Ex Vitro Digestion Study of *Artemisia annua* as a Whole Plant Treatment for Malaria

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Abstract

While many artemisinin (AN) based combination therapies (ACTs) have long been effective in treating malaria, resistance has emerged driving the need for new treatment and prevention strategies. AN is produced by the plant *Artemisia annua* and recent studies suggested that the orally delivered dried leaves of the whole plant (pACT) that also contains AN synergistic flavonoids (FLVs) may be a more effective malaria treatment. In this study, an *ex vitro* digestion system was used to simulate the digestion of pACT to gain insight into how AN and synergistic FLVs become bioavailable as the pACT moves through the digestive system. Various delivery methods (e.g. capsules) and staple foods were combined with pACT and digested in order to investigate their impact on the bioavailability of AN and FLVs. The digested material was collected at the end of the oral, gastric and intestinal stages, filtered into solid and liquid fractions, and extracted for AN and FLV quantification. The intestinal liquid fraction was expected to contain high levels of AN and FLVs in order to be available for absorption into the bloodstream. This study found that compared to pACT alone, sucrose, canola oil, and white rice did not reduce the amount of AN released in the intestinal liquid fraction while the two types of capsules that were tested showed a significant reduction in AN release. The sucrose and canola oil pACT combinations also exhibited significantly greater FLV release than from pACT alone. These results have improved our understanding of how FLVs and AN are affected and released at different stages of digestion and will aid the development of pACT as a therapeutic.

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1. Introduction

In 2011, there were roughly 215 million cases of malaria, mostly among African children, and an estimated 655,000 deaths in 2010 (World Health Organization [WHO] 2010). In 2012, 3.3 billion people, or half of the world population, were at risk of contracting malaria (WHO 2012b). The key drug fraction in malaria treatment is artemisinin (AN) (Figure 1), which is produced and stored in glandular secretory trichomes in the leaves of *Artemisia annua.*

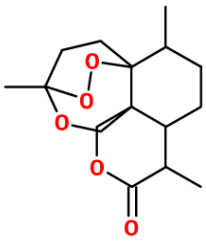


Figure 1: The structure of artemisinin.

Currently, AN based combination therapy (ACT) is the best available treatment for *Plasmodium falciparum* malaria. Malaria treatments contain AN in combination with an older antimalarial drug to prevent AN drug resistance from emerging. While ACTs are an effective treatment, they are expensive and unattainable to many suffering from malaria in developing countries. Resistance to antimalarial medications has undermined malaria control efforts and continues to be a threat (WHO 2010). Emerging research on *A. annua* whole plant treatment suggests that it may be an effective solution for the treatment of malaria (Weathers *et al*., 2011; Elfawal *et al.*, 2012). Whole plant treatment, however, is still being investigated and is not a currently approved treatment for malaria but it may overcome some of the obstacles associated with current malaria treatment such as affordability, AN drug resistance, and accessibility.

A. annua contains flavonoids (FLVs) that act synergistically with AN (Liu *et al.*, 1992). This can increase the potency of AN, lowering the required dosage for treatment, which can decrease negative side effects caused by the drug. Mouse studies have shown that the dried plant provides more bioavailability of AN than the pure drug (Weathers *et al.*, 2011) and is more effective than the pure drug in reducing parasitemia (Elfawal *et al.*, 2012). Bioavailability in particular can be validated with a better understanding of the progression of the drug through the individual stages of the human digestive system.

Variables such as the drug capsule type and the presence of specific staple foods should be studied in order to determine whether they inhibit or enhance the bioavailability of AN and FLVs. Often, victims of malaria are unable to consume food or certain types of food, which limits their treatment options. For the most effective malaria treatment, the type of capsule and food necessary, in conjunction with *A. annua*, has not been studied. This study uses a stimulated *ex vitro* digestion to provide insight into what happens to the plant material as it is processed through the digestion system. It also identifies how dietary supplements and delivery methods impact the bioavailability of AN and FLVs, the key components of the proposed malaria treatment.

1 Literature Review

1.1 Malaria

Malaria is a parasitic, infectious, mosquito born disease caused by *Plasmodium*. Humans receive and pass malaria by the bite of an infected *Anopheles* mosquito. This disease is highly prevalent in tropical and subtropical environments where the climate provides optimal living and breeding conditions for mosquitoes. The WHO has supported significant research in the field of malaria prevention and treatment. Deaths due to malaria have significantly decreased from 2002 to 2012 due to ACTs, vector control, insecticides and the distribution of mosquito nets (WHO 2012b). However, there is no single effective solution in preventing the spread of malaria and a devastating number of deaths due to malaria continue to occur globally every year. Malaria is most prevalent in developing countries and areas with low income where people are more exposed to mosquito bites (WHO 2012a). Malaria treatment is unaffordable to those suffering the most from it. Malaria can be contracted by the same person multiple times so treatment can be very demanding.

There are four types of common malaria parasites: *Plasmodium falciparum, vivax, malariae*, and *ovale* (Davis and Shiel, 2010). Recently, a fifth type, *Plasmodium knowlesi*, was discovered in Malaysia and areas of Southeast Asia. More than one species of *Plasmodium* can infect a patient at the same time. Among five malaria plasmodium species, *P. knowlesi* and *P. falciparum are* the most dangerous and life threatening.

Malaria occurs mainly in tropical and subtropical climates where it is hot and humid and mosquitoes thrive. Malaria parasites can live and complete their life cycles by incubating in the mosquito body (Dugdale, 2011). The Southern Sahara and parts of Oceania, e.g. Papua New

Guinea and Southern Africa, have the highest malaria transmission rate (WHO, 2012a). While parts of Africa have a dry environment, they still have oases with scarce water pools that serve as the main water source for humans as well as a breeding ground for mosquitoes. Malaria does not propagate well in very dry environments in the desert or very cold regions such as Western Europe and the United States (WHO, 2012b).

1.1.1 Treatment and Drug Delivery

Treatment of malaria is dependent on a variety of factors including the severity of the disease, the species of the malaria parasite, the origin of the infection, and its drug resistance status (WHO, 2010). There are many AN-based anti-malarial medications and current treatments contain a derived compound, e.g. artesunate or artemether. Oral AN-based mono-therapies are only used in severe cases because of the risk of developing resistance. ACTs are expensive and therefore difficult to acquire in most developing countries. Most often they are acquired through national and donor funding organizations. Cloroquine and sulfadoxine-pyrimethamine are safe, accessible, and much more affordable (US$0.10 - $0.20), but parasites are often now resistant, which is why they are replaced by a more expensive drug such as artemether in combination with lumefantrine, known as Coartem® (WHO, 2010). ACTs cost approximately US $1.20 - $3.50 per adult course, which is still too expensive for the developing world (WHO, 2010). They are delivered orally in water soluble co-formulated tablets and co-blistered tablets. Co-formulated tablets, as noted in Table 1, have both of the drugs of the ACT in the same dosage form. Co-blistered tablets have each medicine packaged together in separate blisters so that they may be administered as a single dose or as divided doses; half the dose is taken in the morning and the other half is taken at night. Dosage is based strictly according to body weight (WHO, 2010).

Currently, whole plant treatment is not used as a treatment for malaria but it has potential in battling the issues of drug resistance, availability, and costs.

Table 1: Recommended Treatments for Malaria (WHO, 2010)

Active pharmeceutical ingredient(s) in available formulation(s)	Uncomplicated *falciparum* malaria	Used when treatment fails	Severe malaria
Artemether-lumefantrine oral	√	√*	
Artesunate + - amodiaquine oral	√		
Artesunate + mefloquine oral	√	√*	
Artesunate + solfadoxine-pyrimethamine oral	√		
Dihydroartemisinin-piperaquine oral	√	√*	
Artesunate (or quinine) combined with tetracycline or doxycycline or clindamycin, oral		√	
Artesunate, intravenous or intramuscular			√
Artemether, intramuscular			√
Quinine, intravenous or intramuscular			√
Artemotil, intramuscular			√**
Artesunate, rectal			√***
Artemisinin, rectal			√***

Abbreviations:
+ co-packaged products;
- co-formulated products;
+- co-packaged FPPs also available as co-formulated tablets, since some API-API incompatibilities have been resolved
* only if not used as first-line treatment;
** used if no alternatives is available, as few clinical trials have been conducted;
*** for patients with severe malaria before referral to a facility where complete parenteral treatment with artesunate, quinine or artemether can be administered
* for areas where amodiaquine or sulfadoxine-pyrimidine cure rate ≥ 80%

In 2011, a study conducted by Verret *et al.*, on post-response of ACTs in malaria-infected Ugandan children showed that there was a recurrence of malaria parasites in patients who previously treated with dihydroartemisinin-piperaquine (DP). The study took 292 malaria infected children, ages 4 to 12 months, and treated 145 of them with DP and the other 147 with artemether-lumefantrine (AL) for 2 years; 99 % of the subjects were able to clear all parasites by day 3. However, the DP treated children showed a significantly higher risk for recurrence of *P. falciparum* parasite 42 days post treatment. Additionally, the study demonstrated that a

trimethoprim-sulfamethoxazole prophylaxis reduced parasite recurrence in patients with mild to chronic malnutrition (Verret *et al*., 2011). Recurrence of malaria parasites in *A. annua* whole plant treatment has not yet been studied.

1.2 Whole Plant Treatment: Tea Infusion

A. annua has been used for medicinal purposes in traditional Chinese medicine dating back to 168 B.CE. It was not until 1972 that the active drug, AN, was identified as the key anti-malarial agent of *A. annua* (Efferth, 2009). A study conducted by Hsu (2006) suggests that using some of the more traditional preparation methods, pressing fresh juice from the plant, or extracting the plant in cold water, can provide a higher concentration of AN. In information from the history of Chinese medicine, Hsu also noted that soaking the fresh plant to obtain the plant's juices created water-emulsion compounds such as FLVs and ether oils (Hsu, 2006). These compounds likely enhance the amount of AN that can be extracted from the plant.

Recent studies by Carbonara *et al*. (2012) suggested that tea infusion (boiling) is an ineffective method for preparing *A. annua* because it destroys most of the AN. They prepared the tea by infusing *A. annua* for different times: 1, 24, and 48 hours. The AN content of each tea was quantified by HPLC. They also tested various extraction solvents: water, acetonitrile (less polar), and hexane (non-polar). The measured AN content of each sample was very low. Also, there was no detection of phenolic compounds (Catbonara *et al*, 2012). Therefore, they concluded that boiling is not an effective way to prepare *A. annua* tea infusion to obtain AN.

Other studies, however, have shown that tea infusion prepared with high temperatures yield increasing levels of AN, but may destroy some FLVs. van der Kooy and Verpoorte (2011) used three methods to prepare *A. annua* tea infusion: i) applied water with different temperatures from room temperature, 40, 50, 60 … 100˚C ii) used boiling water and autoclaved at 105˚C for 1, 3,

and 10 minutes and iii) added varying volumes of water to the plants and boiled for 5 minutes. According to their study, AN concentration was increased with boiling temperatures, but AN was degraded at 105˚C. By keeping the tea at 100˚C for 2 minutes, the AN extraction was 90% efficient (van der Kooy and Verpoorte, 2011).

A recent study conducted by Weathers and Towler (2012) demonstrated that two flavonoids, casticin (CAS) and artemetin (ART), are poorly extracted and unstable in *A. annua* tea infusion. They used different cultivars of *A. annua*: non-clonal *A. annua* L. (Chinese origin), SAM clone, #15 clone, and FLV5 (Brazilian origin). After infusion in boiling water the tea was extracted based on the optimal extraction method reported by van der Kooy and Verpoorte (2011) and quantified for AN, CAS and ART by GC/MS (Gas Chromatography – Mass Spectrometry) analysis. From this extraction, the measured flavonoids, CAS and ART, were very low. Also, after storage of the tea infusion at room temperature for more than 24 hours, while AN was stable, CAS and ART further decreased more than 40% (Weathers and Towler, 2012). Due to the reported synergistic properties of FLVs with AN, tea infusion does not seem to be an advantageous method for treating malaria. Conversely, a recent study conducted by Ogwang *et al*. (2011) showed that malarial patients in Kenya and Uganda who consume one or two cups of *A. annua* tea a day may develop immunity against malaria.

1.3 Whole Plant Treatment: Dried Leaf Consumption

In addition to AN, dried *A. annua* leaves contain therapeutic compounds that ACTs do not provide. In the study by Rath *et al.* (2004), AN was extracted from dried *A. annua*. L with petrol ether at 60 - 90˚C for 3 hours. They found that AN only made up 1.39% of the dried plant material. Even though dried *A. annua* contained AN content less than 1.5%, other active ingredients were present (Rath *et al*., 2004). Wilcox *et al.* (2004) pointed out that *A. annua*

contains many different classes of compounds that provide anti-malarial characteristics, such as monoterpenes, sesquiterpenes, triterpenoids, flavonoids, and aromatic compounds. Furthermore, they noted that in dried *A. annua*, AN remains stable over a long period of time. Since AN is produced and stored in glandular trichomes, which contain oil, AN should remain stable. Also, with properly dried storage methods, there should be no fungal growth.

Rath *et al.* (2004) found that *A. annua*, delivered in either the form of tea or dried leaves, created equal bioavailability of AN. They detected 240 µg/L of AN in the bloodstream after preparation of a tea from 5 g dried *A. annua*. The study suggested that tea and dried leaves provide similar bioavailability of AN, but that tea may be absorbed faster into the bloodstream. The minimum requirement of AN needed to be effective against malaria in the bloodstream is 10 µg/L (Alin and Bjorkman, 1994). Therefore, the whole plant treatment could provide more than enough AN needed for treatment.

Weathers *et al.* (2011) examined bioavailability of the AN present in the bloodstream of mice that were administered either dried *A. annua* leaves or pure AN mixed with mouse chow. They found that when the mice were fed plant material containing 31µg of AN, the maximum concentration in the blood occurred after 30 minutes, while 1,400 µg of the pure drug with mouse chow took 60 minutes to reach the same concentration in the blood. Also, 31 µg of the pure drug with mouse chow produced undetectable levels of AN in the blood. These results were consistent with the findings of Rath's experiments in which dried plant provided more serum-available AN than the pure drug.

A more recent study provided further evidence that whole plant *A. annua* can be used as an effective antimalarial therapy. Elfawal *et al.* (2012) used *Plasmodium chabaudi* infected mice in

their study and found that a low dose of dried *A. annua* leaves can kill more malaria parasites than a comparable dose of pure AN drug from 12-72 hours after treatment. The authors coined the term 'pACT' to denote whole plant *A. annua* use as a therapeutic wherein the plant contains its own endogenously produced combination drugs.

1.4 The Synergy of Artemisinin and Flavonoids

There are many cultivars of *A. annua* that contain varying concentrations of FLVs and AN. In an *A. annua* cultivar with an Italian origin, eupatin, chrysoplenetin, casticin, and artemetin were identified as the key FLVs present in the plant, however not all FLVs could be separated and validly identified (Baraldi *et al*., 2008). Ferreira (2008) found that a Brazilian cultivar had high levels of antioxidants, which indicated it contained high FLV content. Each cultivar has different types and concentrations of FLVs. However, after they quantified the amount of AN and FLVs from three cultivars of *A. annua*, FLV5 (Brazilian origin), Clone SAM, and Clone 15 by GC-MS, Weathers and Towler (2012) found that the Brazilian FLV5 cultivar does not provide the higher level of FLVs than the other *A. annua* cultivars. Table 2 shows the measured amounts of AN, ART and CAS based on the GC/MS results. According to Table 2, the FLV5 cultivar yielded the lowest amount of ART and CAS. Clone 15 yielded higher amounts of FLV levels but lower AN levels than FLV5. Among the three cultivars, Clone SAM yielded the highest levels of AN, CAS, and ART.

Table 2: AN and FLVs in Three *A. annua* cultivars (Weathers and Towler, 2012)

Cultivar	Artemisinin (mg/g DW)	Artemetin (mg/g DW)	Casticin (mg/g DW)
FLV5	6.79	0.019	0.141
Clone 15	5.71	0.080	0.211
Clone SAM	14.89	0.210	0.410

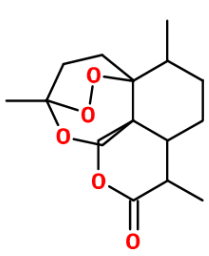
Both AN and FLVs in *A. annua* have been studied for their effectiveness in treating malaria and various types of cancer. Liu *et al.* (1992) measured the level and antimalarial efficacy of 6 types of FLVs that were found in *A. annua* L.: artemetin, casticin, chrypsoplenetin, chrysosplenol-D, cirsilineol, and eupatorin (Figure 2). They also measured the antiparasitic ability of each FLV, present with AN. The IC_{50} represents the concentration of compound required to reduce a pathogen population in an infected individual by half. AN alone produced an IC_{50} of 3.3×10^{-8}M, which was 1,000 times better than any of the studied FLVs acting alone against malaria which had an IC_{50} of $2.4\text{-}6.5 \times 10^{-5}$ M (Table 3). When these FLVs, especially chrysosplenol-D and cirsilineol, were combined with AN, the IC_{50} of AN dropped from 3.3×10^{-8} M to as low as 1.5×10^{-8} M, indicating a major increase in the potency of the drug (Table 3). These results support the potential effectiveness of whole plant treatment since FLVs are found naturally in *A. annua*.

1.5 *Ex Vitro* and *In Vivo* Digestion Studies

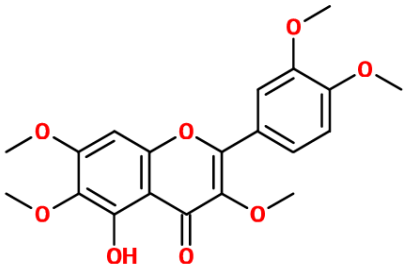
Previous studies validated the effectiveness of using a simulated *ex vitro* digestive system to mimic the breakdown of compounds in human digestion as shown, for example by Barlow *et al*. (2003) in a study on *Ginkgo* digestion. *Ginkgo biloba* is well known for its FLV content, and it was used to test the absorption of polyphenols under gastrointestinal conditions. The study used HPLC analysis to identify the resulting breakdown products and intact FLVs that were likely available for absorption into the bloodstream. After HPLC analysis, the results showed that generally glycosides were hydrolyzed to aglycones. Subsequently the aglycones were further degraded and it was suggested that further research was needed to investigate the reported benefits of Ginkgo flavonoids (Barlow *et al*., 2003).

Table 3: Antimalarial Activity of Flavonoids (Liu *et al.*, 1992)

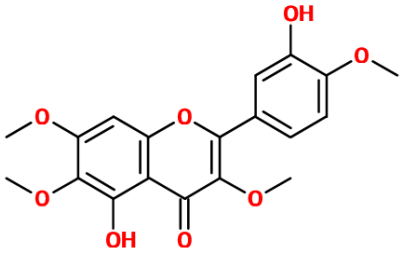
Compounds	IC_{50} of FLV alone (M)	AN IC_{50} + 5 ug of FLV(M)
Artemisinin	-	3.3×10^{-8}
Artemetin	2.6×10^{-5}	2.6×10^{-8}
Casticin	2.4×10^{-5}	2.6×10^{-8}
Chrypsoplenetin	2.3×10^{-5}	2.25×10^{-8}
Chrysosplenol-D	3.2×10^{-5}	1.5×10^{-8}
Cirsilineol	3.6×10^{-5}	1.6×10^{-8}
Eupatorin	6.5×10^{-5}	3.0×10^{-8}



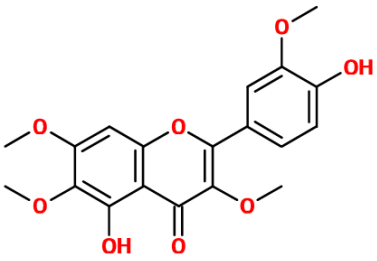
Artemisinin



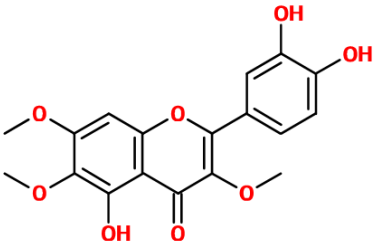
Artemetin



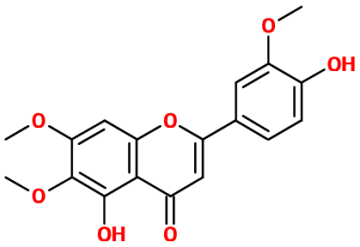
Casticin



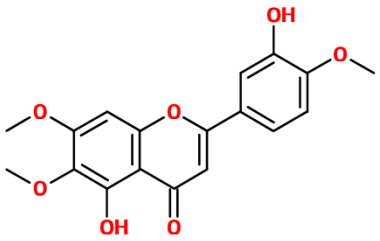
Chrysoplenetin



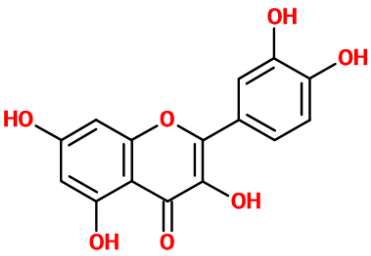
Chrysoplenol-D



Cirsiliol



Eupatorin



Quercetin

Figure 2: Structures of artemisinin and flavonoids reported to be present in *A. annua*.

A recent study by Megalhaes and his colleague (2012) found that *A. annua* infusion tea from different cultivars exhibited a potent anti-inflammatory effect, which they attributed to phenolics. The study used human intestinal Caco-2 cells to mimic an *in vivo* inflammatory environment. By ELISA quantification, they showed that the pro-inflammatory cytokines, IL-8 and IL-6, decreased inflammatory conditions in the cell lines. Using different *A. annua* cultivars of known AN content, Bra (Brazil) and Lux (Luxembourg), they demonstrated that decreased inflammation was not due to the AN in the tea, but rather to the phenolics. Additionally, by measuring cellular metabolic activity, they demonstrated that *A. annua* infusion tea does not affect toxicity of the intestinal Caco-2 cells (Magalhaes *et al*., 2012). This suggested that phenolics may have a beneficial anti-inflammatory affect in the intestine *in vivo*.

1.6 The Digestive System

Studies conducted on *A. annua* in the past have provided information on the efficacy of pACT treatment based on the concentration of the drug found in the bloodstream. More information on how this plant breaks down during the digestive process can provide insight into how endogenous therapeutic chemicals in the plant are released and passed through the digestive tract and into the bloodstream, where parasites reside after infection. The human digestive system consists of three main phases: oral, gastric, and intestinal. Each phase works in sequence to break down food and other compounds that then become available for absorption into the bloodstream during the intestinal phase.

1.6.1 Oral Phase

Human digestion begins in the mouth, known as the “buccal cavity,” where food or drugs are swallowed and sent to the esophagus. Although when swallowing an encapsulated oral drug the oral phase of the digestive system is very short, several enzymes are involved in the process.

When food, or a drug, is present in the mouth, saliva is produced and helps the food to form a bolus to aid in swallowing. Mucin is a protein of mucus that acts as a lubricant which helps to form the bolus. Uric acid and urea are naturally present with α-amylase; the latter is an enzyme present in saliva. The α-amylase catalyzes breakdown of starch into sugars. Once the bolus has formed, the tongue pushes it to the back of the mouth. Upon reaching the pharynx the bolus triggers an involuntary swallowing reflex preventing the bolus from entering the lungs and directing it down the esophagus by peristalsis where it enters the gastric phase of the digestive system (Hu and Li, 2011). The oral phase is the first place that starches begin to break down in the digestive system.

1.6.2 Gastric Phase

The gastric phase mainly hydrolyzes proteins in an acidic condition into smaller units by the work of the enzyme pepsin. The esophagus, which brings food from the oral phase to the stomach, causes mechanical stimulation of the gastric wall via distention and stretching of smooth muscle. There is a secretion of gastric juices, which include HCl, pepsin, intrinsic factor, mucus, and HCO^{-3}. Pepsinogen hydrolyzes to pepsin in order to digest proteins (Koeppen and Stanton 2010). Due to presence of HCl, the pH of gastric juice is approximately 2. The acidic condition in the stomach is critical for killing many microbes that are ingested during a meal (Blair, 1996). The stomach mainly digests proteins; neither fats nor carbohydrates are digested by pepsin.

1.6.3 Intestinal Phase

The intestinal phase digests most of the remaining ingested meal into small absorbable units by the work of various enzymes under slightly basic conditions. As the mucous-containing

food, called chyme, enters the duodenum, a signal is sent from the brain to stop acidic gastric juices produced in the stomach from passing through to the intestinal system (Koeppen and Stanton, 2010). Due to halted production of gastric juices, a slightly less acidic environment develops (Baron, 2009). This condition increases the functionality of enzymes secreted from the pancreas. Chyme stimulates pancreatic and bile secretion (Baron, 2009).

Pancreatin is composed of several enzymes secreted from the pancreas that are needed to digest vital nutrients in order to make them absorbable. Pancreatin contains amylase, lipase, and protease. Amylase helps to break down starches into glucose and lipase works by hydrolyzing lipids in the presence of bile (Koeppen and Stanton, 2010). Bile is an important alkaline that is made by the liver and stored in the gallbladder. Bile acts as an emulsifier for lipid and lipase interaction by hydrolyzing lipids into fatty acids so they can be absorbed by the duodenum part of the small intestine. Bile contains mostly cholesterol, bile acids (bile salt), and bilirubin, which is a breakdown product of red blood cells. Bile also contains water, potassium, sodium, and other metals such as copper (Dugdale, 2010). Lastly, the other important enzyme in pancreatin is protease. Protease works to digest protein by breaking down the peptide bonds between amino acids, the building blocks of proteins (Koeppen and Stanton, 2010). Amino acids are more useful and absorbable than intact proteins and thus more nutritional. By the end of the intestinal phase, digestion is completed and ingested food has been broken down into absorbable and more useful compounds.

1.7 Capsule Delivery Methods and Dietary Supplements

Capsules and food incorporated in the delivery of *A. annua* might affect the bioavailability of AN and certain FLVs. This study tests the effects of gelatin and vegetarian capsules and the staple foods millet, white rice, canola oil, cornmeal, and sugar. These staple

foods were chosen based on their affordability and availability in the countries that are suffering the most from malaria. Vegetarian capsules are plant-based and contain cellulose or plant fiber and are commonly starch, gluten, and preservative free. Gelatin capsules, on the other hand, contain denatured collagen protein derived from connective tissues of vertebrate animals (Jain and Gupta, 2008). Both types of capsules dissolve readily in the stomach.

A. annua is a very bitter tasting plant and may be difficult to ingest for patients suffering from malaria. Dietary supplements may be beneficial in making drug delivery easier, especially for children who have trouble swallowing pills. Sugar is a common staple and addition to tea that could counteract the bitterness of the plant. However, glucose concentration plays an important factor in *P. falciparum* parasite growth. *P. falciparum* converts glucose to lactate through glycolysis, which is the primary source of energy for the parasite (Humeida *et al*., 2011).

A study conducted by Fang *et al.* (2003) showed that glucose starvation represses transcription involved with asexual development of the parasite while upregulating transcription involved in sexual development of the parasite. People suffering from malaria therefore experience hypoglycemia. Even though high blood sugar levels can intensify parasitic growth, if sugar significantly increases the bioavailability of AN, sugar in small quantities may provide a net benefit.

2 Hypothesis

By using simulated digestion system this study will establish the levels of AN and FLVs that become available for absorption at the end of each of the human digestive phases: oral, gastric and intestinal.

Objectives

1. Measure the amount of AN and FLVs, from the *A. annua* pACT, present in the liquid and solid fractions at the end of each digestive phase.

2. Measure the amount of AN and FLVs, from the *A. annua* pACT combined with each palatable delivery method, present in the liquid and solid fractions at the end of each digestive phase. The palatable delivery methods include gelatin and vegetarian capsules, sugar, and canola oil.

3. Measure the amount of AN and FLVs, from the *A. annua* pACT combined with each dietary supplement, present in the liquid and solid fractions at the end of each digestive phase. The dietary supplements include meal of millet, cornmeal, and white rice.

3 Methodology

3.1 Plants

Artemisia annua L., SAM cultivar (Weathers and Towler, 2012), was field grown from rooted cuttings planted in May 2012 and harvested in mid September 2012 from either Professor Weathers garden or the small farm in Stow, MA. All plants were watered regularly and no herbicides or pesticides were used. After harvest, plants were dried under a greenhouse cover and then the dried leaves were removed and pressed through a 600 μm brass sieve. A single homogeneous batch of SAM plant material was used for the entire study.

3.2 Chemicals

The solvents CH_2Cl_2 (D37-3), H_2SO_4 (A300-212), CH_3OH (T324-1), and $CH_3COOCH_2CH_3$ (E145-1), were purchased from Fisher Scientific Company; CH_3CO_2H was purchased from EM Science Company (AX0073-9). Along with all enzymes, $C_8H_8O_2$ (*p*-anisaldehyde) was purchased from Sigma-Aldrich.

3.3 *Ex Vitro* Three Stage Digestion

The *ex vitro* digestion experimental setup in this study used the SOP provided by the Ferruzzi Lab at Purdue University and based on methods of Kean *et al*. (2011) modified from the original protocol of Garrett *et al*. (1999) (See Appendix C). This is a test tube study that simulates food processing through the three phases of the digestive system: oral, gastric, and intestinal. Since this experiment involves the ingestion of a drug, as opposed to porridge used in the Kean *et al*. (2011) protocol, the procedures were slightly modified (See Appendix C). The material was scaled down fourfold based on the mass of plant material needed and each simulation was run in triplicate.

For the oral phase, an oral base solution was prepared containing 0.1792 g potassium chloride, 0.1776 g sodium phosphate, sodium sulfate 0.1140 g, 0.0596 g sodium chloride, and 0.3388 g sodium bicarbonate in 100 mL of water. A 10 mL aliquot of the oral base solution was mixed in a beaker with 4 mg urea, 0.3 mg uric acid, and 0.5 mg mucin. A 1.5 mL aliquot of this solution was added to a 50 mL centrifuge tube containing 0.36 g of dry *A. annua* and 1.64 mL of distilled water. In the capsule delivery experiments, two capsules were used per reaction tube with each capsule containing 0.18 g of *A. annua*. In the palatable delivery methods or dietary supplements experiments, an additional 0.36 g of millet, cornmeal, or white rice meal, canola oil, or sugar was added to the reaction tube directly following the addition of 0.36 g of the *A. annua*. In the experiments containing the cereal meals, the addition of water was doubled. To each reaction tube, 46.875 mg of α-amylase was added and the tube was vortexed for 2 minutes. The tubes were blanketed with N_2 gas, capped, and then placed in a 37°C water bath and shaken at 90 rpm for 10 minutes. During the 10 minute incubation, a 10 mg/mL pepsin in 0.1 M HCl solution was prepared for the gastric phase.

The reaction tubes were removed from the bath and immediately placed on ice. A 4 mL aliquot of saline (0.9% NaCl) was added to bring the volume to 7.5 mL. Using 1M HCl, the pH of each reaction tube was adjusted to 4.0±0.1 and 0.5 mL of the pepsin solution was added. The pH was readjusted using 1M HCl to a pH of 2.5±0.1, and the volume of each reaction tube was brought to 10 mL with saline. The tubes were blanketed with N_2 gas, capped, placed in the 37°C water bath and incubated at 90 rpm for 1 hour. During this time, a 2.25 mL solution containing 30 mg/mL bile extract in 100 mM $NaHCO_3$ solution was prepared and sonicated for 30 minutes; 45 minutes into the incubation period, 1.5 mL of a pancreatin-lipase solution was prepared containing 20 mg/mL pancreatin and 10 mg/mL lipase in 100 mM $NaHCO_3$.

After the hour long gastric phase incubation the reaction tubes were removed and immediately placed on ice for preparation of the intestinal phase. The pH was adjusted to 4.0 ±0.1 using 1M $NaHCO_3$, and 0.5 mL of the pancreatin-lipase solution was added followed by 0.75 mL of the bile extract solution. The pH was then adjusted to 6.5±0.1 using 1M $NaHCO_{3,}$ and each test tube volume was brought to 12.5 mL with saline. The samples were blanketed with N_2 gas, capped, and incubated in the 37 °C water bath at 90 rpm for 2 hours for completion of the *ex vitro* digestion system.

3.4 Filtration and Extraction of Digesta (DG)

The resulting digesta (DG) from each of the oral, gastric, and intestinal phases were vortexed and filtered to separate the liquid and solid fractions. To measure the amount of AN and FLVs present in each digestive phase, four separate samples were extracted including the undigested plant material (Figure 3). For the first sample, the tubes were extracted after the oral phase, for the second sample the tubes were extracted after the gastric phase, and for the third sample the tubes were extracted after the intestinal phase was completed. The solid and liquid fractions at the end of each phase were extracted with methylene chloride and sonicated for 30 minutes in a water bath at a cool temperature. After sonication, the solid fractions were vacuum filtered and dried with N_2 gas and the methylene chloride component of the liquid fraction, which contains the extracted AN and FLVs, was isolated and dried with N_2 gas.

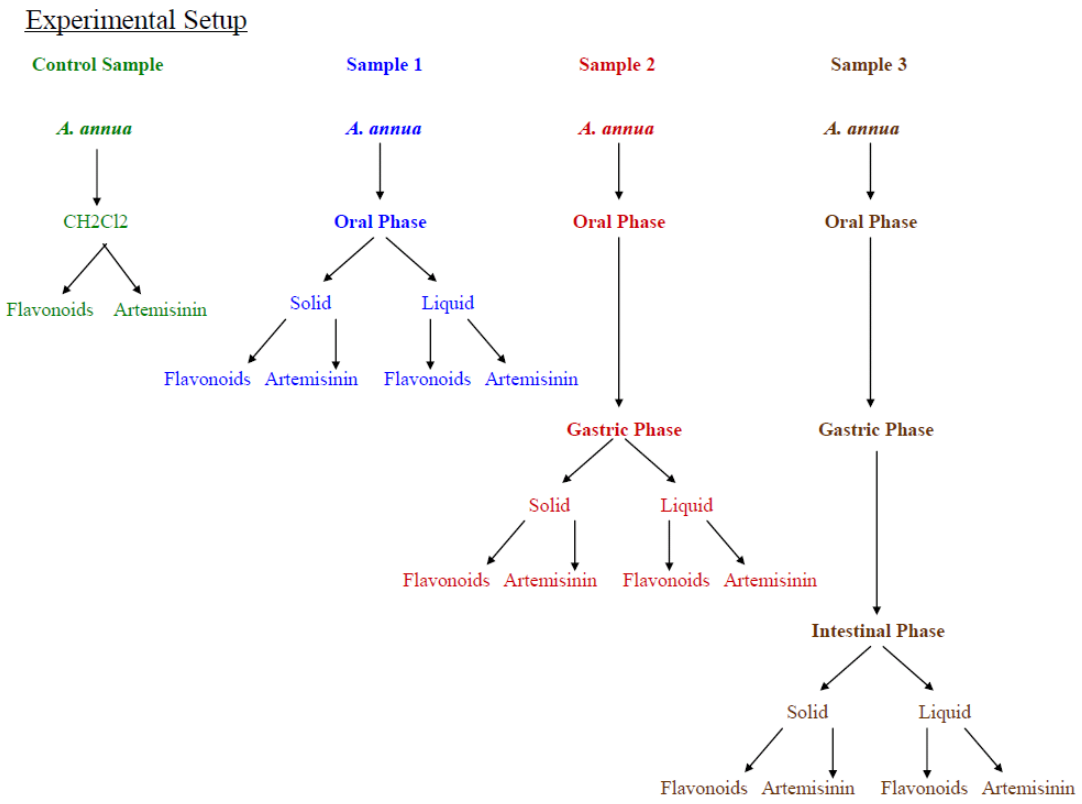


Figure 3: The experimental set-up for *ex vitro* digestion and extraction.

3.5 Thin Layer Chromatography (TLC) Testing

Each DG extraction (solid and liquid) was analyzed using Thin Layer Chromatography (TLC) to provide a visual profile of AN and FLVs released during each digestive stage under different conditions and also to define the appropriate amount of sample that would be needed for further quantification by GC/MS. TLC was performed by using GF-254 Si Gel plates from Sigma-Aldrich Company. The solvent system used was toluene – ethyl acetate (2:1). The TLC was run for the extracted DG along with standard compounds, AN and CAS. CAS was visible as a dark purple dot under UV light. AN stained hot pink after spraying the plate with 50 mL glacial acetic acid containing 1 mL of concentrated sulfuric acid and 0.5 ml *p*-anisaldehyde and heated for at least 10 minutes at 105 °C. The Rfs of AN and CAS were 0.70 and 0.25, respectively. Since AN ran well above all the fluorescing material, which contain FLVs and other materials,

the top strip was used to stain for AN detection (Figure 4). It was determined that 10% of each extract would be used for GC-MS analysis.

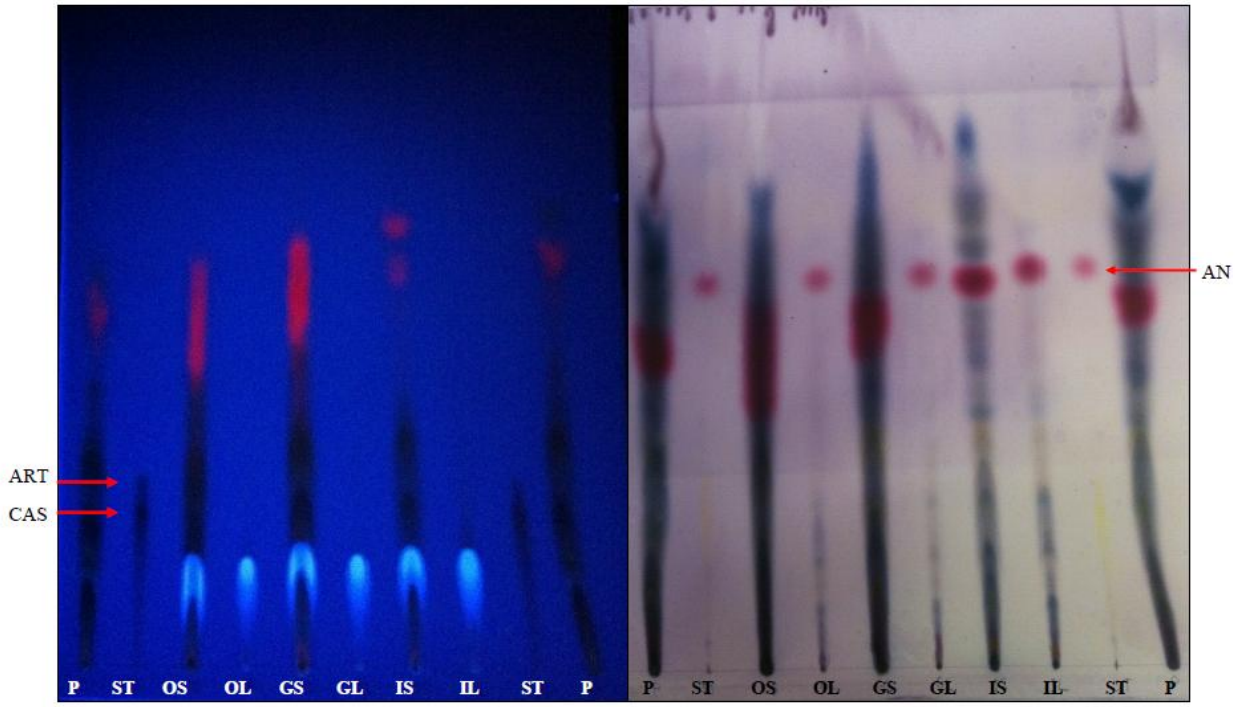


Figure 4: Sample TLC plate under UV light (left) and the same plate after anisaldehyde staining (right).

P, plant: control, SAM, 0.02 g DW
ST, standard compound
O, oral phase
G, gastric phase
I, intestinal phase
S, solid fraction
L, liquid fraction

3.6 AN Quantification by GC-MS

AN was quantified in extracts by GCMS according to the method detailed in Weathers and Towler (2012).

3.7 $AlCl_3$ Assay for Flavonoid Quantification

Using quercetin as the standard, a standard curve was obtained by dissolving 10, 20, 30, 40, and 50 µg of quercetin in MeOH. The solvent was evaporated and then a 1:1 solution of 2 % (w/v) $AlCl_3$ to MeOH was added for a total volume of 3 mL and incubated for 25 minutes. Absorbance (OD), at 415 nm wavelength, was obtained using a Spec20 spectrophotometer. OD 415nm versus the quercetin concentration (µg/mL) was plotted to generate a standard curve and linear equation. A 1:1 ratio of sample to $AlCl_3$ reagent was preparaed; that is, $AlCl_3$ solution added to a fraction of liquid or solid DG containing 5% of the starting plant material and diluted in methanol for a total volume of 3 mL and incubated for 25 minutes. The OD of each sample was measured at 415 nm and FLV content of each DG extract was calculated by using the quercetin standard curve.

3.8 Statistical Analysis

Raw data from GC/MS analysis provided the AN content for each solid and liquid fraction of each digestion phase. These data were converted to µg AN/g dry weight (DW) of digested dry leaf material. FLV raw data from the $AlCl_3$ colorimetric assay were converted to µg FLVs/g DW of digested dry leaf material. Each experiment was replicated three times; the averages, standard deviations, and standard errors were calculated for the AN and FLV content of each DG fraction (See Appendix A and B). Using the statistical software SPSS, post hoc Tukey tests and T-tests were used to determine statistical differences between samples.

4 Results

The AN content data from GC/MS analysis were graphically analysed displaying standard error. Experimental fractions were statistically compared to their digested phase/fraction pACT equivalent by standard T-test analysis. Each experiment and condition were given abbreviations indicated in Table 4.

Table 4: Experimental Data Abbreviations

s= Solid Fraction l= Liquid Fraction
O= Oral Phase G= Gastric Phase I= Intestinal Phase
PE= Plant Extract Control, pACT (undigested) WP= Whole Plant Control, pACT (digested)
VC= Vegetarian Capsule (digested) VC CTRL= Vegetarian Control (undigested) gC= Gelatin Capsule (digested) gC CTRL= Gelatin Capsule Control (undigested)
S= Sucrose (digested) Co= Canola Oil (digested)
M= Millet (digested) WR= White Rice (digested) CM= Cornmeal (digested)
*= Statistically Significant Compared to WP Equivalent, $p \leq 0.05$

4.1 pACT Digestion Compared to pACT Extract Undigested

When the whole plant material (WP ≈ pACT) was extracted, it contained 7.7 mg AN/g DW of *A. annua* (Table 5). After digestion the amounts of released AN found in the liquid fraction of each digestion phase decreased with about half of the originally available AN appearing in the liquid fraction of the intestinal phase (Table 5). The amount of AN in the solid and liquid fractions was totaled for each phase of these simulated digestion experiments (Table

5). These totals reflected the potentially accessible AN in the 0.36 g of *A. annua* digested in each sample and allow us to determine by comparison if AN was lost or unnaccounted for when all fractions of an experiment were summed. The greatest loss in total AN content recovered after digestion of pACT is, as might be expected, in the intestinal phase (Table 5).

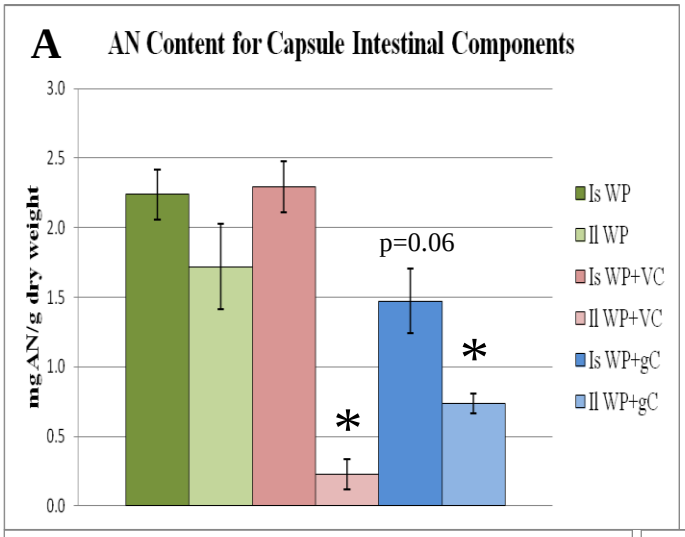
Table 5: Of the total AN present in pACT intestinal digestive phase, 52% was unrecovered compared to the plant extract control.

Experimental Averages	**AN (s + l) Fraction Totals (mg AN/g D)** [1]
Plant Extract	7.7
Digested samples of *A. annua* (pACT)	
pACT Oral Phase	6.1
pACT Gastric Phase	6.4
pACT Intestinal Phase	4.0

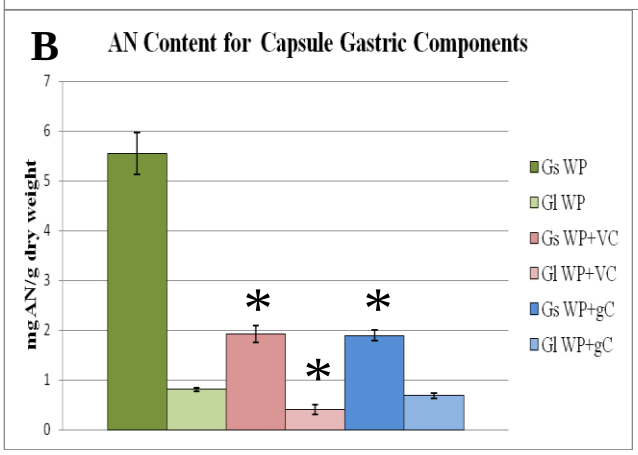
[1] For each digestion phase s is the solid fraction AN, and l is the liquid fraction AN.

4.2 pACT versus Two Capsule Types

When pACT was encased in either a vegetarian or gelatin capsule, results showed a statistically lower yield of AN in the intestinal liquid fraction (0.2-0.7 mg AN/g DW) compared to the DG of the whole plant (1.7 mg AN/g DW; pACT DG) (Figure 5A). DG of pACT in a vegetarian capsule showed a 43% reduction in the average AN content in the liquid portion of the DG from the gastric to the intestinal phase (Figure 5A&B). Much of the AN in pACT seems inaccessible when encapsulated (Figure 5); no particular digestive phase is more liberating or inhibiting for AN release.



s= Solid Fraction l= Liquid Fraction
O= Oral Phase G= Gastric Phase I= Intestinal Phase
WP= Whole Plant, pACT (digested) VC= Vegetarian Capsule (digested) gC= Gelatin Capsule (digested)
*= Statistically Significant Compared to WP Equivalent, p≤0.05



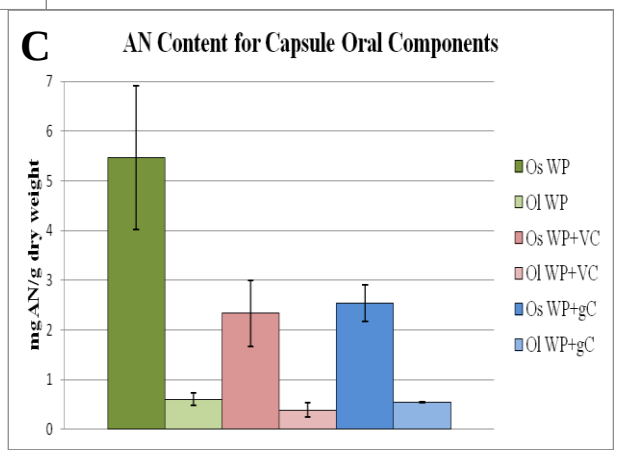
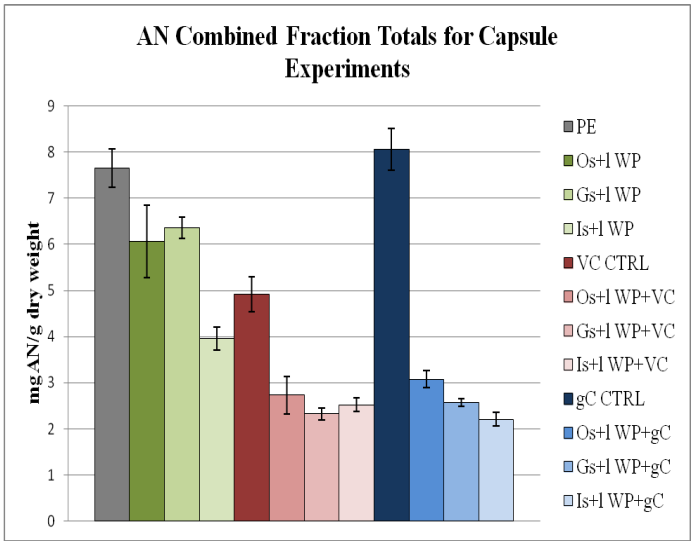


Figure 5: Capsules decrease AN release. A) AN content for the solid and liquid fractions of the intestinal phase for the capsule experiments in mg AN/g D B) AN content for the solid and liquid fractions of the gastric phase for the capsule experiments in mg AN/g DW C) AN content for the solid and liquid fractions of the oral phase for the capsule experiments in mg AN/g DW.

To examine if the reduction in AN when pACT was encased in a capsule was due to the presence of cellulose or gelatin, the main material comprising vegetarian and gelatin capsules, respectively, each type of capsule was added to the unencased pACT and then immediately extracted with no digestion. The gelatin capsule control (undigested) produced AN values equivalent to the undigested plant extract control (Figure 6, dark blue bar vs. gray bar) suggesting that it was not the gelatin alone with *A. annua* that made AN unrecoverable but the combination of digestive material and gelatin (Figure 6, dark blue bar vs. light blue bars). In contrast, the undigested vegetarian capsule control (Figure 6, dark red bar) produced AN values

that were ~30% less than the plant extract control (PE, Figure 6, gray bar) suggesting that cellulose, added to *A. annua*, partially degrades or masks AN (Figure 6, dark red bar).



s= Solid Fraction
l= Liquid Fraction

O= Oral Phase
G= Gastric Phase
I= Intestinal Phase

PE= Plant Extract Control (undigested)
VC CTRL= Vegetarian Control (undigested)
gC CTRL= Gelatin Capsule Control (undigested)
WP= Whole Plant, pACT (digested)

VC= Vegetarian Capsule
gC= Gelatin Capsule

*= Statistically Significant Compared to WP Equivalent, $p \leq 0.05$

Figure 6: Up to 70% of the original AN in the plant material was unrecovered from the solid and liquid fractions after digestion of the pACT with each capsule type.

When the total flavonoid content of the liquid fraction of each intestinal phase was measured, there was no significant difference in the amount of FLVs present (Table 6). However, for the pACT + vegetarian capsule experiment, the oral liquid fraction revealed significantly higher FLV content that increased further in the gastric liquid fraction but decreased by nearly 2-fold into the intestinal phase (Table 5). This suggested that a component of the intestinal phase interacts with material in the vegetarian capsule in a way that degrades FLVs.

Table 6: In the intestinal phase, capsules had no negative effect on FLV content.

Experiment Averages (mg FLVs/g DW)	Oral Liquid	Gastric Liquid	Intestinal Liquid
pACT	0.089	0.118	0.101
pACT + gelatin Capsule	0.105	0.154	0.150
pACT + Vegetarian Capsule	0.193*	0.244	0.130

* Indicates statistically significant at $p \leq 0.05$; n = 3.

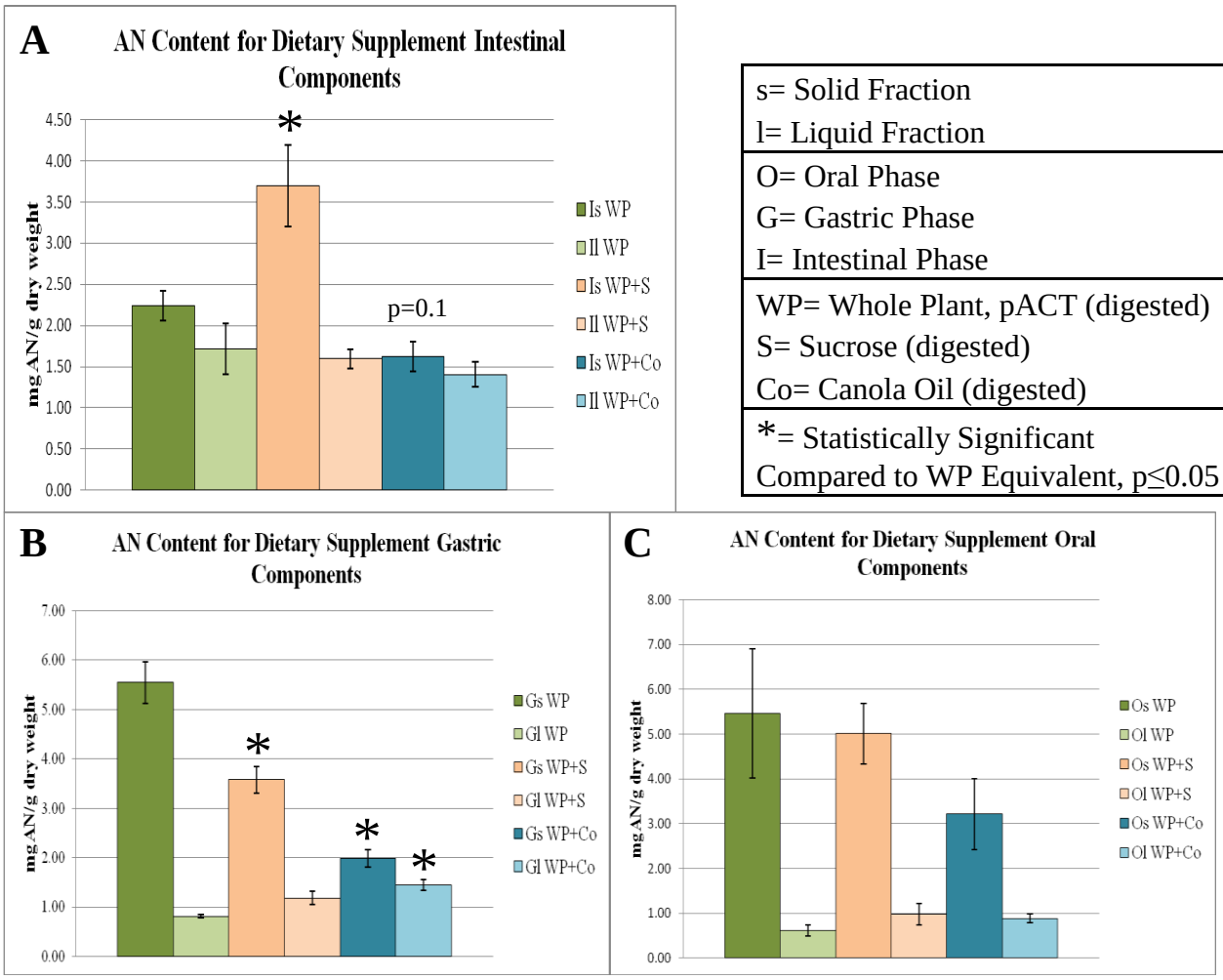
4.3 pACT versus Dietary Inclusions

When sucrose and canola oil were included in digestions with pACT they yielded statistically similar AN levels: 1.6 mg AN/g DW when sucrose was present (WP+S) and 1.4 mg AN/g DW in the presence of canola oil. (WP+Co). These results were not statistically different from the intestinal liquid fraction of pACT digestions (Figure 7A). Moreover, the pACT + canola oil experiment showed a significant increase in AN content in the liquid fraction from the oral to the gastric phase, with no increase from the gastric to the intestinal phase (Figure 7). In contrast, the pACT + sucrose experiment demonstrated little change in AN content in the liquid fraction from the oral to the gastric phase, but a significant increase from the gastric to the intestinal phase (Figure 7).

Both the pACT + sucrose and pACT + canola oil digestions had a positive effect on FLV content. The intestinal liquid fractions for these experiments contain more than twice the FLV content than the pACT alone (Table 7).

Addition of different grain meals to pACT yielded varying AN levels for the intestinal liquid fractions. The pACT + white rice combination showed no significant decrease in the release of AN (Figure 8, light pink bar). However, pACT + millet and pACT + cornmeal combinations decreased AN release in the intestinal liquid fraction (Figure 8, light purple bar and light brown bar). The pACT + cornmeal combination showed the greatest reduction with an average 0.7 mg AN/g DW in the intestinal liquid fraction, a 50% decrease compared to the pACT alone (Figure 8, light brown bar vs. light green bar).

The ‘meal’ experiments showed no statistically significant variance in FLV content present in the liquid fraction in all phases when compared to the pACT (Table 8).



s= Solid Fraction
l= Liquid Fraction

O= Oral Phase
G= Gastric Phase
I= Intestinal Phase

WP= Whole Plant, pACT (digested)
S= Sucrose (digested)
Co= Canola Oil (digested)

*= Statistically Significant Compared to WP Equivalent, $p \leq 0.05$

Figure 7: Sucrose and oil had no negative effect on AN release. A) AN content for the solid and liquid fractions of the intestinal phase for the sucrose and canola oil experiments in mg AN/g DW B) AN content for the solid and liquid fractions of the gastric phase for the sucrose and canola oil experiments in mg AN/g DW C) AN content for the solid and liquid fractions of the oral phase for the sucrose and canola oil experiments in mg AN/g DW.

Table 7: The intestinal liquid fraction of the pACT + sucrose and pACT + canola oil experiments showed significantly higher FLV content than the pACT.

Experiment Averages (mg FLVs/g DW	Oral Liquid	Gastric Liquid	Intestinal Liquid
pACT	0.089	0.118	0.101
pACT + Sucrose	0.113	0.229	0.207*
pACT + Canola Oil	0.084	0.091	0.203*

* Indicates statistically significant at $p \leq 0.05$; n = 3.

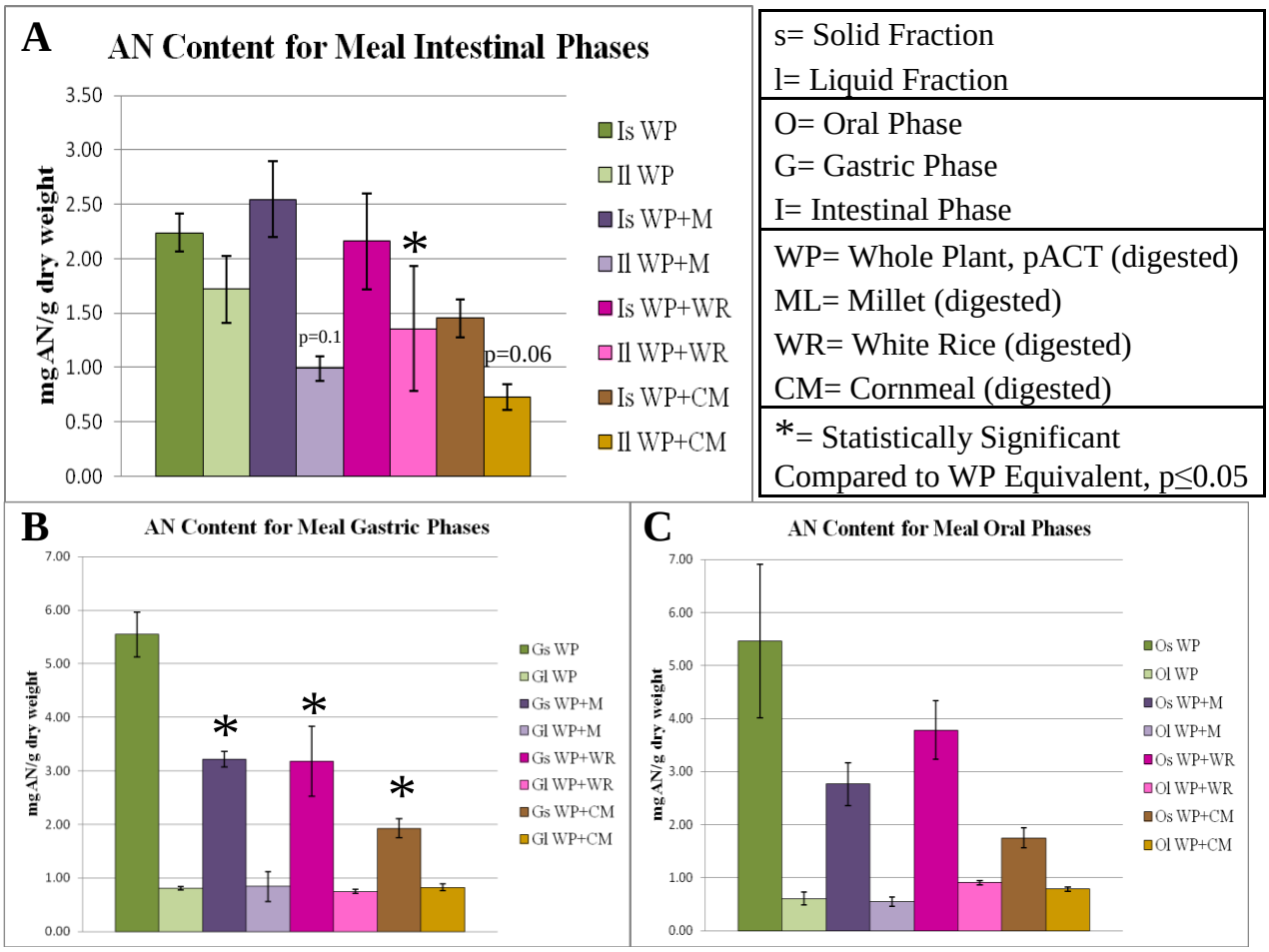


Figure 8: Rice had no negative effect on AN release, but millet and cornmeal decreased AN release A) AN content for the solid and liquid fractions of the intestinal phase for the meal experiments in mg AN/g DW B) AN content for the solid and liquid fractions of the gastric phase for the meal experiments in mg AN/g DW C) AN content for the solid and liquid fractions of the oral phase for the meal experiments in mg AN/g DW.

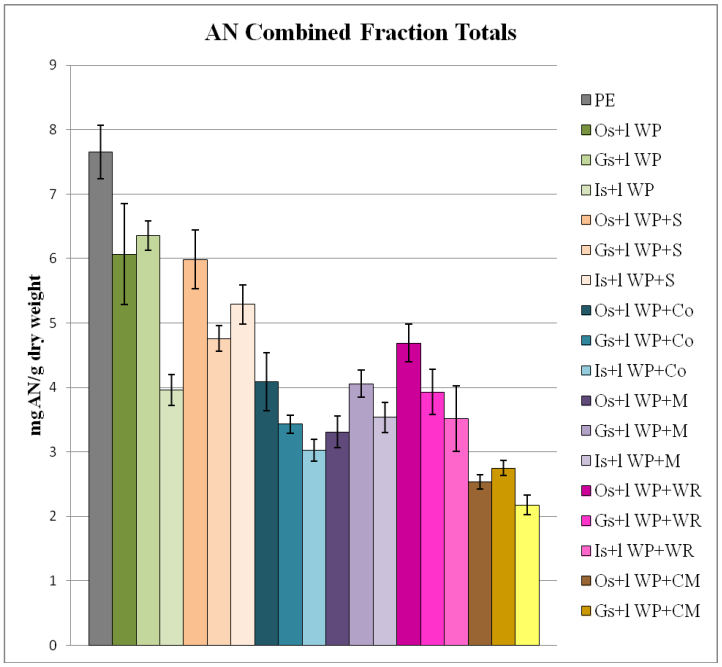
Table 8: The 'meal' + pACT digestion experiments demonstrated no significant effect on FLV release compared to pACT.

Experiment Averages (mg FLVs/g DW)	Oral Liquid	Gastric Liquid	Intestinal Liquid
pACT	0.089	0.118	0.101
pACT + Millet	0.108	0.067	0.116
pACT + White Rice	0.162	0.061	0.156
pACT + Cornmeal	0.129	0.100	0.205

* Indicates statistically significant at p≤0.05; n = 3.

4.4 AN Losses During Digestion

The combined liquid and solid fractions for both the oral phase and gastric phase of the pACT (Figure 9, green bars), and the oral, gastric, and intestinal phases of the pACT + sucrose (Figure 9, orange bars) experiment samples have AN values similar to the total AN present in the undigested plant extract control (Figure 9, gray bar). However, in all of the other experiments, between 30 and 70% of the original AN in the plant material was unrecovered from the sum of the solid and liquid phases after digestion (Figure 9), including the gelatin capsule experiments (Figure 6).



s= Solid Fraction l= Liquid Fraction
O= Oral Phase G= Gastric Phase I= Intestinal Phase
PE= Plant Extract Control, pACT (undigested) WP= Whole Plant Control, pACT (digested)
S= Sucrose (digested) Co= Canola Oil (digested)
M= Millet (digested) WR= White Rice (digested) CM= Cornmeal (digested)

Figure 9: Up to 70% of the original AN in the plant material was unrecovered from the sum of the solid and liquid phases after digestion. Combined AN totals for the solid and liquid fractions of each digestive phase of the dietary inclusion experiments.

5 Discussion

The digestive system is a complex process involving the release and interaction of many molecules as material becomes digested. Compounds become absorbed into the bloodstream during the intestinal phase making bioavailability critical and more probable for material that becomes soluble in the liquid fraction. Therefore, the pACT + sucrose, pACT + canola oil, and pACT + white rice combinations demonstrated the greatest potential for bioavailability suggesting that they may provide the more effective food combinations for oral delivery of dried leaves of *A. annua.* Results indicated that the use of vegetarian or gelatin capsules are not recommended delivery methods for pACT because they reduced AN availability in the intestinal liquid fraction by more than 50%. On average, the pACT + millet and pACT + cornmeal combinations also demonstrated less AN release in the intestinal liquid fraction compared to pACT alone. However, this reduction was not statistically significant therefore no definitive recommendation can be made regarding these dietary inclusions.

The previous results revealed that up to 50% of the original AN in the plant material was unrecovered from the sum of the solid and liquid phases after digestion. A study conducted by Iskra (2000), characterized peroxidases in hairy root cultures of *A. annua* L. and studied their involvement in the degradation of AN. He found that the total degradation of AN required the presence of both absorbic acid and iron (III). He also showed that AN degradation was affected by pH and that peroxidase has a maximum activity for AN degradation around neutrality. During digestion, the processed material reaches a neutral pH during the intestinal phase, which is also the longest period in the overall digestion process. Therefore, if peroxidase is present in the plant material, it is possible that AN becomes degraded during digestion. This could account for the unrecovered AN that was noted. Peroxidase assays will be run in future studies to determine if

the enzyme plays a role in AN degradation that is evident in this study, and to determine if degradation can be reduced.

A recent study conducted by Weathers *et al*. (2001) demonstrated the efficacy of pACT bioavailability in mice. When 31 μg of pure AN was administered to mice, the drug was undetectable in the blood up to 60 minutes whereas feeding mice dried *A. annua* leaves, containing an equivalent 31 μg of AN, produced a maximum concentration of 0.087 mg l^{-1} of AN in blood at 30 minutes. It took 1,400 μg of pure AN to reach a blood concentration of 0.074 mg l^{-1}. When pure AN was combined with glandless *A. annua* (data not shown), a mutant that produces no AN, significantly less AN was released into the intestinal liquid fraction compared to pACT. This reduction in the bioavailablity of pure AN is consistent with the findings of the mouse study. Additionally, pure AN does not contain any plant material and thus cannot be degraded by peroxidase. However, the AN totals for the pure AN digestion were relative to the pACT totals suggesting that it is not peroxidase but rather an element of the digestive process that is reducing the recovery of significant amounts of AN from the total that is available in the intact plant.

As noted earlier, the capsule experiments demonstrated that nearly 70% of the total AN was unrecovered. When gelatin capsule material was added to pACT and immediately extracted without any digestion, AN yield was equal to that of the plant extract indicating that its loss was not a function of the capsule, but rather a result of the digestive process. Gelatin capsules mainly consist of proteins from animal byproducts and are easily digested and absorbed. Urea, which is released in the oral phase, affects the pH behavior of gelatin by increasing the intrinsic pk of the acidic groups of the proteins by 0.45 units (Jana *et al*., 1993). AN can be degraded at extremes of pH (Iskra *et al.*, 2000), so gelatin in combination with urea may influence AN degradation. On

the other hand, when the pACT + vegetarian capsule was extracted without any digestion, there was significant loss of AN. Vegetarian capsules are mainly made of cellulose and these molecules bind strongly to each other. Although most mammals have a limited ability to digest cellulose, this does not explain the loss of AN in the presence of these capsules (Terry *et al.*, 2006).

Flavonoid analysis revealed that the digestion supplements, in combination with pACT, did not have a negative effect on FLV release. On the other hand, FLV release was significantly enhanced after digestion of pACT with sucrose or with canola oil. Considering that FLVs are reported to act synergistically with AN in killing malaria parasites (Liu *et al.*, 1992) this is a favorable result. The amount of FLVs released in the intestinal liquid fraction of the pACT + canola oil experiment was more than two-fold that of the pACT. Quercetin is normally present in canola oil (Chen *et al*., 1996). Quercetin was used to generate the standard curve for the colorimetric assay analysis of the FLV content, so it is possible that the quercetin present in the canola oil may be slightly skewing the results to a higher level and that the actual FLVs released form pACT are a bit lower than we measured. Palm oil, peanut oil, and sunflower oil are accessible in Africa while canola oil is not. However, studies have shown that palm, peanut and sunflower oils, like canola oil, naturally contain quercetin (Arsic *et al.* 2010).

6 Conclusions and Future Work

Sucrose demonstrated comparatively high AN and FLV content in the intestinal liquid fractions in this study. Since *A. annua* is a very bitter tasting plant, sucrose can make the delivery much more palatable, especially for pediatric patients. It is also commonly used in tea as a prophylactic which may be useful against malaria. Small amounts of sucrose may be an effective palatable delivery method if further research supports that it will not significantly increase the parasitic growth of malaria. White rice also showed no negative reduction in the release of AN. White rice is a relatively cheap and available staple food and may be an effective dietary supplement used in the delivery of *A. annua* treatment. Canola oil was also found to release relatively high amounts of AN as well as FLVs in the intestinal liquid fraction, compared to pACT alone. It has the potential for use as a binder in the production of *A. annua* tablets for drug delivery. Although canola oil, specifically, is not used in malaria endemic countries, the data do suggest that oil is not problematic in AN and FLV release. Oils such as sunflower oil, peanut oil, and palm oil more common to malaria infested areas should be tested.

This *ex vitro* study is an indication of how *A. annua* will be processed through the digestive tract and can suggest what contents become bioavailable. However, these conclusions cannot be verified until tested *in vivo*. Future studies are recommended to test AN and FLV bioavailability using a human intestinal Caco-2 cell line.

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Appendices

Appendix A: Digestion results for AN analysis.

pACT: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	7.38	0.43	5.20	0.87	2.01	1.79
Replicate 2	2.63	0.54	6.38	0.78	2.59	2.22
Replicate 3	6.37	0.85	5.06	0.78	2.12	1.15
Average	5.46	0.61	5.55	0.81	2.24	1.72
Standard deviation	2.50	0.21	0.73	0.05	0.31	0.54
Standard Error	1.44	0.12	0.42	0.03	0.18	0.31

WP+VC: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	3.51	0.63	2.25	0.54	2.65	0.23
Replicate 2	2.31	0.12	1.68	0.23	2.17	0.23
Replicate 3	1.20	0.41	1.84	0.44	2.05	0.56
Average	2.34	0.39	1.92	0.41	2.29	0.23
Standard deviation	1.15	0.26	0.29	0.16	0.31	0.19
Standard Error	0.67	0.15	0.17	0.09	0.18	0.11

WP+gC: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	3.28	0.52	2.10	0.77	1.92	0.77
Replicate 2	2.16	0.55	1.80	0.70	1.33	0.70
Replicate 3	2.19	0.53	1.77	0.59	1.16	0.53
Average	2.54	0.54	1.89	0.69	1.47	0.74
Standard deviation	0.64	0.02	0.19	0.09	0.40	0.12
Standard Error	0.37	0.01	0.11	0.05	0.23	0.07

WP+S: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	3.71	1.43	3.29	1.31	3.00	1.41
Replicate 2	5.32	0.64	4.10	1.32	4.65	1.78
Replicate 3	6.01	0.86	3.34	0.92	3.44	1.48
Average	5.01	0.97	3.57	1.18	3.70	1.59
Standard deviation	1.18	0.41	0.45	0.23	0.85	0.20
Standard Error	0.68	0.24	0.26	0.13	0.49	0.11

WP+M: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	3.23	0.71	2.99	1.39	3.00	0.96
Replicate 2	3.10	0.40	3.48	0.51	1.87	1.20
Replicate 3	1.96	0.53	3.19	0.62	2.78	0.81
Average	2.76	0.55	3.22	0.84	2.55	0.99
Standard deviation	0.70	0.15	0.25	0.48	0.60	0.20
Standard Error	0.40	0.09	0.14	0.27	0.35	0.11

WP+WR: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	3.34	0.86	3.02	0.67	2.97	2.50
Replicate 2	3.12	0.87	2.13	0.78	2.06	0.92
Replicate 3	4.89	0.98	4.39	0.80	1.45	0.65
Average	3.78	0.91	3.18	0.75	2.16	1.36
Standard deviation	0.96	0.07	1.14	0.07	0.77	1.00
Standard Error	0.56	0.04	0.66	0.04	0.44	0.58

WP+Co: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	1.62	0.90	2.05	1.67	1.28	1.71
Replicate 2	4.05	0.70	2.25	1.35	1.70	1.25
Replicate 3	3.96	1.06	1.64	1.33	1.88	1.25
Average	3.21	0.88	1.98	1.45	1.62	1.40
Standard deviation	1.38	0.18	0.31	0.19	0.31	0.26
Standard Error	0.79	0.10	0.18	0.11	0.18	0.15

WP+CM: mg AN/g DW	Os	Ol	Gs	Gl	Is	Il
Replicate 1	1.38	0.74	1.91	0.95	1.12	0.68
Replicate 2	1.93	0.75	2.25	0.79	1.71	0.77
Replicate 3	1.95	0.86	1.62	0.74	1.53	1.08
Average	1.75	0.78	1.93	0.83	1.45	0.73
Standard deviation	0.32	0.07	0.31	0.11	0.30	0.21
Standard Error	0.19	0.04	0.18	0.06	0.17	0.12

Appendix B: Digestion results for FLV analysis.

pACT: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.058	0.211	0.074
Replicate 2	0.063	0.069	0.142
Replicate 3	0.145	0.074	0.087
Average	0.089	0.118	0.101
Standard Deviation	0.049	0.081	0.036
Standard Error	0.028	0.047	0.021

WP+S: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.098	0.174	0.227
Replicate 2	0.082	0.193	0.190
Replicate 3	0.161	0.322	0.206
Average	0.113	0.229	0.207
Standard Deviation	0.042	0.080	0.019
Standard Error	0.024	0.046	0.011

WP+gC: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.076	0.158	0.148
Replicate 2	0.129	0.200	0.121
Replicate 3	0.111	0.103	0.182
Average	0.105	0.154	0.150
Standard Deviation	0.027	0.049	0.030
Standard Error	0.015	0.028	0.018

WP+VC: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.224	0.138	0.086
Replicate 2	0.178	0.026	0.105
Replicate 3	0.178	0.567	0.198
Average	0.193	0.244	0.130
Standard Deviation	0.027	0.285	0.060
Standard Error	0.015	0.165	0.035

WP+CM: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.150	0.142	0.134
Replicate 2	0.116	0.079	0.206
Replicate 3	0.121	0.079	0.274
Average	0.129	0.100	0.205
Standard Deviation	0.018	0.037	0.070
Standard Error	0.011	0.021	0.040

WP+M: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.105	0.098	0.100
Replicate 2	0.100	0.037	0.142
Replicate 3	0.119	0.066	0.105
Average	0.108	0.067	0.116
Standard Deviation	0.010	0.030	0.023
Standard Error	0.005	0.018	0.013

WP+WR: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.134	0.053	0.158
Replicate 2	0.164	0.079	0.156
Replicate 3	0.187	0.050	0.153
Average	0.162	0.061	0.156
Standard Deviation	0.026	0.016	0.003
Standard Error	0.015	0.009	0.002

WP+Co: mg FLV/g DW	Ol	Gl	Il
Replicate 1	0.087	0.074	0.251
Replicate 2	0.087	0.095	0.148
Replicate 3	0.079	0.103	0.211
Average	0.084	0.091	0.203
Standard Deviation	0.005	0.015	0.052
Standard Error	0.003	0.009	0.030

Appendix C: The *ex vitro* digestion protocol for *A. annua* developed from the SOP of the *In vitro* Digestion for Porridge protocol by Ferruzzi lab at Purdue University based on the methods of Kean *et al*. (2011).

<table>
<tr><td>Ex vitro digestion of A. annua</td><td>In vitro digestion for Porridge</td></tr>
<tr><td colspan="2">Stock solutions:

0.9% NaCl; 100 mM $NaHCO_3$; 1.0 M HCl; 0.1 M HCl; 1.0 M NaOH; 0.1 M NaOH</td></tr>
<tr><td colspan="2">Enzymes and bile solutions:

- Pepsin solution: 10 mg/mL Pepsin in 1.0M HCl
- Pancreatin-Lipase solution: 20 mg/mL Pancreatin (in 100 mM $NaHCO_3$), 10 mg/mL Lipase (in 100 mM $NaHCO_3$)
- Bile solution: 30 mg/mL Bile Extract (in 100 mM $NaHCO_3$</td></tr>
<tr><td colspan="2">Preparation of oral phase base solution (q.s. to 1 L with DI water):

- Potassium Chloride 1.792g
- Sodium Phosphate 1.776g
- Sodium Sulfate 1.140g
- Sodium Chloride 0.596g
- Sodium Bicarbonate 3.388g</td></tr>
<tr><td>Prepare material:

- Make 0.36 g of A. annua + 1.64 mL DI water (equivalent to 25% of the porridge)
- Use all the prepared for digestion</td><td>

- Porridge =10 g flour + 45 mL DI water
- Use 8 g of the prepared porridge for digestion</td></tr>
</table>

Oral phase 1. Warm bath to 37°C. 2. Prepare oral phase base solution (use lesser): with 10 mL base solution in beaker with stir bar, add 4mg/ 0.004 g urea, 0.3 mg/ 0.0003 g uric acid, and 0.5 mg/ 0.0005 g mucin. 3. Weigh 0.36 g of A. annua + 1.64 ml of water into a 50 ml centrifuge tube. 4. Add 1.5 mL oral phase solution 2. 5. Add 46.875 mg/ 0.0469 g of α-amylase. 6. Vortex for 2 minutes 7. Blanket with nitrogen gas and cap tightly. 8. Place vertically in 37 °C water bath for 10 minutes. 9. Meanwhile, prepare pepsin solution	1. Warm bath to 37°C. - Thaw canola oil and porridge. 2. Prepare oral phase base solution: with 100 mL base solution in beaker with stir bar, add 40 mg urea, 3 mg uric acid, and 5 mg mucin. 3. Weigh 8 g of porridge into a 50 mL centrifuge tube. - Add 0.4 g (5%w/w) canola oil using needle and syringe. 4. Add 6 mL oral phase solution 2. 5. Add 0.190 mg of α-amylase. 6. Vortex for 2 minutes 7. Blanket with nitrogen gas and cap tightly. 8. Place horizontally in 37 °C water bath for 10 minutes. 9. Meanwhile, prepare pepsin solution
Gastric Phase 10. Remove from water bath, place immediately on ice. 11. Bring to 7.5 mL with saline (Assume 1 g food material = 1 mL). 12. Adjust pH to equal 4.0±0.1 using 1.0 M HCl. 13. Add 0.5 mL of 10 mg/mL Pepsin solution. 14. Adjust pH to 2.5±0.1 using 1.0 M HCl. 15. Bring up to 10 mL with saline 16. Blanket with nitrogen gas, cap tightly, and place vertically in 37 °C water bath. 17. Incubate at 90 opm for 1 hr. 18. Meanwhile, prepare bile extract (sonicate for 30 minutes). 45 minutes into incubation, prepare Pancreatin-Lipase solution.	10. Remove from water bath, place immediately on ice. 11. Bring to 30 mL with saline (Assume 1 g food material = 1 mL). 12. Adjust pH to equal 4.0±0.1 using 1.0 M HCl. 13. Add 0.5 mL of 10 mg/mL Pepsin solution. 14. Adjust pH to 2.5±0.1 using 1.0 M HCl. 15. Bring up to 10 mL with saline 16. Blanket with nitrogen gas, cap tightly, and place horizontally in 37 °C water bath. 17. Incubate at 90 opm for 1 hr. 18. Meanwhile, prepare bile extract (sonicate for 30 minutes). 45 minutes into incubation, prepare Pancreatin-Lipase solution.

Intestinal phase: 19. Remove from water bath, place immediately on ice. 20. Adjust pH to 4.0 ±0.1 using $NaHCO_3$. 21. Add 0.5 mL Pancreatin-Lipase solution. 22. Add 0.75 mL Bile extract solution. 23. Adjust pH to equal pH 6.5±0.1 using $NaHCO_3$. 24. Bring to 12.5 mL with saline 25. Blanket with nitrogen gas, cap tightly, and place vertically in 37 °C water bath. 26. Incubate at 90 opm for 2 hr	19. Remove from water bath, place immediately on ice. 20. Adjust pH to 4.0 ±0.1 using $NaHCO_3$. 21. Add 2 mL Pancreatin-Lipase solution. 22. Add 3 mL Bile extract solution. 23. Adjust pH to equal pH 6.5±0.1 using $NaHCO_3$. 24. Bring to 50 mL with saline 25. Blanket with nitrogen gas, cap tightly, and place horizontally in 37 °C water bath. 26. Incubate at 90 opm for 2 hr