to dry in the hood. The NEP acid hydrolysis fraction was collected and stored in -20° for further studies.

f. Determination of Total Phenolic Content (TPC)

The total phenolic content test uses the polyphenol gallic acid as a standard for comparison. For the total phenolic content test, standards were prepared at the concentrations 0, 6.25, 12.5, 25, 50, 100, and 200 µg/mL of gallic acid and placed in a 96-well plate with six replicates per standard concentration. The extractable polyphenol and three non-extractable polyphenol extractions were prepared in 50% methanol at a dilution of 0.4 µg/mL. The experiment began by adding 20 µL of each specific polyphenol extraction sample, 20 µL water and 20 µL of Folin's reagent. After waiting 10 minutes, 140 µL of 7% Na_2CO_3 was added to each well. After an additional 90 minutes, the plate was read in the Synergy 2 Multi-mode BioTek reader at an absorbance of 760 nm.

g. Determination of Anti-Oxidant Capacity

The Oxygen Radical Absorbance Capacity Assay (ORAC) was used to determine the anti-oxidant capacities of the EP and all three NEP extractions. This assay uses trolox as a standard for comparison. Trolox is a cell permeable, water soluble derivative of vitamin E and has potent antioxidant properties (Dictionary of Food and Nutrition, 2005). This standard was created at multiple concentration levels (0, 6.25, 12.5, 15, 50, 100 µg/mL) and seeded at 20 µL per well in a 96-well fluorescence plate. The polyphenol extract samples were also added at 20 µL per well in the remaining wells within the plate at a dilution of 0.2 µg/mL. After this, fluorescein sodium salt was added at a concentration of 75 µM at a volume of 40 µL per well. The plate was then shaken for 3 minutes, and 2,2'-Azobis(2-amidinopropane) dihydrochloride, also known as AAPH, was added at a volume of 140 µL per well. This chemical compound is commonly used to study the chemistry of oxidation and is a free radical-generating azo compound (Betigeri,

2005). After the addition of these chemicals, the plate was read in the Synergy 2 Multi-mode BioTek reader at an absorbance of 485 nm for excitation and 528 for emission and data collection.

h. Determination of Anti-Inflammation Capacity

To investigate the anti-inflammatory effects of EP and NEP, their toxicity was first tested through a cell viability assay. Neither the EP or NEP fractions showed cellular toxicity at the concentration range of 50 to 300 μg/mL. After establishing the nontoxic concentration range of 50 to 300 μg/mL, the effects of the EP and NEP fractions were compared to NO production induced by LPS (1μg/mL) in macrophages.

The experiment began with cell seeding. The Raw 264.7 cells were seeded at a volume of 200 μL per well in a 96-well plate at a concentration of 50 x 10^4, using RPMI + Hepes as the media. After 24 hours in the incubator, the media was removed from the plate and new media was added containing 3 mL of fresh media, multiple concentrations of specific polyphenol extract ranging from 50 to 300 μg/mL and 3 μL of lipopolysaccharide (LPS), which was used to induce inflammation in the cells. After these treatments rested for an additional 24 hours in the incubator, the plate was removed and 150 μL was removed from each well of the plate into a fresh blank corresponding plate. The original plate then had the remaining 50 μL removed, and was treated with 100 μL of MTT solution per well. This solution was a mixture of RPMI + Hepes Media with MTT powder at a concentration of 0.1 μg/mL. This plate was placed back in the incubator for 1-2 hours to allow the MTT treatment to reveal the cell vitality post treatment colorimetrically. When the plate receiving the MTT treatment had changed to a dark purple color, after roughly 1-2 hours, the media was removed and the plate received

100 μL of DMSO in each well before being read in the Synergy 2 Multi-mode BioTek reader at an absorbance of 570 nm. The fresh plate, now with 150 μL of solution from the first plate, was treated with 100 μL of two reagents, A and B at a 1:1 ratio. Reagent A was composed of 100 mL of double distilled water, 2 grams of sulfanilamide and 2.77 mL of 85% phosphoric acid. Reagent B was 100 mL double distilled water and 0.2 grams N-1-naphthylethylenediamine. This plate was read in the Synergy 2 Multi-mode BioTek reader at an absorbance of 540 nm.

CHAPTER 3

RESULTS AND DISCUSSION

a. Total Phenolic Content

The total phenolic content test revealed the phenolic content levels of the EP, NEP enzyme, NEP alkaline and NEP acid for comparison. These results were crucial in supporting that the NEP has untapped potential in disease prevention research. Table 1 shows each fraction and their respective total phenolic content value.

Table 1: Total Phenolic Content (mg GAE/100g powder)

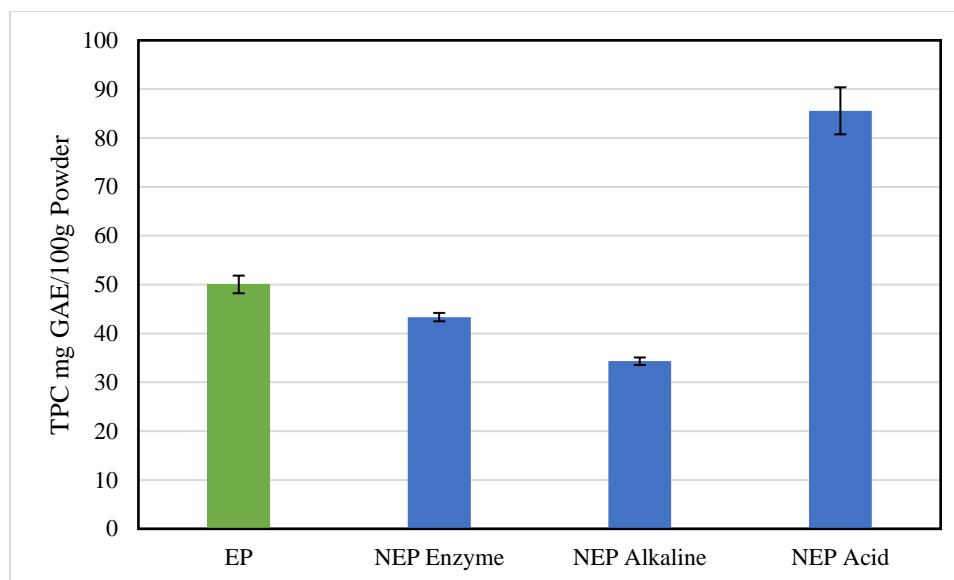| Fraction	Content (mg GAE/100g powder)
EP	50.03 ± 1.80
NEP Enzyme	43.34 ± 0.86
NEP Alkaline	34.31 ± 0.77
NEP Acid	85.57 ± 1.60

Figure 1: Total Phenolic Content of EP and NEP Fractions

The NEP acid-fraction had the highest total phenolic content with a value of 85.57± 1.60 mg GAE/100 grams powder (Table 1, Figure 1). This result was unexpected, as the extractable polyphenol fraction is the most widely studied type of extract and is known to show higher phenolic content. This fraction was predicted to have the highest phenolic content, but had the second highest TPC with a value of 50.03 ± 1.80 mg GAE/100 grams powder. The values of the last two NEP extractions were close to that of the EP extraction, with a NEP enzyme TPC value of 43.34 ± 0.86 mg GAE/100 grams powder and a NEP alkaline TPC value of 34.31 ± 0.77 mg GAE/100 grams powder. These results showed that this secondary extraction did in fact yield more phenolic content from the extract and these findings could be applied to further disease prevention research.

b. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC Assay was used to determine each polyphenol extract's anti-oxidant capacity. The table below compares the EP, NEP enzyme, NEP alkaline and NEP acid extractions anti-oxidant capacity values.

Table 2:Oxygen Radical Absorbance Capacity µmol TE/gram powder

Fraction	Content (µmol TE/gram powder)
EP	10.84 ± 0.54
NEP Enzyme	3.64 ± 0.17
NEP Alkaline	2.56 ± 0.78
NEP Acid	13.20 ± 0.64
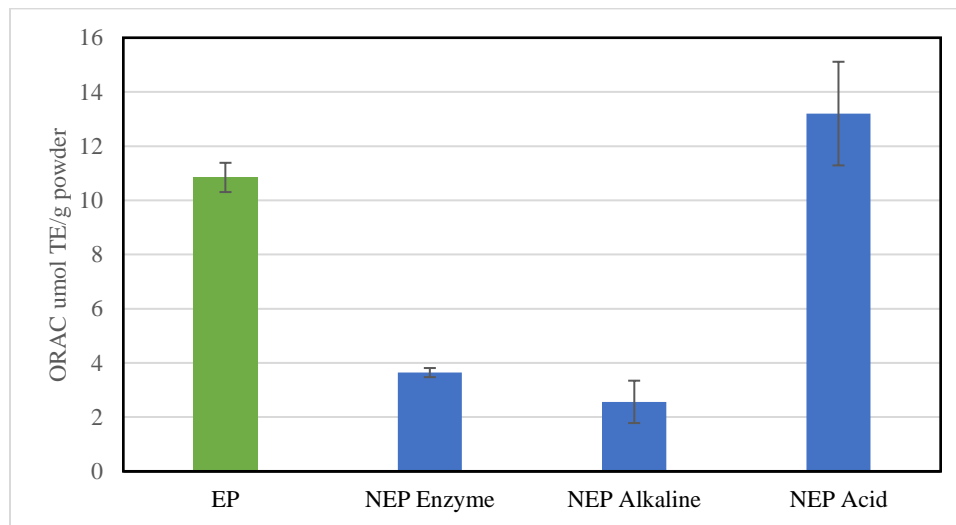
Figure 2: Oxygen Radical Absorbance Capacity µmol TE/gram Powder
TE = Trolox Equivalent

Table 2 and Figure 2 both show that the NEP acid extract had the highest oxygen radical absorbance capacity of the four extracts with a value of 13.20 ± 0.64 µmol TE/gram powder. This aligns with the total phenolic content reading where the NEP acid extract also had the highest value. The EP has the second highest reading with a value of

10.84 ± 0.54 µmol TE/gram powder, which also correlates with the previous TPC findings. The last two types of NEP extracts also have oxygen radical absorbance capacity values that are lower, yet still prevalent. This supports the hypothesis that the non-extractable polyphenol can be utilized in the fight against disease prevention through its anti-oxidant abilities under the right conditions and treatments.

c. Anti-Inflammation Assay

The anti-inflammation assay was vital in understanding the in vitro anti-inflammatory effects of all four polyphenol extracts. An insight on in vitro studies can be applied to real life dietary benefits, which supports the focus of disease prevention through further antioxidant and anti-inflammatory research of the extractable and non-extractable polyphenol. The chart below shows each extract at concentrations of 50, 100, 200 and 300 µg/mL, and a positive and negative control value for comparison.
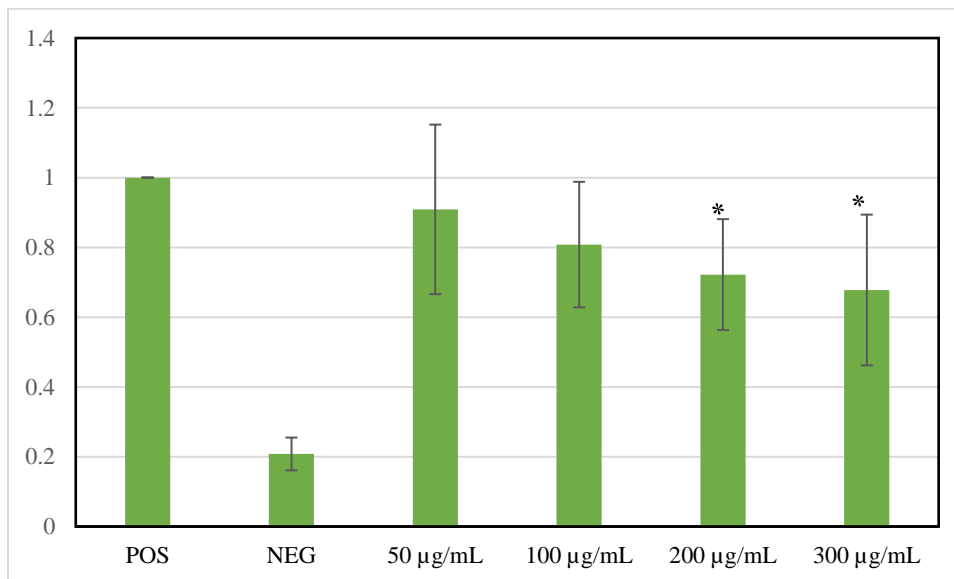

Figure 3: Anti-inflammatory Effects of EP Fractions

(The stars in Figure 3 represent that these values are statistically significant when compared to the positive control, with a p value <0.05)
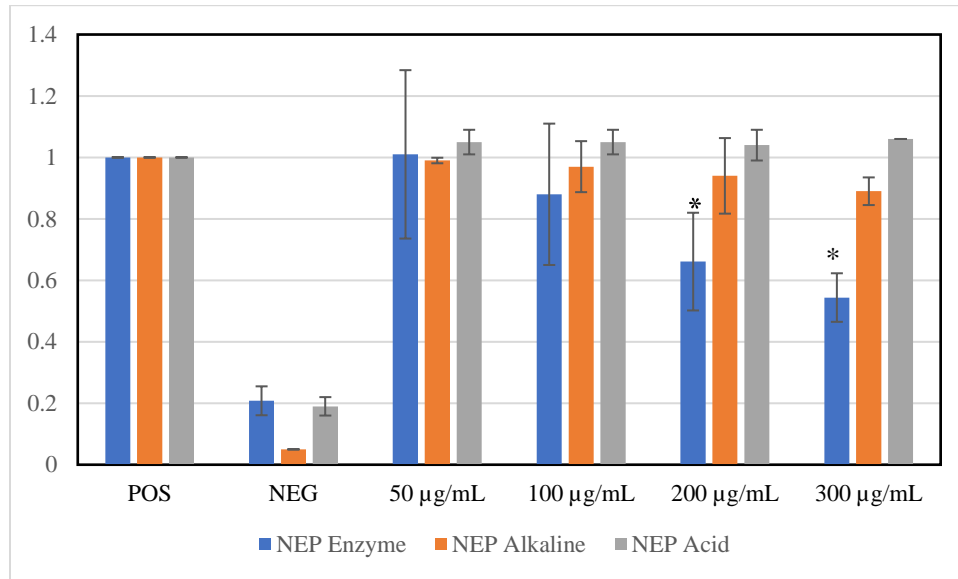

Figure 4: Anti-inflammatory Effects of NEP Fractions

(The stars in Figure 4 represent that these values are statistically significant when compared to the positive control, with a p value <0.05)

The positive control in this assay was strictly 3 μL of the inflammatory inducing agent (LPS) with 3 mL of RPMI + Hepes media and no polyphenol extract. This control shows the inflammation capacity of the cells, which is shown as a value of one. The negative control receives 3 μL DMSO with 3 mL RPMI + Hepes media and no inflammatory agent.

As Figure 3 and 4 show, the increase in sample concentration should correlate to show a lower nitric oxide (NO) percentage. A lower standardized reading shows that the cells endured less inflammatory stress with the phenolic treatment present, which can be used to suggest that these phenolic extracts can protect against inflammation in vitro. The EP and NEP enzyme extractions both show a strong negative relationship between

increased sample concentration and inflammation protection to the cells. This can be seen in the downward trend in NO% value as the sample concentration increases. The EP and NEP enzyme extraction also show statistically significant anti-inflammatory effects at the concentrations of 200 and 300 μg/mL when compared to the positive control, meaning that they were found to have a p value < 0.05. These results merit further research into the potential of the non-extractable polyphenols.

The NEP Enzyme extraction showed a strong inverse relationship between the NO percentage and increase in concentration levels of polyphenol extract. Additionally, the higher concentrations of 200 and 300 μg/mL were found to be statistically significant when compared to the positive control. It is important to understand that for each specific hydrolysis method, specific fibers and bonds within the residue will be broken or degraded, which will release a variety of different polyphenols and other active compounds or reducing agents. These differences in what is released during each extraction process is important in understanding the functionality and overall bioactivities of each extraction. For this fraction, pectinase and viscozyme broke down plant cell walls and specific fibers within the residue. The process of this hydrolysis was the slowest and most gentle extraction, which could possibly explain why this extraction had such low results in the TPC and ORAC assays, yet yielded strong results in vitro for anti-inflammation capacity. HPLC-MS analysis would be crucial in understanding which exact compounds were or were not released by this hydrolysis process and then their bioactivities could be analyzed.

The NEP alkaline extraction follows the same downward trend, but was barely effective in comparison to the EP and NEP enzyme extracts in protecting the cells against

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inflammatory damage. Research has shown that certain phenolics such as caffeic, chlorogenic and gallic acids are not stable at high pH levels and that the damages these pH levels cause to these phenolics are irreversible after a certain period of time (Friedman and Jurgens, 2000). These changes occur in these phenolics due to their structural features. Friedman and Jurgens suggest that the phenolic -OH groups on the caffeic, chlorogenic and gallic acid compounds are responsible for the observed spectral changes and their instability. Other compounds, such as catechin, epigallocatechin, and rutin also have phenolic -OH groups, but their multiring aromatic structures were much more complex than the monoring phenolic compound, which is believed to reduce the susceptibility these compounds face to the effects of pH. This specific extract was treated with 2 M sodium hydroxide for one hour during hydrolysis, a strong base, which could have led to severe irreversible damage to these phenolic compounds. This is a potential explanation to why these phenolics are detected in the TPC test, but lose their functionality in the cellular-based assay. The fact that these changes are irreversible also can potentially explain why even after the pH correction to a value of 2 before the ethyl acetate wash, the phenolics were unable to regain their functionality. Performing HPLC-MS on this extract would be crucial in future research studies to know exactly which phenolic acids are present and understand their stability conditions under certain pH levels.

Additionally, the NEP acid extract showed no anti-inflammatory effects in vitro for this assay. This can be seen in the graph above by the values of each sample concentration remaining consistent around the positive control value of one. This result was unexpected due to the high performance that was seen in the TPC and ORAC assays.

It is important to remember that this extract was treated in an extremely acidic environment for 1 hour before being adjusted to a pH level of 2, where it would remain for further experimentation. Although phenolic compounds remain stable in acidic environments, this does not mean that other compounds will also remain stable. There is a possibility that due to the acidity of the environment of this hydrolysis that more of the residue suffered degradation than the other NEP hydrolysis methods, which could have led to a release of a wider variety of polyphenols or additional reducing agents. If any additional reducing agents were released during this hydrolysis process, there is also a possibility that the chemical Folin's reagent used in the total phenolic content test could have detected these reducing agents and recognized them as phenolic content although they were not. This chemical is known for detecting all reducing agents, not just polyphenols and the possibility of false positives in the overall reading could be a potential explanation for why the TPC level for this fraction was so incredibly high. The only way that these reducing agents and other potential compounds can be recognized would be through HPLC-MS analysis, and after they are detected they can be studied and their biological activities can be understood.

d. Conclusion

The results of the total phenolic content reading, anti-inflammation assay and oxygen radical absorbance capacity (ORAC) assay show tremendous potential regarding the disease-fighting abilities of non-extractable polyphenols. The EP extraction had the second highest performance in all three experiments conducted, which supports that this extract has been consistent with previous research findings, but the anti-inflammation

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results have shown that there is potential that was previously unseen for the NEP extractions.

Out of each of the NEP extractions, the NEP enzyme extraction had the most meaningful results. Although it performed at the third lowest for both the TPC reading and ORAC assay, it showed the strongest anti-inflammation capacity at the concentrations of 200 and 300 μg/mL in vitro in the anti-inflammation assay. These cellular-based assay results are very meaningful when realistically considering the NEP in the diet for human consumption. The success of the NEP enzyme extraction in the cellular-based assay could be due to the fact that this extract did not receive any excessive pH altercation and strictly received enzymatic treatment to the residue during the extraction process.

While the NEP alkaline extraction performed poorly in the TPC reading and both the anti-oxidant ORAC assay and anti-inflammation assay, the NEP acid extraction had the highest TPC and ORAC assay values, but no noticeable anti-inflammation assay performance. The NEP alkaline extraction had most likely lost phenolic function and content due to the basic pH treatment it underwent during the extraction process. Multiple phenolic compounds are not stable in high pH environments, and this is a potential explanation for why this extraction had the lowest TPC value, ORAC assay results and very poor anti-inflammation capacity performance.

The results regarding the NEP acid extraction were hard to comprehend, as this extract seemed to have promising results for the anti-inflammation cellular-based assay due to its top TPC and ORAC assay performances, but did not show any anti-inflammation capacity in vitro. It is important to remember that this hydrolysis was

incredibly acidic, and this acidic environment could have allowed additional compounds to be released that were not polyphenols. This possibility could also have led to a false positive reading in the TPC detection, which could have been misleading in the overall data collection.

The non-extractable polyphenol is rarely studied, however with further research it has the potential to be utilized as an anti-inflammatory and anti-oxidant agent in disease prevention studies. For future research studies, HPLC-MS results on each extract will allow researchers to understand the exact compounds present in each extract, their functionality levels and how resistant they are to pH changes. Once these compounds are analyzed, experiments can be manipulated to put these compounds in their ideal environments to see if these conditions can be mimicked in the human body for disease prevention.

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