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COLLEGE OF AGRICULTURE AND LIFE SCIENCES

Department of Nutrition and Food Science

Final Data Report

Objective 1: Colon & Breast Cell Culture Study

A data report submitted to the National Mango Board

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Introduction

There are extensive epidemiological and clinical studies that explain the potential benefits of polyphenolics on human health [1]. Mango (*Mangifera Indica* L.) is the most popular fruit consumed world-wide, with over 1000 known varieties and commercial production in 87 countries [2]. Many of the world's most popular commercial varieties were actually developed in Florida. These varieties include Kent, Keitt, Palmer, Haden, and Tommy Atkins which are superior for exportation because of their firmer texture, are less fibrous, and are more suited to long-distance travel than other mango varieties. [3, 4]. Mango contains high concentrations of phytochemicals, including gallic acid, mono galloyl glucosides, gallotannins, flavonol glycosides and benzophenone derivatives [5], some of which are unique to the plant and have been proposed for use in creating phytochemical rich dietary supplements [6]. Fundamentally, gallotannins are composed of gallic acid moieties esterified to a core polyol, most commonly glucose, [7, 8]. Gallic acid esters of glucose have been reported to range from one to twelve degrees of polymerization. In addition, penta-galloylglucose, a gallotanin with galloyl moieties esterified to each of the five available hydroxyls of glucose, is considered the standard for gallotannins. Since glucose has a maximum of five aliphatic hydroxyl groups, larger gallotannins are only created by gallic acid linking via a *para (p)*or *meta (m)*-depside bonds on a phenolic hydroxyl of gallic acid [9]. The *p*- or *m*-depside bond between gallic acid moieties is generally weaker than the ester bond between glucose and the first gallic acid moiety, which allows penta-galloyl glucose to be easily synthesized using commercial tannic acid through the addition of a mild acid and methanol (known as methanolysis) which preferably cleaves the depside linkages [10].

Relating Polyphenol-Content to Health-Benefits: Mango polyphenolics are reported to have anti-oxidant [11] and anti-inflammatory activities in *in vivo* and *in vitro* experiments [12-14]. Gallic acid, the most abundant polyphenol in mango, has been shown to have anti-inflammatory and chemopreventive effects [15, 16]. In addition, gallotannins have exhibited antioxidant and anti-inflammatory properties [17]. However, the effectiveness of mango polyphenolics may vary according to the types and contents of the specific compounds, such as gallic acid, galloyl-glucosides, and gallotannins. The polyphenolic contents of mango are influenced by varieties, ripening, cultivar, environmental, and handling factors [18]. Currently, the golden standard for the determination of polyphenol content of mango and other fruits is the Folin's Ciocalteu assay. This assay measures how polyphenols interact with metal ions and does not directly relate to the health-benefits of polyphenols. Across different studies there is great variability whether the total polyphenol content of a certain fruit does or does not correlate with the determined health benefits. For this reason, it is difficult to compare different studies and come to unified conclusions about the health benefits of polyphenols.

Overall Study Objective: The objective of this work was to quantify the polyphenolic content of mangos as it relates to their health-benefits (inflammation). We performed a bioefficacy-guided fractionation to mango polyphenolic extracts of five different varieties

where we fractionated mango polyphenols into smaller classes and groups according to their anti-inflammatory efficacy.

Major Findings: The anti-inflammatory activities of polyphenolic extracts from five mango cultivars (Ataulfo, Keitt, Kent, Haden, and Tommy Atkins) were investigated and tested at the same concentration of total polyphenols (Gallic Acid Equivalents). Keitt and Kent extracts showed overall higher anti-inflammatory effects compared to the other varieties, and when the Keitt and Kent extracts were separated into low and high molecular weight fractions, the fraction containing the high molecular weight compounds had a higher anti-inflammatory activity.

In addition, gallic acid, one of the predominant compounds in the low molecular weight fraction, was higher in anti inflammatory efficacy compared to penta-galloyl glucose at the molar level. In the correlation analysis, gallic acid and penta-galloyolglucose showed a trend to have a higher correlation for anti-inflammatory activity than other compounds in mango. As we discovered total polyphenols is not an accurate method for measuring anti-inflammatory properties, we are recommending to determine the concentration of polyphenols in mango preparations by quantifying low molecular weight compounds using gallic acid or mono-galloylglucoside and quantifying the higher molecular weight fraction (gallotannins) using penta-galloylglucoside. This will allow for a more accurate representation of potential in vivo benefits.

Finally, in context with Objective 2 we have discovered that the bioactivities of mango polyphenols are not solely driven by the composition of monomers versus polymers. We are finding that upon consumption certain gallotannin bonds can undergo both enzymatic and non enzymatic hydrolysis of *m*-depside bonds to yield free gallic acid. This shows a complex relationship between the polyphenolic content in the native fruit to what happens when it is consumed as the human intestinal track.

The first and second manuscripts derived from this objective have been published:

Krenek KA, Barnes RC, Talcott ST. *Phytochemical composition and effects of commercial enzymes on the hydrolysis of gallic acid glycosides in mango (*Mangifera indica L. *cv. 'Keitt') pulp*. J Agric Food Chem. 2014 Oct 1;62(39):9515-21. doi: 10.1021/jf5031554. Epub 2014 Sep 18.

Barnes RC, Krenek KA, Meibohm B, Mertens-Talcott1 SU, Talcott ST. *Urinary Metabolites from Mango (Mangifera indica L. cv. Keitt) Galloyl Derivatives and In Vitro Hydrolysis of Gallotannins in Physiological Conditions.* Mol Nutr Food Res. 2015 Dec 7. doi: 10.1002/mnfr.201500706. [Epub ahead of print] PMID: 26640139

Benefits to the Mango Industry: By defining a more relevant standard of quantification of mango polyphenols, it is possible to perform studies investigating the health benefits of mangos where the quantified amount of polyphenols relates more to the magnitude of the observed health benefits. This will help scientific studies to **a)** be more consistent in quantifying mango polyphenols and **b)** will yield research data that are expected to be more consistent regarding the determination of the amount of mango polyphenols required for a certain health benefit, e.g. anti-inflammatory effects**, c)** will help to better compare scientific research studies. More consistent research studies will help mango

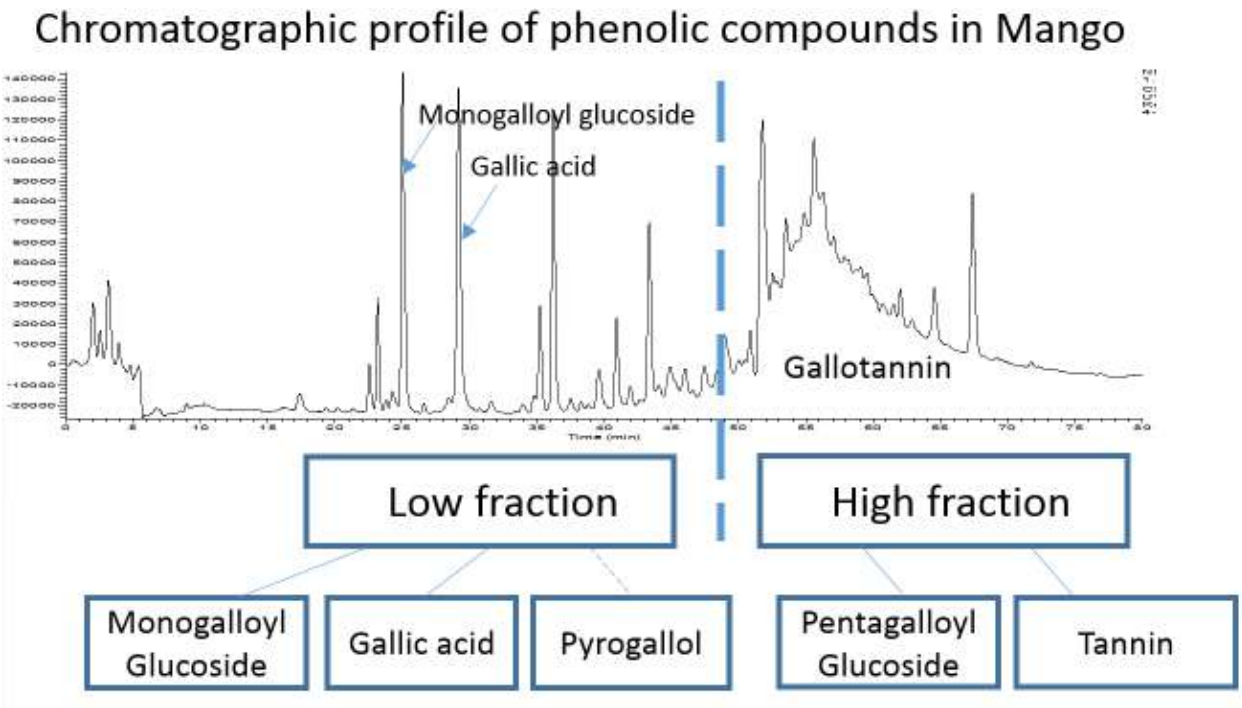
fruit to be more readily recognized as fruit with superior health benefits by consumers and researchers.

Future Studies:
If able we would like to continue to investigate the hydrolysis of galloyl derivatives, specifically the gallotannins, due to enzymes generated by bacteria in the human colon. Some bacteria in the colon are hypothesized to produce tannase an enzyme capable of cleaving *m* or *p* – depside and ester bonds from gallotannins. Thus the human microbiome has the potential to influence the poylphenols found native in fruit and could hydrolyze large tannins (6GG – 12GG) to produce penta-galloyl glucose along with gallic acid which we have shown to exhibit anti-inflammatory activites. In addition these enzymes may degrade the penta-galloyl glucose into tannins with 2-4 galloyl groups at different hydroxyl positions on glucose which could give tannins different bioactive properties.

STUDY DETAILS

Fruit Source: Five cultivars of mango (Ataulfo, Keitt, Kent, Haden, and Tommy Atkins) were acquired and delivered to the Texas A&M Department of Nutrition and Food Science. Fruit were allowed to ripen under ambient conditions, and fruits of the same cultivar were then manually peeled, deseeded, and homogenized.

Chemical Analysis: Chemical analysis was performed parallel to investigating the anti-inflammatory activities of mango polyphenols. For analysis of polyphenolics in mango pulp, three 10g samples of pulp were extracted three times with 30mL of 1:1 methanol:acetone. The different samples were run in independent reaction vessels, and solvents were evaporated under vacuum at 45 ^{o}C. High and low molecular weight fractions were separated by the use of Sephadex LH-20. Mango extracts were loaded onto the column in ethanol. High molecular weight polyphenols stuck to the column and were eluted off with 80% acetone. Final extracts were brought up to a known volume in water acidified with 0.1% formic acid. Total Soluble Phenolics (TSP) was quantified by use of the Folin-Ciocalteu Assay with results reported in Gallic Acid Equivalents. More detailed quantifications of gallic acid, mono-galloylglucose, and penta-galloylglucose derivatives were quantified using HPLC-MS/MS.



Bio-molecular assays: The anti-inflammatory activities of polyphenolics from five mango cultivars were investigated. Mango fractions produced from the previous chemical analysis were treated to lipopolysaccharide (LPS)-treated human CCD-18Co colon-myofibroblast cell lines. The mRNA levels encoding the inflammatory markers, NFkB, TNFa, IL-1B, and IL-6 were measured using RT-PCR.

RESULTS

Chemical Analysis

	Gallic Acid	Ester-MGG	Ether-MGG	Gallotannins
Ataulfo	4.33 ± 0.91	37.9 ± 0.42	0.5 ± 0.07	608 ± 193
Tommy Atkins	2.26 ± 0.43	5.07 ± 0.2	2.29 ± 0.38	274 ± 12
Keitt	1.14 ± 0.02	5.94 ± 0.32	1.02 ± 0.07	61.9 ± 7.3
Haden	2.02 ± 0.16	7.16 ± 0.45	2.18 ± 0.06	80.3 ± 8.2
Kent	2.15 ± 0.14	8.26 ± 0.52	0.93 ± 0.06	25.4 ± 6.2

Table 1. Quantification of Individual Polyphenolics in five mango varieties reported as mg / 200 g of fruit ± SEM.

Quantitation of individual phenolics using standards seems to be superior for measuring potential bioactivity compared to an overall chemical reaction assay such as the Folin's Ciocalteu. Using HPLC-MS techniques, individual phenolics can be separated, characterized and quantified. (Table 1) show the concentrations of gallic acid, mono galloyl glucosides, and gallotannins found in the five varieties of mango studied.

A detailed characterization and quantification of the phytochemistry of the mango pulp is important to truly understand and possibly predict in-vivo mechanisms behind the potential health benefits of mango consumption. Once the mechanisms of action are understood involving mango polyphenols and health knowing how much of specific polyphenol is in mango will give consumers and idea of how many they would need to consume to get appropriate levels of the compound.

ANALYSIS of INFLAMMATION BIOMARKERS

When five cultivars of mango extracts (Ataulfo, Keitt, Kent, Haden, and Tommy Atkins) were treated to LPS-treated CCD-18 cell lines, Keitt and Kent extracts significantly suppressed the expression of NF-kB, TNF-a, and IL-1B mRNA, whereas, Ataulfo and Haden suppressed the expression of IL-6 mRNA (Figure 4).

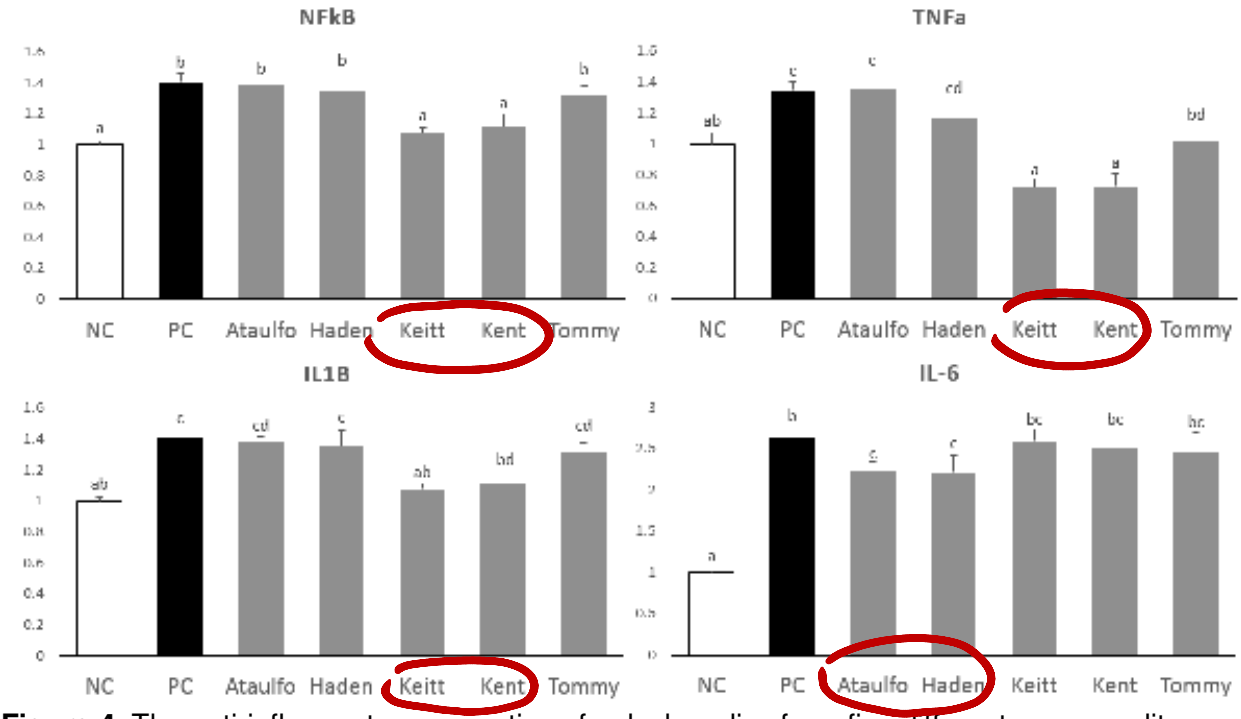


Figure 4. The anti-inflammatory properties of polyphenolics from five different mango culitvars. The cells were treated with the mango extract (20 mg GAE/L) and LPS (1ug/ml) for 3h. The results were expressed as the mean ± SEM (n=3). The different letters indicate significance at $p<0.05$.

The most effective Keitt and Kent polyphenolics were fractionated into low and high molecular weight fractions. Gallic acid, mono-, di, and tri-galloyl glycosides were included in the low fraction, and tetra-, penta- galloyl glycosides, and tannins with six to twelve galloyl groups were present in the high fraction. When the low and high fractions

of Keitt and Kent were treated, the low fraction suppressed the levels of TNFa and IL-6 mRNA levels, whereas high fraction suppressed the levels of all the markers compared to LPS-treated cells (**Figure 5**). When the predominant compounds, gallic acid in the low fraction and penta-galloyl glycosides in the high fraction, were treated to LPS-treated cells, penta-galloyl glycosides showed more suppressive effects on mRNA expression of inflammatory markers.

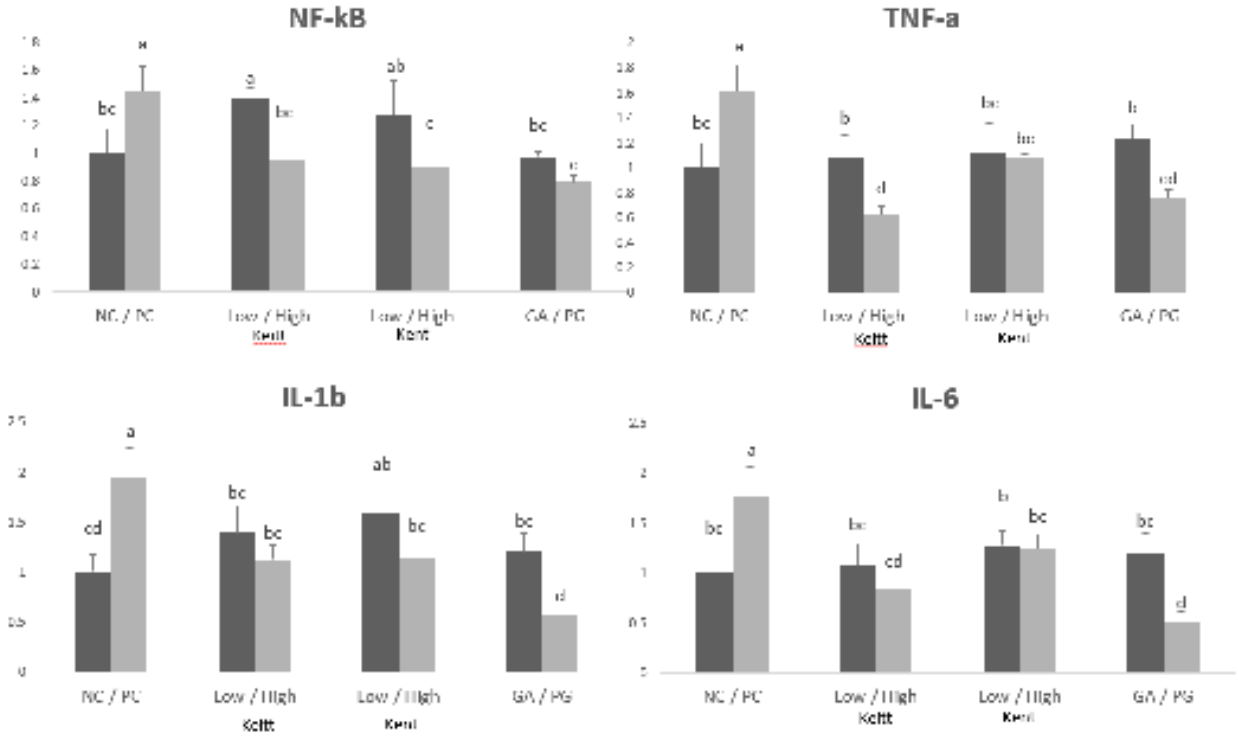


Figure 5. The anti-inflammatory properties of mango polyphenolic fractions separated by molecular weight. Cells were treated with the low and high fractions of mango Keitt and Kent, gallic acid (GA), and penta-galloylglucose (PG), (20 mg GAE/L) and LPS (1ug/ml) for 3h. The results were expressed as the mean ± SEM (n=3). The different letters indicate significance at $p<0.05$.

In order to determine additional details regarding the correlation of polyphenol composition and magnitude of health benefits, single primary mango polyphenolics including gallic acid, mono-galloyl glycoside, pyrogalloyl, penta-galloyl glycosides, and a complex tannin mixture were treated at the same ppm or same molar concentration to LPS-treated cells. Penta-galloylglucose showed the most suppressive effects among the compounds when the same ppm was treated. When tested at the same molar concentration treatment, the effectiveness of penta-gallo-glucoside was decreased, and gallic acid, monogalloyl glucoside, and pyrogallol suppressed the levels of IL-1B and IL-6.This indicates that on a molecular level the low molecular weight species are more

effective (**Figure 6**)/We are going to propose to investigate this metabolite and other metabolites more closely in our follow-up proposal.

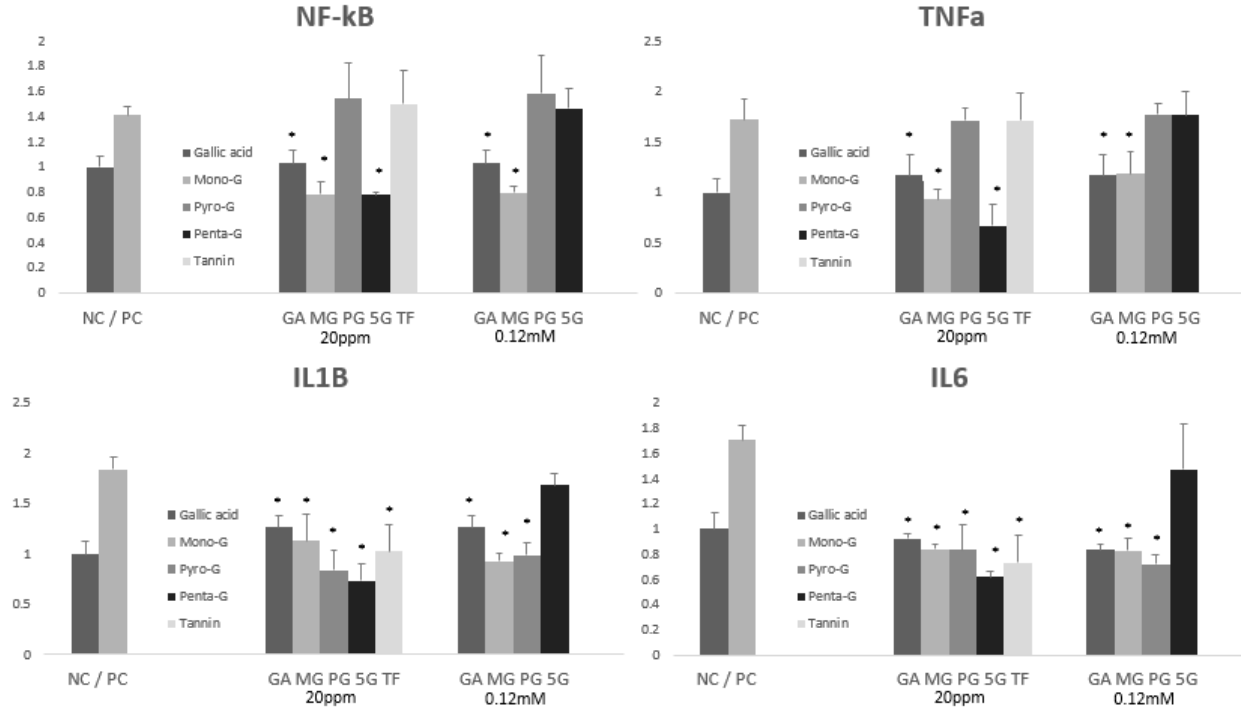


Figure 6. The anti-inflammatory properties of primary mango polyphenolics. The cells were treated with the primary mango polyphenolics (20 mg GAE/L or 0.12mM) and LPS (1ug/ml) for 3h. The results were expressed as the mean ± SEM (n=3). The different letters indicate significance at $p<0.05$.

Statistical correlation analyses was performed between the content of polyphenols in the five mango varieties and the levels of inflammatory markers and it was found that there was not one single compound solely responsible for the anti-inflammatory efficacy of mango. In contrast, likely synergistic effects seem to take place. When synergy analysis of selected single compounds was performed only mild synergistic or additive effects were observed. However, the compounds found to most highly correlate with reduction of all biomarkers were 5GG and 6GG. For individual biomarkers gallic acid correlated with NFKB, 5GG, 4GG, and gallic acid correlated with TNFa, and 6GG, 5GG, and gallic acid with IL-1B (Table 2). Overall, gallic acid and penta-galloyol glycoside seem to be most correlated to anti-inflammatory effects than other compounds. For this reason, we will in future report any mango extract treatment quantified as gallic acid (low molecular compounds) and penta-galloylglucoside (tannins).

Table 2. Pearson correlation between the contents of five mango varieties and the reducing rate of levels of inflammatory markers

	NFkB	TNFa	IL-1B	IL-6
Gallic acid	-0.69	-0.80	-0.69	0.66
Mono-G	-0.50	-0.70	-0.50	0.56
3GG	-0.54	-0.73	-0.54	0.55
4GG	-0.65	-0.80	-0.65	0.60
5GG	-0.70	-0.81	-0.70	0.58
6GG	-0.71	-0.69	-0.71	0.41
7GG	-0.68	-0.79	-0.68	0.53
8GG	-0.64	-0.78	-0.64	0.55
9GG	-0.59	-0.76	-0.59	0.56
10GG	-0.56	-0.74	-0.56	0.56

Non-Enzyamtic Hydrolysis of Gallotannins: The stability of mango gallotannins (312 mg/L) were evaluated *in vitro* to investigate the influence of a low acid environment at pH 7.4 and 37°C on the chemical hydrolysis of gallotannins and subsequent release of free gallic acid. After 4 h exposure to these conditions, the average molecular weight distribution of gallotannins had shifted from a majority of relatively higher molecular weight species (≥8GG) at 63% of the total ion count to that of lower molecular weight tannins ranging (4GG – 7GG) at 68% of the total ions (Table 3). For individual gallotannins, a significant difference ($p<0.05$) was observed in the percentage of total ion count for 5GG (7.57% to 14.8%), 6GG (11.5% to 19.6%), and 7GG (16.8% to 21.2%) along with a precipitous decrease in 9GG (21.3% to 9.77%) and 12GG (1.59% to undetectable).

The production of free GA (23.1 mg/L) and digallic acid (16.0 mg/L) was an additional indication that physiological conditions alone in the human intestines are sufficient to non-enzymatically hydrolyze GTs. GA alone is inherently unstable under human physiological conditions, with oxidation and condensation occurring rapidly under these conditions [19]. The instability of both GA and GTs under low acid environments, even under anaerobic conditions, shows a complex relationship between the release of GA and its potential to be either metabolized, absorbed, or degraded. The presence of both GA and digallic acid after 4 h incubation may indicate hydrolysis of both *m*-depside and galloyl-glucose bonds at physiological pH and temperature conditions. As gallic acid was found to exhibit strong anti-inflammatory effects it may be beneficial that the tannins hydrolysis in physiological conditions. As gallic acid is smaller it is more likely to enter a cell where it can exhbit its anti-inflammtroy effects.

Table 3. Changes in the distribution of individual gallotannin ions expressed as a percentage of total ion count when incubated for 0 and 4 h at pH 7.4 and 37°C.

				% Total Ion Signal[1,2]	
Abb.	**Compound I.D.**	**$[M-H]^-$ (*m/z*)**	**$[M-2H]^{2-}$ (*m/2z*)**	**T_0**	**T_4**
4GG	tetra-*O*-galloylglucose	787	393	0.94 ± 0.2[a]	9.44 ± 1.7[b]
5GG	penta-*O*-galloylglucose	939	469	7.57 ± 1.2[a]	14.8 ± 0.8[b]
6GG	hexa-*O*-galloylglucose	1091	545	11.5 ± 0.8[a]	19.6 ± 0.6[b]
7GG	hepta-*O*-galloylglucose	1243	621	16.8 ± 1.1[a]	21.2 ± 0.5[b]
8GG	octa-*O*-galloylglucose	1395	697	20.7 ± 1.6[a]	17.1 ± 1.2[a]
9GG	nona-*O*-galloylglucose	1547	773	21.3 ± 1.8[a]	9.77 ± 1.0[b]
10GG	deca-*O*-galloylglucose	1699	849	15.3 ± 3.1[a]	6.21 ± 0.9[b]
11GG	undeca-*O*-galloylglucose	1851	925	4.44 ± 1.1[a]	1.91 ± 0.3[a]
12GG	dideca-*O*-galloylglucose	2003	1002	1.59 ± 0.5[a]	nd[b]

Conclusion: From these investigations it was found that all polyphenolics in mango contribute to its anti-inflammatory effects with gallic acid on a molar level contributing the most.

Out of the five varieties studied Keitt and Kent displayed superior anti-inflammatory activities compared to the other cultivars. We recommend that future analyses of mango pulp quantify the amount of gallic acid and penta galloyl glucose found natively in the fruit.

Additionally, it is recommended that the amount of gallic acid that can be hydrolyzed from gallotannins in physiological conditions be quantified in addition to the amount native in the fruit as these can be additional source of in vivo gallic acid that could exhibit anti-inflammatory properties

Our most recent findings indicate that native and bound gallic acid is subject to microbial conversion to pyrogallol-derivatives in the intestinal tract where it has anti-inflammatory and other beneficial effects. For this reason, cultivars that are high in complex tannins may provide beneficial effects after intestinal and microbial digestion. These aspects are currently being investigated in samples from Objective 2 in the currently ongoing metabolism-objective.

References

1. Ross JA, Kasum CM: **Dietary flavonoids: bioavailability, metabolic effects, and safety**. *Annual review of Nutrition* 2002, **22**(1):19-34.
2. Tharanathan R, Yashoda H, Prabha T: **Mango (Mangifera indica L.),"The king of fruits"—An overview**. *Food Reviews International* 2006, **22**(2):95-123.
3. Galán Saúco V: **Mango production and world market: Current situation and future prospects**. In: *VII International Mango Symposium 645: 2002*; 2002: 107-116.
4. Evans EA: **Recent trends in world and U.S. mango production, trade, and consumption** *University of Florida: Cooperative Extension Services in the Institute of Food and Agricultural Sciences* 2008.

5. Schieber A, Ullrich W, Carle R: **Characterization of polyphenols in mango puree concentrate by HPLC with diode array and mass spectrometric detection**. *Innovative Food Science & Emerging Technologies* 2000, **1**(2):161-166.
6. Barreto JC, Trevisan MT, Hull WE, Erben G, de Brito ES, Pfundstein B, Würtele G, Spiegelhalder B, Owen RW: **Characterization and quantitation of polyphenolic compounds in bark, kernel, leaves, and peel of mango (Mangifera indica L.)**. *Journal of agricultural and food chemistry* 2008, **56**(14):5599-5610.
7. Ishimaru K, Nonaka G-I, Nishioka I: **Gallic acid esters of< i> proto</i>-quercitol, quinic acid and (−)-shikimic acid from< i> quercus mongolica</i> and< i> q. myrsin aefolia</i>**. *Phytochemistry* 1987, **26**(5):1501-1504.
8. Masaki H, Atsumi T, Sakurai H: **Hamamelitannin as a new potent active oxygen scavenger**. *Phytochemistry* 1994, **37**(2):337-343.
9. Hagerman AE: **Hydrolyzable Tannin Structural Chemistry**. *Tannin Handbook (http://www users muohio edu/hagermae/tannin pdf)* 2002.
10. Li L, Shaik AA, Zhang J, Nhkata K, Wang L, Zhang Y, Xing C, Kim S-H, Lü J: **Preparation of penta-O-galloyl-β-d-glucose from tannic acid and plasma pharmacokinetic analyses by liquid–liquid extraction and reverse-phase HPLC**. *Journal of Pharmaceutical and Biomedical Analysis* 2011, **54**(3):545-550.
11. Martinez G, Delgado R, Perez G, Garrido G, Nunez Selles AJ, Leon OS: **Evaluation of the in vitro antioxidant activity of Mangifera indica L. extract (Vimang)**. *Phytotherapy research : PTR* 2000, **14**(6):424-427.
12. Garrido G, Gonzalez D, Lemus Y, Garcia D, Lodeiro L, Quintero G, Delporte C, Nunez-Selles AJ, Delgado R: **In vivo and in vitro anti-inflammatory activity of Mangifera indica L. extract (VIMANG)**. *Pharmacological research : the official journal of the Italian Pharmacological Society* 2004, **50**(2):143-149.
13. Marquez L, Perez-Nievas BG, Garate I, Garcia-Bueno B, Madrigal JL, Menchen L, Garrido G, Leza JC: **Anti-inflammatory effects of Mangifera indica L. extract in a model of colitis**. *World journal of gastroenterology : WJG* 2010, **16**(39):4922-4931.
14. Masibo M, He Q: **Major mango polyphenols and their potential significance to human health**. *Comprehensive Reviews in Food Science and Food Safety* 2008, **7**(4):309-319.
15. Al-Halabi R, Bou Chedid M, Abou Merhi R, El-Hajj H, Zahr H, Schneider-Stock R, Bazarbachi A, Gali-Muhtasib H: **Gallotannin inhibits NFkB signaling and growth of human colon cancer xenografts**. *Cancer biology & therapy* 2011, **12**(1):59-68.
16. Kim SH, Jun CD, Suk K, Choi BJ, Lim H, Park S, Lee SH, Shin HY, Kim DK, Shin TY: **Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells**. *Toxicological sciences : an official journal of the Society of Toxicology* 2006, **91**(1):123-131.
17. Erdelyi K, Kiss A, Bakondi E, Bai P, Szabo C, Gergely P, Erdodi F, Virag L: **Gallotannin inhibits the expression of chemokines and inflammatory cytokines in A549 cells**. *Molecular pharmacology* 2005, **68**(3):895-904.
18. Ribeiro S, Barbosa L, Queiroz J, Knödler M, Schieber A: **Phenolic compounds and antioxidant capacity of Brazilian mango (< i> Mangifera indica</i> L.) varieties**. *Food chemistry* 2008, **110**(3):620-626.
19. Friedman, M., Jürgens, H. S., **Effect of pH on the Stability of Plant Phenolic Compounds.** *Journal of Agricultural and Food Chemistry* 2000, *48*, 2101-2110.