UNIVERSITY OF SÃO PAULO
School of Pharmaceutical Science
Graduate Program in Food Science
Field of Study Bromatologie

Biomarker exposure of phenolic compounds from grumixama (*Eugenia brasiliensis* Lam.) in healthy human model: Metabolomic approach

Luciane de Lira Teixeira

Thesis for obtaining DOCTORAL degree in Science
Advisor: Profa. Dra. Neuza Mariko Aymoto Hassimotto

São Paulo
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Thesis for obtaining doctoral degree in Science

Profa. Dra. Neuza Mariko Aymoto Hassimotto

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Resumo

TEIXEIRA, L.L. **Identificação de biomarcadores de exposição de compostos fenólicos de grumixama (*Eugenia brasiliensis* Lam.): Abordagem metabolômica.** 2016. 157f. Tese (Doctoral degree) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

O consumo de frutas e verduras na dieta está associado à redução da incidência de doenças crônicas não transmissíveis entre eles câncer, síndrome metabólica e doenças cardiovasculares. A atividade biológica atribuída à ingestão desses alimentos é relacionada principalmente à presença de compostos bioativos, entre eles os compostos fenólicos, tais como flavonoides e elagitaninos. Contudo, a biodisponibilidade e a influência destes compostos no metabolismo humano não estão estabelecidas. Assim, o objetivo geral do presente estudo foi investigar as alterações no metaboloma humano decorrente da ingestão de uma fonte rica em compostos fenólicos, o suco da grumixama roxa (*Eugenia brasiliensis* Lam.), buscando identificar possíveis pontos de regulação do metabolismo. Para isto, a grumixama, variedades amarela e roxa, foram caracterizadas quanto ao seu perfil de compostos fenólicos e administradas na forma de suco, em dose única, a voluntários saudáveis. A grumixama roxa se mostrou rica em antocianinas e elagitaninos, principalmente cianidina 3-*O*-glicosídeo e a strictinina, respectivamente. Para o ensaio clínico, 15 voluntários saudáveis consumiram, em dose única, suco de grumixama roxa (10 ml de suco/kg de peso corporal). Amostras de plasma e urina foram coletadas em diferentes tempos durante 24 h após ingestão e analisados por CG-MS e LC-ESI-MS/MS. Os metabólitos exógenos excretados na urina foram identificados como urolitinas e ácidos fenólicos, derivados da degradação, principalmente, pela microbiota dos elagitaninos e das antocianinas, respectivamente. Quatro urolitinas (A, B, C e D) foram encontradas na urina, principalmente como metabólitos de fase II, detectados a partir de 4 h após a ingestão do suco com aumento na concentração observado até 24h. Além disso, quatro ácidos fenólicos foram identificados, destes o ácido hipúrico como majoritário. 114 metabólitos, entre eles, 17 aminoácidos, 47 ácidos orgânicos, 7 outras classes de compostos e 43 compostos desconhecidos foram identificados por CG-MS, para os tempos de coleta de urina antes da ingestão de suco de grumixama (T0) e para os períodos de 1-2 h e 2-4 h após a ingestão. A OPLS-DA foi utilizada para descriminar os metabólitos alterados pela ingestão de suco de grumixama. A análise das vias metabólicas mostrou que a ingestão do suco de grumixama influenciou principalmente em três vias metabólicas: metabolismo do glioxilato e dicarboxilato (*up-regulated*), da beta-alanina (*down-regulated*) e da fenilalanina (*up-regulated*), sendo essas direcionadas ao metabolismo energético. Além disso, os extratos de grumixama roxa e amarela também foram testados em modelo animal (camundongo C57BL/6J) de obesidade e resistência à insulina induzido por uma dieta rica em lipídeos e açúcares. O tratamento com os extratos, concomitante à dieta e durante 8 semanas, promoveu modulação significativa do metabolismo lipídico. Como conclusões, a grumixama roxa mostrou ser uma boa fonte de antocianinas e elagitaninos, e a interação entre metabólitos oriundos da ingestão do fruto e dos metabólitos endógenos podem estar relacionados com alterações nos metabolismos de aminoácidos e energético. No entanto, mais estudos são necessários para elucidar e validar as hipóteses geradas.

Palavras-chave: grumixama, antocianinas, elagitaninos, urolitinas, aminoácidos, ácidos orgânicos, metabolômica.

ABSTRACT

TEIXEIRA, L.L. **Biomarker exposure of phenolic compounds from grumixama (Eugenia brasiliensis Lam.) in healthy human model: Metabolomic approach.** 2016. 157f. Tese (Doutorado) - School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2016.

The fruits and vegetables intake has been associated to the reduction of chronic non-communicable disease incidence, such as cancer, metabolic syndrome and cardiovascular diseases. The biological activities attribute to them has been related mainly to the phenolic compounds, such as flavonoids and ellagitannins, presents in their composition. However, the bioavailability and influence of these compounds under human metabolism still unclear. Thus, the objective of the present study was investigate changes in human metaboloma as a result of the acute intake of the polyphenol-rich source from purple grumixama juice (*Eugenia brasiliensis* Lam.), searching to identify possible sites of metabolic regulation. In this way, purple and yellow grumixama varieties were characterized to polyphenol profile, and a single dose of the purple grumixama juice was administered to healthy human. The purple grumixama showed be a good source of anthocyanins and ellagitannins, mainly cyanidin 3-*O*-glucoside and strictinin, respectively. In the clinical trial, 15 healthy subjects intake a single dose of purple grumixama juice (10 ml of juice/kg of body mass). Plasma and urine samples were collected, before and after intake (over 24 h), and analyzed by GC-MS and LC-MS. The exogenous metabolites excreted and identified in urine samples by LC-MS were identified as urolithins and phenolic acids, gut microbiota catabolites of ellagitannins and anthocyanins, respectively. Four urolithins were detected beginning excretion 4 h after juice intake, increasing over 24 h. Furthermore, four phenolic acids were identified, being the hippuric acid the majority of them. 114 metabolites were identified to urine collection points before and after intake (1-2 h and 2-4 h) by CG-MS, being 17 amino acids, 7 other classes, 47 organic acids and 43 unknown compounds. A OPLS-DA discriminated the metabolites changed by the grumixama juice intake. The pathway analysis showed that juice intake influenced mainly three metabolic pathways: glyoxylate and dicarboxylate metabolism (up-regulated), beta-alanine metabolism (down-regulated), and phenylalanine metabolism (up-regulated), being these pathways related to energetic metabolism. Furthermore, the purple and yellow grumixama fruits extracts were evaluated in animal model of obesity and insulin resistance (C57BL/6J mice) induced by high fatty and high sugar diet. The treatment, during 8 weeks, promoted lipid metabolism modulation. As conclusions, purple grumixama showed to be a good source of anthocyanins and ellagitannins, and the interaction among the metabolites from fruits and endogenous metabolites can be related to changes in energetic metabolism and amino acid metabolism. However, more studies are necessary to elucidate and validate these hypotheses.

Key-words: grumixama, anthocyanins, ellagitannins, amino acids, organic acids, metabolimic.

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List of Abreviations

GJ	Grumixama juice
ET	Ellagitannins
LC-MS	Liquid chromatography coupled to mass spectrometry
C3G	Cyanidin 3-*O*-glucoside
HPLC-DAD	High performance liquid chromatography coupled to a diode array detector
LC-ESI-MS/MS	Liquid chromatography coupled to electrospray ionization mass spectrometry
LC-qTOF-MS	Liquid chromatography coupled to Q Time-of-flight mass spectrometry
VA	Vanillic acid
PCA	Protocatechuic acid
iVA	Isovanillic acid/homovanillic acid
HA	Hippuric acid
EA	Ellagic acid
CFSE	Carboxyfluorescein diacetate succinimidyl ester.
DMSO	Dimethyl sulfoxide
AUC	Area under curve
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
GC-MS	Gas chromatography-mass spectroscopy
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ORAC	Oxygen radical absorbance capacity
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
PLS-DA	Partial Least Squares Discriminant Analysis
NAFLD	Non-Alcoholic Fatty Liver Disease
HFHS	High Fat and High Sugar
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
VLDL	Very Low Density Lipoprotein

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
DPPH	2,2-diphenylpicrylhydrazyl
ET-Yb	Ellagitannins-Ytterbium
FW	Fresh Weight
DW	Dry Weight
HHDP	Hexahydroxydiphenoyl group
BA	Benzoiz acid
HHA	Hydroxyhippuric acid
HPA	Hydroxyphenylacetic acid
FA	Ferulic acid
CouA	Coumaric acid
3-HBA	3-Hydroxybenzoic acid

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1 INTRODUCTION

The healthy conditions have been associated to the food quality intake during the life. The intake of fruits and vegetables has been related to the reduction of incidence of chronical diseases such as cancer, metabolic syndrome and cardiovascular diseases (PAREDES-LÓPEZ *et al.*, 2010). The health benefits associated to fruits and vegetables-rich diets have been attributed to bioactive compounds that can be essential nutrients, such as ω3-fatty acids and vitamin C, and non-essential nutrients, such as carotenoids and phenolic compounds (CLIFFORD and BROWN, 2006; SCALBERT *et al.*, 2014).

Nutritional intervention studies have been looking for explanations to biological activities attributed to nutrients and non-nutrients bioactive compounds and their role on health promotion (SCALBERT *et al.*, 2014). These bioactive compounds can interact with human organism playing an important role by modulating enzyme systems (SZOTAKOVA *et al.*, 2013), transcription factors (SALAMONE *et al.*, 2016), proteins expression, signaling cascade (EDWARDS *et al.*, 2015) and showing more directly antioxidant activity (DÍAZ-RUBIO *et al.*, 2015).

The metabolomic approach shows to be a powerful tool to helpful to explain these biological activities, attributed to nutrients and non-nutrients compounds.

Among the “omics” (Proteomic, Genomic, Metabolomic and Transcriptomic), the metabolomic approach showed to be the most directly related to possible biological effects of the nutrition intervention (HERRERO *et al.*, 2012; SCALBERT *et al.*, 2014), due to it search for metabolites, which could express an end product of the biological system. Metabolomic studies are used to identify biomarker of exposure from food and endogenous metabolome, trying to understand the interaction among them (SCALBERT *et al.*, 2014) and their interaction with also gut microbiota (VERNOCCHI *et al.*, 2012). Usually, the metabolites are separated in endogenous and exogenous compounds (SCALBERT *et al.*, 2014), but it also possible to identify metabolites that can be both or be formed from gut microbiota (VERNOCCHI *et al.*, 2012, JACOBS *et al.*, 2012). The metabolomic studies can be classified in targeted and untargeted studies, being the search for metabolites specific and unspecific, respectively. This classification is a direct consequence of sample preparation and extraction which will direct the findings to specific (targeted) or unspecific metabolites (untargeted).

Among the non-nutrients, bioactive compounds, the phenolic compounds present in high amount in fruits and vegetables have been associated to the biological activities, such as

flavonoids and ellagitannins (ETs) (PAREDES-LÓPEZ *et al.*, 2010, LLORACH *et al.*, 2014; DÍAZ-RUBIO *et al.*, 2015). Flavonoids have showed modulate some important pathways as amino acid metabolism (JACOBS *et al.* 2012) and steroid biosynthesis (MEDINA *et al.*, 2013). However, most of the studies involving metabolomic approach search for exogenous metabolites (LLORACH *et al.*, 2014).

Several biological activities have been attributed to phenolic compounds, including anti-inflammatory, antioxidant and anti-proliferative activities and apoptosis (LI *et al.*, 2009; LI *et al.*, 2011). However, the biological relevance of anthocyanin and ETs intake have been discussed extensively due to the low bioavailability of the native structure, and the beneficial effects have been attributed to the catabolites formed by gut microbiota degradation (KAY, KROON and CASSIDY, 2009; WILLIAMSON and CLIFFORD, 2010). The main catabolites associated with anthocyanins and ETs metabolism are phenolic acids (VITAGLIONE *et al.*, 2007; CZANK *et al.*, 2013) and urolithins (GONZALEZ-BARRIO *et al.*, 2010; GARCÍA-VILLALBA *et al.*, 2013; GARCÍA-MUÑOZ *et al.*, 2014), respectively, which shows the importance of gut microbiota in polyphenol bioavailability assessment.

Anthocyanin bioavailability has been found to be less than 1% of intake (DEL RIO *et al.*, 2013) (considering 690 mg intake) (WU *et al.*, 2002). Overall, 70% disappeared from the gastrointestinal tract after 4 h through gut microbiota action and also by spontaneous degradation by neutral pH (VITAGLIONE *et al.*, 2007; FANG, 2014). When the catabolites, such as phenolic acids, were considered, the bioavailability increased to approximatly 12% (CZANK *et al.*, 2013).

Similarly, ETs were not bioavailable and were metabolized by gut microbiota to form dibenzopyranone compounds, which are also known as urolithins (GONZALEZ-BARRIO *et al.*, 2010; GARCÍA-VILLALBA *et al.*, 2013; TULIPANI *et al.*, 2012; SELMA *et al.*, 2014; LUDWIG *et al.*, 2015). The urolithins can be transformed by the ellagic acid (EA) that is already present in the food matrix or by the EA formed by the action of gut microbiota under the ETs (GARCÍA-VILLALBA *et al.*, 2013; GARCÍA-MUÑOZ *et al.*, 2015).

The grumixama (*Eugenia brasilienses* Lam.) is a cherry, approximately 2.0 cm in diameter, containing one or many seeds, and is somewhat sweet. It belongs to the Myrtaceae family and is a native fruit of the southern and southeastern regions of the Atlantic Forest of Brazil. Three varieties were recognized by Cambessèdes (1832−1833) according to their fruit colors: The α-variety or purple fruit is the most common and is known as the Brazilian cherry (MORENO *et al.*, 2007; FLORES *et al.*, 2012). The β-variety has red fruit, and the γ-variety has white fruit, although it was also described by Mattos (1984) as being yellow (MORENO

et al., 2007). Despite belonging to the same species, the yellow and purple varieties show distinct chemotypes with respect to the terpene profiles in the leaves and fruits (MORENO *et al.*, 2007). Grumixama is rarely consumed as a fresh fruit, but is commonly used to produce fruit juice and frozen pulp, with the pulp frequently containing a mixture of the purple and yellow forms. Although still limited, commercial cultivation has begun in recent years, and this plant represents economic potential due to the attractive sensory attributes and phenolic compounds in its composition, including ellagitannins and flavonoids (REYNERTSON *et al.*, 2008; FLORES *et al.*, 2012; ABE *et al.*, 2012).

In general, fruits belonging to this family are known to be good sources of bioactive compounds (REYNERTSON *et al.*, 2008; FRACASSETTI *et al.*, 2013). The purple grumixama fruit was shown to be rich in anthocyanins, mainly cyanidin-3-glucoside, and carotenoids, mainly monohydroxy carotenoids such as all-trans-β-cryptoxanthin (Flores *et al.*, 2012; SILVA *et al.*, 2014). High antioxidant capacity (REYNERTSON *et al.*, 2008) and anti-inflammatory activity (FLORES *et al.*, 2012) have been demonstrated. However, no information about phenolic composition, the antioxidant capacity, or other biological activities of the yellow fruit was previously available.

As a member of the Myrtaceae family, in addition to the flavonoids, grumixama would also be expected to be rich in ETs and EA derivates, another class of phenolic compounds. This class of phenolics is relatively uncommon in dietary fruits and vegetables, although a few berries, such as strawberry, raspberry, blackberry, and pomegranate, have substantial contents of these compounds (KASIMSETTY *et al.*, 2010; LANDETE *et al.*, 2011; SANGIOVANNI *et al.*, 2013). It has been suggested that ETs as well as flavonoids may prevent chronic diseases such as cancer and cardiovascular diseases. These protective activities are attributed to both classes of phytochemicals, which are thought to provide antiproliferative (ASCACIO-VALDÉS *et al.*, 2011), anti-inflammatory, and antioxidant activities (SANGIOVANNI *et al.*, 2013; MORTON *et al.*, 2000; NIJVELDT *et al.*, 2001; CLIFFORD and BROWN, 2006) and to function as glycemic regulators (ASCACIO-VALDÉS *et al.*, 2011; ŞTEFĂNUŢ *et al.*, 2013; SEPÚLVEDA *et al.*, 2011).

Several biological activities have been attributed to both phenolic compounds, including anti-inflammatory, antioxidant and anti-proliferative activities and apoptosis (LI *et al.*, 2009; LI *et al.*, 2011). Among the biological activities, the antiproliferactive activity of metabolites in breast cancer cells and the effects of poplyphenols against non-alchoolic fatty liver disease had been explored in this thesis.

The anti-tumor activity of anthocyanins and ETs from many fruit and vegetable extracts have been explored in mammal cells. Berries such as blackberry, raspberry, blueberry and strawberry have demonstrated inhibitory activity and apoptosis in oral (KB, CAL-27), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) cancer cells (SEERAM *et al.*, 2006). Anthocyanin has been found in raspberry extract, but the anti-proliferative activity on HeLa cancer cells was mainly associated with ETs (ROSS *et al.*, 2007). In addition, EA also demonstrated anti-proliferative activity against lung (NCI 460), breast (MCF-7) and ovary (OVCAR03) cancer cells, and the activity was more efficient compared to strawberry and strawberry' ETs extracts (PINTO *et al.*, 2010). Additionally, urolithins strongly inhibited both androgen-dependent and androgen-independent prostate cancer cells (CaP) (KASIMSETTY *et al.*, 2010).

Non-alcoholic fatty liver disease (NAFLD) is extensively reported worldwide and it is related to metabolic syndrome associated to insulin resistance and obesity (ROY *et al.*, 2013; CUI *et al.*, 2014). NAFLD can carry out to development of liver inflammation, steatohepatitis, hepatic fibrosis and cirrhosis (PAN *et al.*, 2015). The pathogenesis of NAFLD has showed be deeply associated to metabolism disorders that promote the energy storage as triglycerides in steatotic hepatocytes in adipocyte-packed vacuoles. Insulin resistance and fatty acid metabolism disorders and inflammatory disorders have been usually associated with NAFLD (BERLANGA *et al*., 2014).

Polyphenol-rich sources can be helpful to inhibit NAFLD disease progression as well as health melioration (SALAMONE *et al.*, 2012; ANHÊ *et al.*, 2014). Phenolic compounds can act by modulation the antioxidant enzymatic systems (ANHÊ *et al.*, 2014), modulation of lipogenesis (SALAMONE *et al.*, 2012), inflammation inhibition (SALAMONE *et al.*, 2016), directly action as antioxidant (DIAZ-RUBIO *et al.*, SALAMONE *et al.*, 2016) and also by change gut microbiota. The gut microbiota composition has been also appointed as an important factor in NAFLD development. Awhere, a gut microbiota more responsive to a high fatty diet increasing de-novo lypogenesis and triglycerides in liver (ROY *et al.*, 2013). However, the mechanisms of polyphenol activity in NAFLD prevention and inhibition progression still under-recognized with much unknowns.

2 OBJECTIVE

2.1 GENERAL OBJECTIVE

Indentify biomarkers exposure of phenolic compounds from grumixama (*Eugenia brasiliensis* Lam) in healthy humans.

2.2 SPECIFIC OBJECTIVES

- Chemical and phenolic characterization of grumixama varieties;
- Study the phenolic compounds bioavailability of purple grumixama in healthy human model;
- Identify endogenous metabolites and their changes after purple grumixama juice intake;

3 PART 1: CHEMICAL AND PHENOLICS CHARACTERIZATION OF GRUMIXAMA FRUITS

3.1 MATERIAL AND METHODS

3.1.1 Chemicals

Fluorescein, 2,2′-azobis(2-methyl-propanimidamide) dihydrochloride (AAPH), 2,2-diphenylpicrylhydrazyl (DPPH), trifluoroacetic acid, ytterbium acetate, sucrose, glucose, fructose, catechin, epicatechin, epigallocatechin gallate, gallocatechin gallate, epicatechin gallate, quercetin, gallic acid, ellagic acid, and proanthocyanidin B1 and proanthocyanidin B2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin 3-glucoside, myricetin, cyanidin-3-glucoside, cyanidin, and delphinidin were purchased from Extrasynthese (Genay, France). Methanol and acetic acid were purchased from Merck Millipore (Darmstadt, Germany). All other chemicals used were of analytical or HPLC grade.

3.1.2 Plant Materials

The fruits of the yellow and purple grumixama were collected in the southeastern and southern regions of Brazil. Twelve kilograms of purple grumixama was acquired by local producers in the Vale do Paraiba (Sítio do Bello farm) located in São Paulo state, and 4 kg of grumixama was provided by Epagri-Itajaı́ located in Santa Catarina state. Both samplings were collected in December 2012 and January 2013. Yellow grumixama (4 kg) was acquired by Sitio do Bello farm in December 2012. The fruits of the purple and yellow grumixama were separated into flesh (F) and seed (S) and named F1 and F2 (from São Paulo state), F3 and F4 (from Santa Catarina state), and S1, S2, S3, and S4, respectively. The samples designated F5 and S5 corresponded to the yellow variety. All fruits were harvest at ripening stage and stored at -70 °C until analysis. Samples were thoroughly homogenized by powdering in liquid nitrogen to perform the analysis.

3.1.3 Physicochemical Analyses

The analysis of the protein content was performed according to the Kjedahl method, using the nitrogen conversion factor of 5.75 for vegetable proteins (AOAC, no. 950.48). The moisture and lipid contents were analyzed according to the methods described by AOAC (AOAC, no. 925.40 and 948.22, respectively) (1995) (VENKATACHALAM *et al.*, 2006). The fiber content was measured according to the method described by AOAC (no. 985.29) (AOAC, 1995; Venkatachalam *et al.*, 2006). The soluble solids (°Brix) were determined using a hand-held refractometer (AOAC, no. 932.12) (AOAC, 1990).

3.1.4 Soluble Sugars Content

The samples were extracted with 80% ethanol (v/v) at 80 °C for 30 min under stirring. The soluble sugars were analyzed by high-performance liquid chromatography (HPLC) coupled to a pulse amperometric detector (PAD) using a one-Dionex DX500 system (Dionex, Thermo Scientific, Waltham, MA, USA), equipped with a CarboPac PA1 column (4 mm × 250 mm) (Dionex), using a mobile phase consisting of 18 mM NaOH, at a flow rate of 1 mL min^{-1} for 25 min. The quantification was based on an external calibration using sucrose, glucose, and fructose (SHIGA *et al.*, 2011).

3.1.5 Total Phenolic Content, Monomeric Anthocyanins, and Antioxidant Capacity

Three replicate samples (0.5 g) were extracted with 70% methanol containing 5% acetic acid (25 mL) using an ultraturrax (Polytron Kinematica GnbH, Kriens-Luzern, Switzerland), followed by 30 min of stirring at 5 °C and then filtration. These extracts were used to assess total phenolic content, monomeric anthocyanin content, and antioxidant capacity. The assays were carried out in triplicate. The total phenolic content was measured using the Folin−Ciocalteu method, and the absorbance was measured at 763 nm (SWAIN *et al.*, 1959). The results were expressed as gallic acid equivalents per 100 g of sample. The monomeric anthocyanin content was determined using the pH differential method (LEE *et al.*, 2005). The monomeric anthocyanin pigment concentration was calculated using the molar absorptivity of cyanidin 3-glucoside (ε = 26900) and expressed as milligrams of cyanidin 3-glucoside equivalents per 100 g of sample. The antioxidant capacity was analyzed using the DPPH (BRAND-WILLIAMS, CUVELIER and BERSET, *et al.*, 1995) and oxygen radical

absorbance capacity (ORAC) methods (PRIOR *et al.*, 2003) and expressed as millimoles of trolox equivalents per 100 g of sample. All results were expressed as fresh weight.

3.1.6 Ellagitannin Content

The samples were extracted with 80% acetone and dried under nitrogen. An aliquot of the extract was added to 2 M trifluoroacetic acid and hydrolyzed at 120 °C for 120 min, according to the method of Giner-Chávez *et al.* (1997) with a slight modification (GINER-CHÁVEZ *et al.*, 1997; PINTO *et al.*, 2010). The hydrolyzed samples were evaporated to dryness under nitrogen, redissolved in methanol, and filtered through a 0.43 μm PTFE filter (Millipore Ltd., Bedford, MA, USA) for HPLC-DAD analysis as described for the phenolic compounds. Ellagic acid (EA) was used to construct the calibration curve, and the results were expressed as milligrams of total EA equivalents per 100 g of sample.

3.1.7 Identification and Quantification of Phenolic Compounds

The samples were extracted with 70% methanol, acidified with 5% acetic acid, using an ultraturrax (Polytron Kinematica GnbH), and filtered. The extract was then concentrated by removal of the methanol under vacuum at 40 °C on a rotary evaporator (Rotavapor RE 120; Buchi, Flawil, Switzerland) prior to application to a solid-phase extraction column. The extract was passed through a polyamide column (1 g) (CC 6, Macherey-Nagel Gmbh and Co., Duren, Germany) previously conditioned with methanol and ultrapure water. The sample was loaded onto the column and washed with ultrapure water. The phenolic compounds were eluted with 0.3% HCl in methanol. The eluates were completely dried using a rotary evaporator under vacuum at 40 °C, resuspended in methanol, and filtered through a 0.45 μm PTFE filter (Millipore Ltd.) for the quantification of flavonoids and phenolic acids by HPLC-DAD analysis (SESTARI *et al.*, 2014).

3.1.8 Analysis of Phenolic Compounds by Reversed-Phase HPLC DAD.

Identification and quantification of the phenolic compounds was performed using a 1260 Infinity Quaternary LC System (Agilent Technologies, USA), with an autosampler and a quaternary pump, coupled to a diode array detector (DAD). The column used was a 5 μm Prodigy ODS3 column (4.60 × 250 mm) (Phenomenex Ltd., UK) with a flow rate of 1

mL/min at 25 °C. The mobile phase consisted of two solvents: solvent A, 0.5% formic acid in water, and solvent B, 0.5% formic acid in acetonitrile. The solvent gradient was applied as follows: 10% B at the beginning, 10% at 5 min, 20% at 15 min, 25% at 25 min, 35% at 33 min, 50% at 38 min, 90% at 43−44 min, and 10% at 45 min. Anthocyanins were detected by monitoring the elution at 525 nm, and other flavonoids were monitored at 270 nm. The phenolic compounds were identified by comparing their retention times, diode array spectral characteristics, and mass spectra, measured using LC-ESI-MS/MS, with the standards and the data available in the literature (SESTARI *et al.*, 2014). The calibration was performed using commercial standards described in item 3.1.1.

3.1.9 Identification of Ellagitannins

Approximately 5 g of sample was extracted with 80% acetone and evaporated to eliminate the acetone. An aliquot of 0.1 M ytterbium acetate was added to the aqueous extract and held overnight at 5 °C. The precipitate (ET-Yb) was centrifuged at 3000g for 10 min at 5 °C. The precipitate was washed three times, twice with 80% acetone and once with pure acetone. The precipitate was subsequently dissolved in 10 mM oxalic acid, and the supernatant was dried under nitrogen, resuspended in methanol and filtered using 0.43 μm PTFE membrane filters (Millipore Ltd.) for LCESI- MS/MS analysis (GINER-CHAVEZ *et al.*, 1997).

3.1.10 LC-ESI-MS/MS Condition

The identification of the flavonoids and ellagitannins was performed by LC-ESI-MS/MS using a Prominence liquid chromatograph (Shimadzu, Japan) linked to an ion trap Esquires-LC mass spectrometer (Bruker Daltonics, Billerica, MA, USA) with an electrospray ionization (ESI) interface. The solvent gradient conditions were the same as those used for the HPLC-DAD process. After passing through the DAD, the flow rate was changed to 0.2 mL/min for application to the mass spectrometer. The ESI was used in the positive mode to detect the anthocyanins and in the negative mode for the other classes of flavonoids. The mass spectrometer operating conditions were as follows: collision energies of 4500 and 4000 V were used for the positive and negative mode, respectively, and the capillary temperature was 275 °C. The analysis was carried out using a full scan from m/z 100 to 1500. The compounds were identified according to comparison with the retention times of authentic standards when

possible, as well as by absorption spectrum similarity, mass spectral characteristics, and comparison with literature data (SESTARI *et al.*, 2014).

3.1.11 Identification and Quantification of Proanthocyanidin

The freeze-dried samples (0.8 g) were homogeneized with extraction solution (acetone/water/acetic acid, 70:29.5:0.5, v/v/v) using an Ultraturrax followed by sonication in an ultrasonic bath for 15 min.The homogenates were centrifuged at 2000g for 10 min. The acetone was evaporated under reduced pressure at 35 °C and passed through a Sep-Pack C-18 solid phase extraction cartridge (Waters, Milford, MA, USA) and washed with Milli-Q water. The phenolic compounds were eluted with methanol. The eluates were completely dried using a rotary evaporator under vacuum at 40 °C, resuspended in methanol, and filtered through a 0.45 μm PTFE filter (Millipore Ltd.) for the quantification of proanthocyanidins by HPLC-DAD analysis. For strawberry proanthocyanidin analysis (cv. Camarosa), used to compare proanthocyanidin profile, the same protocols was applied (BUENDIA *et al.*, 2010).

3.1.12 Normal Phase HPLC-DAD of Proanthocyanidin Extract

The analysis was carried out as reported previously (BUENDIA *et al.*, 2010). The same HPLC equipment described above was used. The column used was a Develosil Diol (4.60 × 250 mm, 5 μm) (Phenomenex Ltd., UK) with a flow rate of 1 mL/min at 25 °C. The mobile phase consisted of two solvents: solvent A, CH_3CN/HOAc (98:2, v/v), and solvent B, CH_3OH/H_2O/HOAc, 95:3:2, v/v/v). The solvent gradient was applied as follows: 0% B at the beginning, 40% at 35 min, and 40% at 45 min. UV detection was set at 280 nm, and fluorescence detection used excitation at 276 nm and emission at 316 nm. The calibration was performed using commercial standards of proanthocyanidin B1 and proanthocyanidin B2.

3.1.13 Statistical Analysis

The results were expressed as the mean values ± standard deviation. Analysis of variance (ANOVA) followed by the Tukey test was performed using Biostat 5.0.

3.2 RESULTS AND DISCUSSION

3.2.1 Chemical Composition

Table 1 shows the chemical composition for the flesh of purple grumixama, collected in two different places and times (F1, F2, F3 and F4), and a single sample of the flesh of the rarer yellow variety (F5). According to the °Brix values, all fruits analyzed were at the same ripening stage, but there were slight differences but not significant in the chemical composition among the fruits harvested at different times and places. Glucose was the main sugar found in these fruits, which is compatible with the results for a ripe berry fruit, due to the hydrolysis of sucrose during ripening (WANG *et al.*, 2003; TALCOTT *et al.*, 2007).

Table 1 – Chemical composition of the flesh of *Eugenia brasiliensis* Lam., purple and yellow grumixama.

Samples	**F1**	**F2**	**F3**	**F4**	**F5**
	g/100 g	g/100 g	g/100 g	g/100 g	g/100 g
Moisture	84.80 (0.18)	88.29 (0.50)	87.60 (0.07)	83.67 (0.33)	85.21 (0.21)
Total Soluble Sugar	2.98 (0.10)	2.69 (0.06)	3.53 (0.22)	2.61 (0.10)	2.97 (0.07)
Glucose	2.85 (0.10)	2.55 (0.06)	3.38 (0.23)	2.26 (0.11)	2.84 (0.10)
Frutose	0.13 (0.00)	0.12 (0.00)	0.12 (0.00)	0.11 (0.00)	0.12 (0.00)
Sacarose	ND	Tr	Tr	0.02 (0.00)	ND
ºBrix	1.35 (0.00)	1.35 (0.00)	1.35 (0.00)	1.35 (0.00)	1.35 (0.00)
Protein[1]	0.66 (0.01)	0.50 (0.00)	0.54 (0.01)	0.61 (0.01)	0.56 (0.02)
Total Fiber	4.65 (0.24)	3.11 (0.11)	3.10 (0.08)	4.18 (0.10)	3.08 (0.12)
Insoluble Fiber	1.70 (0.16)	1.07 (0.03)	1.49 (0.08)	2.04 (0.10)	1.45 (0.07)
Soluble Fiber	2.95 (0.10)	2.04 (0.10)	1.53 (0.03)	2.15 (0.10)	1.75 (0.04)
Lipid	0.26 (0.01)	0.22 (0.00)	0.16 (0.00)	0.18 (0.01)	0.22 (0.00)
Ash	0.56 (0.01)	0.44 (0.00)	0.31 (0.02)	0.61 (0.01)	0.56 (0.01)

[1]Correction factor for vegetable proteins: 5.75; ND: not detected; values are means (Standard Desviation); F1-F4 correspond to purple fruits; F5 corresponds to yellow fruit; F1 and F2: from São Paulo, F3 and F4: from Santa Catarina; Tr: trace.

3.2.2 Soluble Total Phenolic and Monomeric Anthocyanin Contents and Antioxidant Capacity

Figure 1A shows the results for the soluble total phenolic and monomeric anthocyanin contents for the flesh and seeds. All seeds of purple grumixama, collected in different places and times, presented higher content of total phenolic content (varying from 2.51 ± 0.09 to 3.3 ± 0.12 g 100 g−1 fresh weight, FW) than the respective flesh (varying from 0.18 ± 0.01 to 0.75 ± 0.02 g 100 g−1 FW). Among the flesh samples, F1 and F4 showed the highest total phenolic, values similar to those found in Brazilian blackberry cultivars (from 0.34 ± 0.01 to 0.50 ± 0.01 g 100 g−1 FW) (TALCOTT *et al.*, 2007). The flesh of purple grumixama contained high contents of the monomeric anthocyanin, varying from 30 to 200 mg 100 g−1 FW, where F1 and F4 presented values similar to those founded in Brazilian blackberry cultivar Guarani (*Rubus sp*.) (190 mg. 100 g^{-1} FW) (HASSIMOTTO *et al.*, 2008). Differences in flavonoids and phenolic contents were observed in several vegetables and could be due to agronomic conditions, plant tissue, and varieties. Anthocyanins were not detected in the yellow fruit or in its seeds. The yellow fruit presented the lowest total phenolic content (0.18 ± 0.01 g. 100 g^{-1} FW); however, there was no difference in the phenolic content in the seeds of the two varieties. These results indicate that the place and time of fruit harvesting do not influence the phenolic or anthocyanin contents of grumixama.

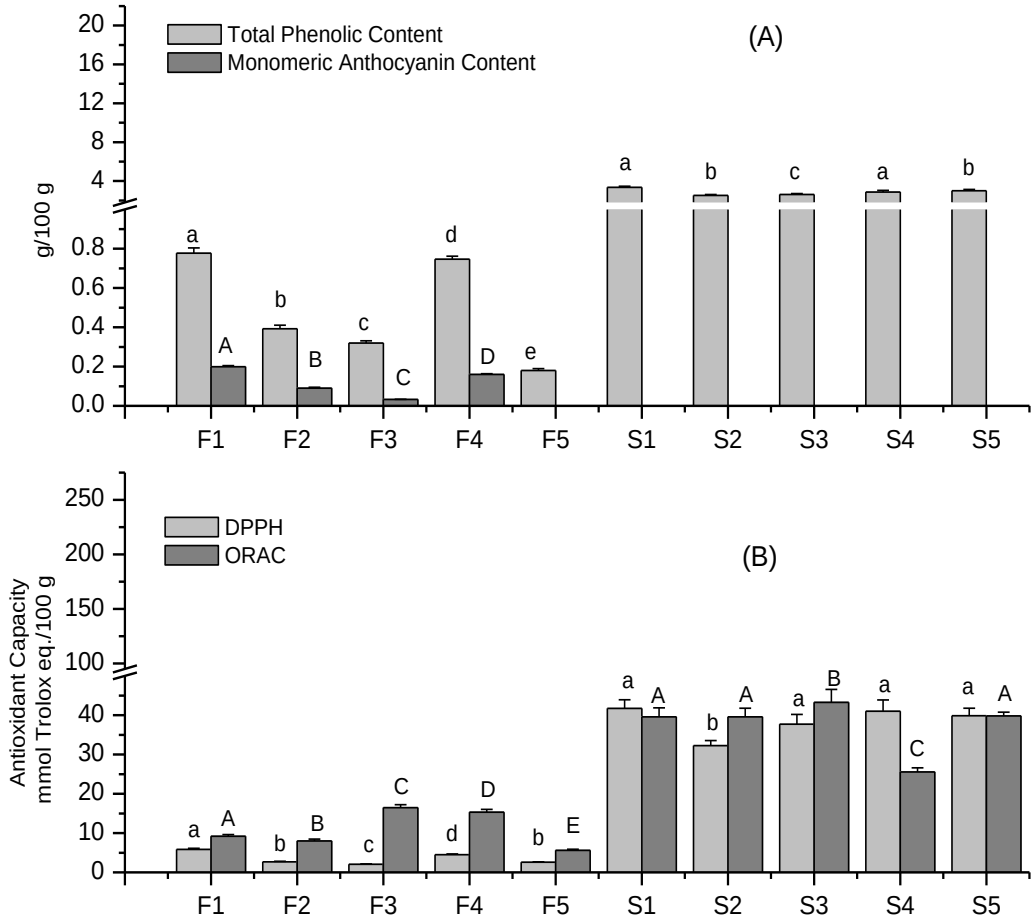

Figure 1 – Total phenolic, monomeric anthocyanin contents and antioxidant capacity of puple and yellow grumixama fruits. (A) total phenolic and monomeric anthocyanin contents (B) antioxidant capacity analyzed by DPPH and oxygen radical absorbance capacity (ORAC) methods for the flesh fruits (F1;F2; F3, F4 and F5) and seeds (S1; S2; S3, S4 and S5) of *Eugenia brasilienses* Lam. F1-F4 and S1-S4 correspond to purple fruits; F5 and S5 corresponds to yellow fruit. The same letters indicate not significant variations between samples ($p \leq 0.05$).

Figure 1B shows the antioxidant capacity evaluated by the DPPH and ORAC methods. The flesh showed the lowest antioxidant capacity values by both methods. No significant differences ($p < 0.05$) with respect to the place or harvest period were observed among the fruits or seeds. Due to their structure, phenolic compounds possess high antioxidant capacities, and these compounds represent one of the main phytochemical classes responsible for the total antioxidant capacity of plant-derived food (CLIFFORD and

BROWN, 2006). Thus, a high correlation (Pearson correlation) was observed between the total phenolic content and the DPPH values (r = 0.93) as well as the ORAC (r = 0.87) values. There was also a strong correlation between the content of monomeric anthocyanins and total phenolic compounds (r = 0.97), suggesting an influence of the phenolic compounds in the antioxidant capacity. In addition, ascorbic acid, an important hydrophilic antioxidant present in fruits, was not detected in the flesh of either the purple or yellow variety.

3.2.3 Identification of Flavonoids and Phenolic Acids

Figure 2 shows the HPLC-DAD chromatograms of the flesh of the purple and yellow grumixama fruits (A and C, respectively) and seeds (B and D, respectively), and the respective identification of the compounds by LC-ESI-MS/MS is shown in **Table 2**.

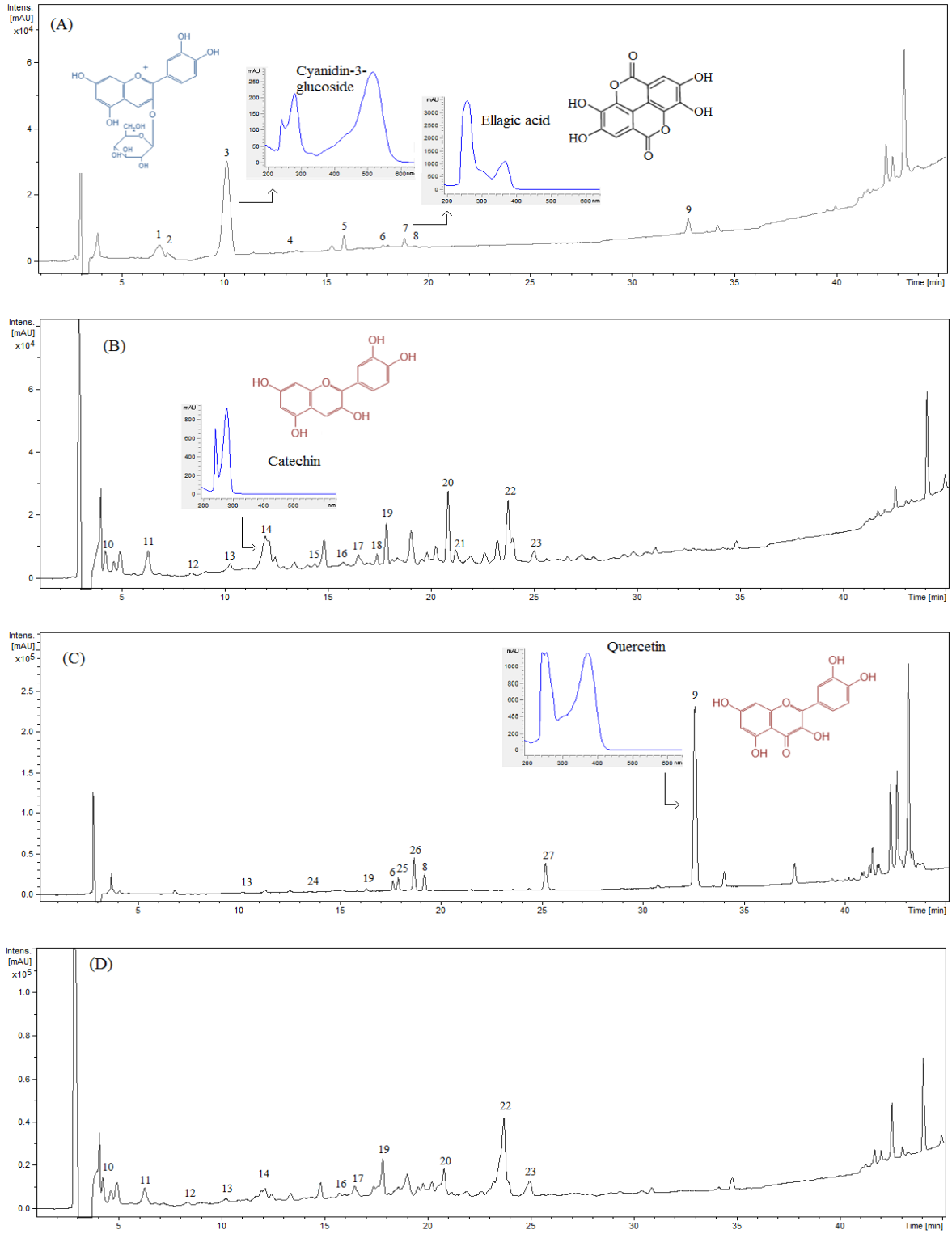

Figure 2 - Chromatograms obtained by HPLC-DAD (λ = 270 nm) of the purple and yellow grumixama. (A) purple and (C) yellow fleshs of grumixama; (B) purple and (D) yellow seeds of grumixama. The identification of the peaks is given in Table 2.

3.2.3.1 Fruits

Eight compounds were identified in the flesh of each of the two varieties, the purple and yellow grumixama (**Table 2**). In the flesh of the purple fruit, two classes of flavonoids (five anthocyanins and two flavonols) and one phenolic acid were identified (**Figure 2**). In the flesh of the yellow fruit, two classes of flavonoids (four flavonols and two flavanols) and two EA derivatives were detected. Among these, two flavonoids were common to both fruits (peak 8, quercetin 3-glucoside at *m/z* 463, and peak 9, quercetin aglycone at *m/z* 301). As expected, anthocyanins were detected only in purple grumixama. Specifically, this fruit contained two anthocyanidins, cyanidin and delphinidin, with characteristic molecular ions at *m/z* 287 and 303, respectively. Peaks 6 (at *m/z* 615), 10 and 12 (at *m/z* 633) were identified as ellagitannins, and their identification is further discussed below. The major class of compounds present in the purple grumixama fruit was anthocyanins (82% of total identified compounds), whereas the yellow fruit was dominated by flavonols (74% of total identified compounds) (**Table 2**).

The major anthocyanins present in the purple grumixama were delphinidin hexoside (peak 1, at *m/z* 465) and Cyanidin 3-*O*-glucoside (peak 3, at *m/z* 449), which were responsible for 30 and 61% of total anthocyanins, respectively. In the yellow grumixama, the major flavonols present were quercetin 3-glucoside (peak 8, at *m/z* 463) and quercetin aglycone (peak 9, at *m/z* 301), which were responsible for 25 and 53% of total flavonols, respectively.

Peak 1 had a molecular ion at *m/z* 465 and two MS/MS fragments at *m/z* 303 $[M - 162]^+$ and at *m/z* 229, suggesting that this peak represents a delphinidin linked to a hexoside, where the loss of 162 corresponds to a hexose unit. This compound was probably delphinidin-3-glucoside, also identified in purple grumixama (Silva *et al.*, 2014). Peaks 2 and 3 had the same molecular ion at *m/z* 449 and mass fragments at *m/z* 287 $[M - 162]^+$ and at *m/z* 213. Peak 3 was identified as cyanidin 3-*O*-glucoside, and its identity was confirmed by coelution with the commercial standard. Thus, considering the elution order in relation to cyanidin 3-O-glucoside, which was consistent with that expected from the reversed phase elution, peak 2 could be cyanidin 3-galactoside, also identified in the purple grumixama (Flores *et al.*, 2013; Silva *et al.*, 2014).

Peak 4 had a molecular ion at *m/z* 419 and an MS/MS fragment at *m/z* 287 $[M - 132]^+$, suggesting that it corresponded to a cyanidin linked to a pentoside: either a xyloside or arabinoside unity (FLORES *et al.*, 2013). The identity of peak 5 was confirmed by coelution

with standard as cyanidin (*m/z* 287). Peak 7 showed absorption spectra similar to EA, and its identity was confirmed as ellagic acid (*m/z* 301) by coelution with commercial standard.

Peak 8, identified as quercetin 3-glucoside, had a molecular ion at *m/z* 463 and one mass fragment at *m/z* 301, corresponding to quercetin after the loss of a hexose unit (−162 from $[M - H]^-$), and a second fragment at m/z 151. Peak 9, with a molecular ion at m/z 301 and MS/MS fragment at *m/z* 151 was identified as quercetin aglycone. Quercetin and ellagic acid demonstrated the same characteristic molecular ion at *m/z* 301. However, on MS/MS analysis, quercetin forms characteristic mass fragments at m/z 179 and 151, whereas the fragments from ellagic acid were observed at *m/z* 257 and at m/z 229 (GATES *et al.*, 2012).

Table 2 – Phenolic compounds identified in the flesh and seed of yellow and purple grumixama by HPLC-ESI-MS/MS.

Compounds Identified (Positive mode)	**RT (min)**	**$[M]^+$ (*m/z*)**	**MS/MS (*m/z*)**	**Peak[1]**	**Purple Fruit (%)**	**Yellow Fruit (%)**	**Purple Seed (%)**	**Yellow Seed (%)**
Anthocyanin								
Delphinidin hexoside	6.4	465	303; 229	1	25	-	-	-
Cyanidin-3-galactoside	7.1	449	287; 213	2	6	-	-	-
Cyanidin-3-glycoside*	10.0	449	287; 213	3	50	-	-	-
Cyanidin-3-xyloside/arabinoside	13.4	419	287; 213	4	Tr	-	-	-
Cyanidin aglycone*	15.8	287	213	5	2	-	-	-
Compounds Identified (Negative mode)	**RT (min)**	**$[M-H]^-$ (m/z)**	**MS/MS (m/z)**	**Peak[1]**	**Purple Flesh (%)**	**Yellow Flesh (%)**	**Purple Seed (%)**	**Yellow Seed (%)**
Flavanols								
Gallocatechin dimeric ion	6.2	611	305/ 220	11	-	-	11	11
Catechin/ Dimeric ion *	12.0	289/579	245/203	14	-	2	14	14
Gallocatechin gallate	14.2	457	305	15	-	-	4	-
Epigallocatechin gallate*	15.6	457	305/ 331/ 169	16	-	-	2	3
(−)-Epicatechin gallate*	21.2	442	289/ 245/169	21	-	-	3	-
Epicatechin*	13.5	289	245/ 203	24	-	3	-	-
Proantocyanidins								
(epi)catechin(epi)catechin	9.2	577	407/ 289	13	-	-	2	2
Phenolic Acids/ Derivadeds								

Table 2 - Continuation

Compounds Identified (Negative mode)	RT (min)	$[M-H]^-$ (m/z)	MS/MS (m/z)	Peak[1]	Purple Flesh (%)	Yellow Flesh (%)	Purple Seed (%)	Yellow Seed (%)
Free Ellagic acid *	18.8	301	301	7	5	-	-	-
Ellagic acid dipentoside	17.4	594/593	447/ 301	18	-	-	2	-
Ellagic acid hexoside	20.7	927	463/ 301/ 257	20	-	-	24	23
Ellagic acetyl rhaminoside/dimeric ion	23.7	978/489	489/ 301/ 257	22	-	-	6	11
Ellagic acetyl rhaminoside	25.0	489	301	23	-	-	7	9
Ellagic acid pentoside	17.7	447	447/ 301	25	-	14	-	-
Ellagic acid derivative	18.7	491	301/ 257	26	-	3	-	-
Flavonols								
Quercetin-3-glucoside*	19.2	463	301/151	8	Tr	21	-	-
Quercetin aglycone/ dimeric ion*	32.4	301/603	301/ 151	9	9	44	-	-
Myricetin galloyl hexoside	16.4	633/631	479/ 316	17	-	-	1	1
Myricetin hexoside dimeric ion	17.8	959	479/ 316	19	-	1	6	8
Myricetin aglycone*	25.4	317	317/ 151	27	-	7	-	-

[1]Peaks are numbered according to Figure 2A (flesh of the purple grumixama), 2C (flesh of the yellow grumixama), 2B (seeds of the purple grumixama), 2D (seeds of the yellow grumixama); Tr: traces < 0.01; *Compounds were identified by comparison with retention time of standards, and mass fragment profile and UV/Vis absorption spectra; RT – retention time. Percentage was calculated from the total area of the chromatograms at 525 nm for anthocyanins and 270 nm for the other compounds.

In the yellow variety, two flavonols and two flavanols were identified (**Table 2**). Peak 27 had a molecular ion at *m/z* 317, characteristic of myricetin. Peak 19, identified as myricetin hexoside dimeric ion, had a molecular ion at *m/z* 959 and MS/MS fragments at *m/z* 479 and at *m/z* 317 ($[M - H]^-$ -162, corresponding to the loss of a hexose unit) (Fracassetti *et al.*, 2013; Gates *et al.*, 2012). Peak 14 showed molecular ions at *m/z* 579 and at *m/z* 289 and an MS/MS fragment at *m/z* 289 ($[M - H]^-$ - 290), which suggested it was a catechin dimeric ion. In addition, this compound showed another fragment at *m/z* 245 (neutral loss of CO_2, 44, from the catechin) (GATES *et al.*, 2012). A dimeric ion is an ion that is formed by the ionization of a dimer or by the association of an ion with its neutral counterpart such as $[M2]^{\bullet-}$ or $[M - H - M]^-$ (MURRAY *et al.*, 2006). Peak 24 had the same molecular ion at *m/z* 289, characteristic of a catechin isomer, and two mass fragments at *m/z* 245 and at *m/z* 203. Peaks 14 and 24 were identified as catechin and epicatechin, respectively, and their identities were confirmed by coelution with the corresponding standards.

The identities of the flavonoids cyanidin 3-*O*-glucoside, cyanidin aglycone, quercetin 3-glucoside, quercetin aglycone, and myricetin aglycone, and free ellagic acid were confirmed by coelution with standards and the similarities of the UV−vis absorption spectra and the mass spectral characteristics to those of the standards.

Flavonoids are rarely present in plants as aglycones. Instead, they are usually linked to various sugars (ERLUND *et al.*, 2006; WU *et al.*, 2006). It is interesting to observe that high concentrations of the aglycone forms of cyanidin and quercetin were detected in the purple grumixama, and aglycone forms of quercetin and myricetin were detected in the yellow variety of these fruits. No aglycone forms were released when isolated cyanidin 3-*O*-glucoside, quercetin 3-glucoside, and quercetin 3-rutinoside were tested in the protocol extraction, excluding the possibility that they were artifacts.

Flores *et al.* (2013) identified nine anthocyanins, of which five were found in the purple grumixama analyzed in this work. In addition, Silva *et al.* (2014) identified three of these and one additional form: cyanidin 3-acetyl-hexoside. However, myricetin, rutin, and kaempferol, also detected by Reynertson *et al.* (2008) in purple grumixama, were not detected in the present study. These differences in the profiles could be due to differences in local plantation and/or agronomic conditions.

The phenolic profile of yellow grumixama had not been previously characterized, most likely because this variety is less common than the purple variety. The flavonoid profile of the yellow variety was very different from that of the purple variety. Specifically, the purple and yellow varieties are good sources of anthocyanins and flavonols, respectively.

3.2.3.2 Seeds

Table 2 presents the compounds identified by LC ESI-MS/MS in the seeds of the purple and yellow grumixama. The peak numbers in this table refer to the numbered peaks in **Figure 2B** and **2D**, respectively, which identified 12 and 9 phenolic compounds in the seeds of the purple and yellow grumixama, respectively. All compounds identified in the seeds of yellow variety were also found in the purple form. Among the nine compounds present in both seeds, three ellagic acid derivates (peaks 20, 22, and 23, at *m/z* 927, 489, and 489, respectively), three flavanols (peaks 11, 14, and 16, at *m/z* 611, 289, and 457, respectively), two flavonols (peaks 17 and 19, at *m/z* 631 and 959, respectively), and one proanthocyanidin (peak 13, at *m/z* 577) were identified. In addition to these, in the seed of purple grumixama, four additional compounds were identified: one ellagic acid derivative (peak 18, at m/z 593), two flavanols (peaks 15 and 21, at *m/z* 457 and 442, respectively), and one flavonol (peak 27, at *m/z* 317 and 959). Peaks 10 and 12, at *m/z* 633, were identified as ellagitannins.

The major compounds identified in the seeds of the yellow and purple grumixama were ellagitannins, expressed as total ellagic acid (42 and 38% of the total compounds identified, respectively) and flavanols (28 and 34% of the total compounds identified, respectively) (**Table 2**).

In addition to myricetin hexoside (at *m/z* 479), which was also found in the flesh, myricetin galloyl hexoside (peak 17) was identified in seeds of both varieties. Peak 17 showed a molecular ion at *m/z* 631 and two mass fragments at *m/z* 479 and 316. The fragment at *m/z* 479 corresponds to a myricetin hexoside, resulting from the loss of a galloyl group (−152 from $[M - H]^-$).

The phenolic acids or derivatives detected and identified in the grumixama (flesh and seed) have common molecular ions at *m/z* 301 and 169, which correspond to EA and gallic acid, respectively. Other structures linked to these phenolic acids included sugars (hexoses and pentoses) and the acetyl group. Examples include ellagic acid hexoside (peak 20, at *m/z* 463 with an MS/MS fragment at *m/z* 301) and ellagic acetyl rhaminoside dimeric ion (peak 23, at *m/z* 489 with an MS/MS fragment at *m/z* 301), respectively. With the exception of free EA, all other phenolic acids were identified by comparison with data from the literature (FRACASSETTI *et al*., 2013; SCOPARO *et al.*, 2012; OLIVEIRA *et al.*, 2014. Among these compounds (**Table 2**), catechin, epicatechin, gallocatechin gallate, epigallocatechin gallate, (-)-epicatechin gallate, free ellagic acid, myricetin aglycone, and quercetin aglycone were identified by coelution with the corresponding standards and the similarity of the UV−vis

absorption spectra and mass spectra characteristics. The compounds in the seeds had not been previously studied.

3.2.4 Identification of Proanthocyanidin of Fruits

Proanthocyanidin are polymeric flavonoid compounds composed of flavonol-3-ol subunits. Proanthocyandins were the main strawberry phenolics (BUENDIA *et al.*, 2010), presenting from dimeric to polymeric forms. Flesh and seed of both grumixama varieties presented dimeric form of proanthocyanidin. None other polymeric form was detected. Peak 13 (**Table 2**) had a molecular ion at *m/z* 577 and two MS/MS fragments at m/z 407 and 289, that were identified as proanthocyanidins B1/B2, a dimer of catechin/epicatechin. The identity was confirmed by coelution with the commercial standard (**Figure 3**).

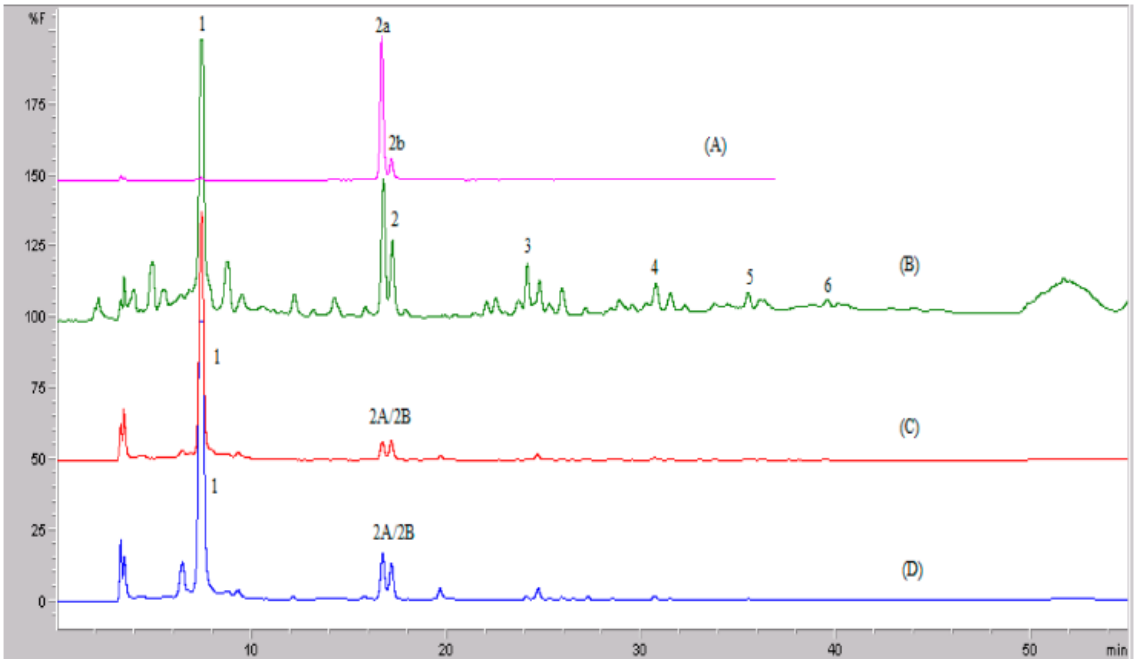

Figure 3 - Chromatogram obtained by HPLC-fluorescence of proanthocyanidins of *Eugenia brasiliensis* Lam, purple and yellow varieties in normal phase diol column. (A) proanthocyanidin B1/B2; (B) strawberry extract; (C) yellow grumixama; (D) purple grumixama.The numbers denote the polymerization degree (1, monomers; 2, dimer; 3, timer; etc.). (A) proanthocyanidin B2; (B) proanthocyanidin B1.

3.2.5 Identification of Ellagitannins of Fruits

ETs are present in berries and a number of other sources, such as nuts (Landete *et al.*, 2011). ETs are hydrolyzable tannins, a class of polyphenols with a structure that consists of esters of hexahydroxydiphenoyl (HHDP) groups with a polyol core (usually glucose or quinic acid) that often contains galloyl groups (ASCACIO-VALDÉS *et al.*, 2011; BAKKALBAŞI *et al.*, 2009; CLIFFORD and SCALBERT, 2000). An ET-enriched fraction was used to identify the ETs by LC-ESI-MS/MS analysis. The ET-enriched fraction was obtained by the precipitation of ETs using ytterbium acetate, which provided an improved separation from other phenolics such as flavonoids (GINER-CHÁVEZ *et al.*, 1997).

Figure 4 shows the chromatograms obtained from the flesh of the yellow and purple grumixama (A and B, respectively). The peak numbers indicate the compounds identified, which are listed in **Table 3**. Seven ellagic acid derivatives were identified in the ET-enriched fraction, as discussed above.

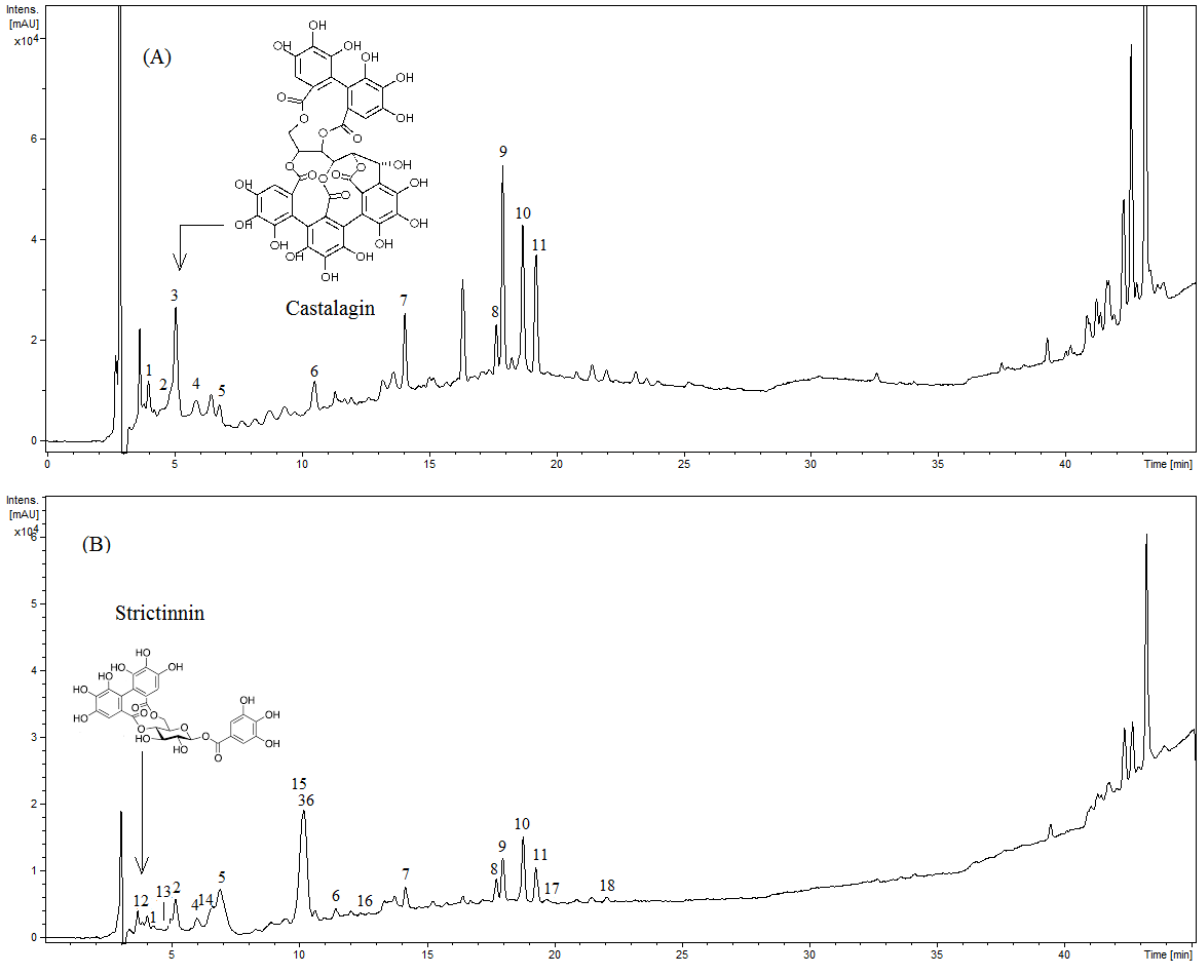

Figure 4 - Chromatograms of the ellagitannins obtained by HPLC-DAD (λ = 270 nm) of the the flesh of grumixama fruits. (A) yellow and (B) purple grumixama. The identification of the peaks is given in Table 3.

In the flesh of the purple and yellow varieties, 10 and 7 compounds, respectively, were identified as ETs (**Table 3**). All of the other numbered peaks in the chromatograms were identified as ellagic acid derivatives and flavonoids and are listed in **Table 2**. Among these, there were six ellagic acid derivatives and cyanidin 3-glucoside in the purple grumixama and four EA derivatives in the yellow one. All of the ETs detected in the flesh of the yellow fruit were also detected in the purple fruit.

Table 3 – Ellagitannins identified in flesh of yellow and purple grumixama by HPLC-ESI-MS/MS.

Identified Compounds (Negative Mode)	Molecular Weight	Retention Time (min)	[MS]- (m/z)	MS/MS (*m/z*)	Number[1]	Purple Flesh(%)	Yellow Flesh(%)
ETs							
Castalagin/Vescalagin	934	4.0	933	915/ 301	1	3	8
Pedunculagin	784	4.9	783	301/257	2	6	4
Castalagin/Vescalagin	934	5.1	933	301	3	-	17
Strictinin	634	5.3	633	301	4	1	3
Pedunculagin	784	6.9	783	481/ 301	5	3	2
Potentillin/Casuarictin	936	10.7	935	633/ 301	6	Tr	4
Ellagic acid galloyl hexoside	616	17.7	615	463/ 301	8	6	3
Strictinin	634	3.8	633	594/ 481/301	12	4	-
Strictinin	634	4.3	633	274/ 203	13	4	-
Strictinin	634	6.4	633	301/ 211	14	4	-
Tellimagrandin I	786	12.0	785	615/483/301	16	2	-

[1]Peak numbered accoridng to Figure 4A (flesh of yellow grumixama), 4B (flesh of purple grumixama); fragmentation profile of MS; MS/MS; absorption spectrum; RT – retention time.

Peaks 1 and 3 were identified as C-glycosidic ellagitannins. The molecular ions for both peaks 1 and 3 were at *m/z* 933, and both produced two mass fragments at *m/z* 915 and 301 and identified as castalgin/vescalagin isomers. The fragment at *m/z* 915 resulted from the neutral loss of water ($[M - H]^- - 18$), and the other, at *m/z* 301, resulted from the neutral loss of 632 (monogalloyl - glucose) (Fracassetti *et al.*, 2013; Romani *et al.*, 2012; Koponen *et al.*, 2007). Castalgin/vescalagin isomers were the major ellagitannins in the flesh of the yellow grumixama, corresponding to approximately 61% of the total ETs identified (**Table 3**). Peaks 2 and 5 was identified as peduncunlagin isomers (2,3- or 4,6-dihexahydroxydiphenoyl [HHDP]-glucose) with a molecular ion at *m/z* 783 and three mass fragments: one at *m/z* 481 resulting from the neutral loss of an HHDP group ([M - H]-302) and two others at *m/z* 301 ($[M - H]^- 482$, resulting from the loss of an HHDP (302), a glucosyl group (162) and H_2O (18) and at *m/z* 257. The fragment at m/z 301 is characteristic of the ellagitannins, and the one at *m/z* 257 (loss of CO_2) is a typical fragment of ellagic acid (ROMANI *et al.*, 2012; KOPONEN *et al.*, 2007; MULLEN *et al.*, 2003).

Peaks 4, 12, 13, and 14, identified as strictinin isomers, all had molecular ions at *m/z* 633 and mass fragments at *m/z* 481, resulting from the neutral loss of a galloyl group ($[M - H]^-$ -152) and at *m/z* 301, resulting from the neutral loss of one hexose unity and one molecule of water ($[M - H]^-$ - 180 from the loss of glucosyl (162) groups and water (18)) from the fragment at *m/z* 481(DOU *et al.*, 2007). Among these four isomers, only compound 4 were present in both varieties.

Peak 8 showed a molecular ion at *m/z* 615 and MS/MS fragments at *m/z* 463, resulting from the loss of a galloyl group from $[M - H]^-$, and at *m/z* 301, resulting from the loss of one hexose unit (162). Compound 8 was identified as ellagic acid galloyl hexoside. Pedunculagin isomers, strictinin isomers, and ellagic acid galloyl hexoside were the major ellagitannins in purple grumixama, corresponding to 20, 30, and 19%, respectively, of the total ETs identified.

Peak 6 had a molecular ion at m/z 935 and yielded MS/MS fragments at *m/z* 633 ($[M - H]^-$ - 302), resulting from the loss of an HHDP group), and at *m/z* 301 ($[M - H]^-$ - 634, resulting from the loss of HHDP (302), gallic acid (170), and glucosyl (162) groups. On the basis of the mass spectra, this peak was identified as a probable casuarictin (galloyl-bis-HHDP-glucoside), an *O*-glucoside isomer (Zhentian *et al.*, 1999). Isomeric ions have the same numbers of each isotopic atom, but the positions of these atoms differ (MURRAY *et al.*, 2006). Thus, these molecules have different retention times, but the same mass spectra characteristics.

Ten ellagitannins were identified in the flesh of the fruits. Characterization of the ellagitannins had not previously been studied in grumixama. However, the ellagitannins have been studied in other plants from the Myrtaceae family. Various ellagitannins such as telligramadin, pedunculagin, casuarictin, strictinin, vescalagin and castalagin (OKUDA *et al.*, 1982; KOPONEN *et al.*, 2007; FRACASSETTI *et al.*, 2013) including their monomeric forms, were also identified in these studies. However, dimeric ellagitannin forms such as sanguinin H-6 and sanguinin H-10, which are common in berries (KOPONEN *et al.*, 2007; ZHENTIAN *et al.*, 1999) were not detected in grumixama. Furthermore, oligomeric forms such as melasquanin A, which are common in other species of the Myrtaceae family, were also not identified here (YOSHIDA *et al.*, 2010).

3.2.6 Quantification of Flavonoids, Phenolic Acids, Proanthocyanidin, and Ellagitannins

The main constituents of the flesh of the purple grumixama were the anthocyanins (32− 180 mg of total anthocyanin equiv 100 g^{-1} FW) and ellagitannins, expressed as total ellagic acid (82−243 mg ellagic acid equiv 100 g^{-1} FW) (**Tables 4** and **5**, respectively). Ellagitannin quantification is based on hydrolysis of these compounds to yield free ellagic acid (EA), which is detected by HPLC.

The cyanidin 3-glucoside contributed 82−89% of the total anthocyanin, of which 3−8% was present as cyanidin aglycone (**Table 4**). The amount of the anthocyanins found in the flesh by HPLC-DAD analysis is consistent with the monomeric anthocyanin content (**Figure 1A**). Thus, the anthocyanin concentrations in purple grumixama vary according to harvest time and local plantation. The lowest concentration was similar to those given for several strawberry cultivars, ranging from 23.5 ± 1.9 mg.100 g^{-1} in cv. Aibión to 46.6 ± 2.9 mg.100 g^{-1} FW in cv. Aguedilla (BUENDIA *et al.*, 2010) and cranberry (66.7 mg.100 g^{-1}) (KOPONEN *et al.*, 2007). Also, the highest concentration was similar to that reported for blackberry cultivars, ranging from 80 to 100 mg. 100 g^{-1} FW (TALCOTT *et al.*, 2007) and blueberry (218 mg 100 g^{-1} FW) (KOPONEN *et al.*, 2007).

The flesh of the yellow grumixama (**Table 4**) is rich in flavonols (quercetin and myricetin derivatives), corresponding to 41 mg.100 g^{-1} FW with only small amounts of the flavanols (catechin and epicatechin). However, the major components were the ellagitannins (91.72 mg 100 g^{-1} FW) (**Table 5**).

Table 4 – Flavonoid content in the flesh and seeds of *Eugenia brasiliensis* Lam, purple and yellow varieties.

Sample	Quercetin	Quercetin-3-glucoside	Delphinidin[a]	Cianidina-3-glucoside	Cyanidin	Total Anthocyanin
F1	18.55 (1.44)[a]	9.49 (0.73)[a]	15.97 (0.64)[a]	152.54 (3.77)[a]	14.71 (3.05)[a]	179.79 (7.71)[a]
F2	7.77 (0.77)[b]	5.45 (0.47)[b]	10.24 (0.86)[b]	71.81 (6.66)[b]	4.88 (0.92)[b]	86.11 (6.25)[b]
F3	21.42 (0.44)[c]	3.71 (0.38)[c]	2.32 (0.05)[c]	28.73 (0.55)[c]	0.86 (0.09)[c]	31.87 (0.71)[c]
F4	35.51 (1.77)[d]	6.22 (0.69)[b]	8.38 (0.30)[d]	124.96 (9.46)[d]	6.71 (0.45)[b]	138.61 (10.77)[d]
	Quercetin	**Quercetin 3-glucoside**	**Myricetin**	**Catechin**	**Epicatechin**	
F5	26.28 (1.20)	13.98 (1.20)	3.46 (0.19)	2.87 (0.41)	2.64 (0.29)	
	Quercetin	**Gallotocatechin[b]**	**Myricetin[c]**	**Catechin**	**Epicatechin**	**Epigallocatechin gallate**
S1	ND	29.92 (1.59)[a]	21.32 (1.17)[a]	36.18 (1.57)[a]	ND	3.69 (0.22)[a]
S2	ND	19.31 (1.51)[b, c]	13.60 (2.45)[b]	19.19 (1.20)[b]	ND	3.66 (0.51)[a]
S3	ND	23.97 (1.76)[c]	13.04 (2.09)[b]	19.75 (2.10)[b]	ND	2.73 (0.26)[b]
S4	ND	21.38 (2.60)[b]	14.38 (2.13)[b]	15.34 (1.70)[c]	ND	1.81 (0.03)[c]
S5	ND	16.17 (1.19)[d]	13.28 (1.24)[b]	20.45 (1.52)[d]	ND	2.60 (0.18)[b]

[a]Delphinidin hexoside was quantified as delphinidin aglycone; [b]gallotocatechin was quantified as catechin; [c]myricetin derivates were quantified as myricetin aglycone; The values shown are the means (standard deviation); ND: not detected; F1 and F2: flesh of the fruit of the purple grumixama from São Paulo, F3 and F4: flesh of the fruit of the purple grumixama from Santa Catarina, F5: flesh of the fruit of the yellow grumixama from São Paulo; S1 and S2: seeds of the purple grumixama from São Paulo, S3 and S4: seeds of the purple grumixama from Santa Catarina, S5: seeds of the yellow grumixama from São Paulo; Tr: trace; Value expressed as mg/100 g. The same letters indicate non-significant differences between samples ($p < 0.05$).

The flavonoids identified in the seeds were primarily flavonols and flavanols. The total flavanol amounts in seeds varied from 39 to 70 mg.100 g^{-1} FW, whereas the flavonol amounts varied from 14 to 30 mg.100 g^{-1} FW and consisted mainly of myricetin. However, the major compound in seeds was the ellagitannin, expressed as total ellagic acid, contributing 2.22−2.95 g.100 g^{-1} FW. The flesh of grumixama presented a low concentration of proanthocyanidins B1/B2 (**Table 5**). The concentration ranged from 0.41 ± 0.04 to 2.01 ± 0.15 mg.100 g^{-1} FW in the purple grumixama and to 0.79 ± 0.02 mg.100 g^{-1} FW in the yellow one. These values were lower than reported for several cultivars of strawberry, ranging from 53.9 to 168.1 mg.100 g^{-1} FW (BUENDIA *et al.*, 2010).

Table 5 - Total ellagic acid and proanthocyanidin contents in the flesh of *Eugenia brasiliensis* Lam, purple and yellow varieties.

Sample	Free ellagic acid	Total ellagic acid	Total proanthocyanidin
F1	13.76 (2.53)[a]	201 (12)[a]	1.99 (0.26) [a]
F2	9.38 (0.28)[b]	82 (4)[b]	0.41 (0.04) [b]
F3	3.97 (1.16)[c]	152 (11)[c]	1.04 (0.11) [c]
F4	5.14 (0.40)[d]	243 (9)[d]	2.01 (0.15) [a]
F5	13.08 (1.52)	92 (11)	0.79 (0.02)
S1	64.68 (5.53)[a]	2905 (179)[a]	na
S2	60.81 (7.28)[a, b]	2734 (152)[b]	na
S3	37.50 (1.95)[c]	2225 (126)[c]	na
S4	69.21 (3.43)[a]	2240 (113)[d]	na
S5	53.95 (2.69)[b]	2323 (105)[e]	na

The values shown are the means (standard deviation); na = not analyzed; F1 and F2: flesh of the fruit of the purple grumixama from São Paulo, F3 and F4: flesh of the fruit of the purple grumixama from Santa Catarina, F5: flesh of the fruit of the yellow grumixama from São Paulo; S1 and S2: seeds of the purple grumixama from São Paulo, S3 and S4: seeds of the purple grumixama from Santa Catarina, S5: seeds of the yellow grumixama from São Paulo; Value expressed as mg/100 g FW. The same letters indicate non-significant differences between samples (p).

The flesh of purple and yellow grumixama fruits was shown to be a good source of anthocyanins and flavonols, respectively, and both were good sources of ellagitannins. Given the biological activities attributed to these classes of compounds (flavonoids and ellagitannins), the purple and yellow fruits show considerable potential as functional foods. The seeds, although inedible, could be used as sources of bioactive compounds, mainly

ellagitannins. However, studies regarding the biological activities of these materials are also necessary to elucidate the mechanisms by which the biological effects attributed to this plant could occur.

4 PART 2: BIOAVAIBILITY OF ANTHOCYANINS AND ELLAGITANNINS FROM PURPLE GRUMIXAMA IN HUMAN MODEL

4.1 MATERIAL AND METHODS

4.1.1 Chemicals

Cyanidin 3-*O*-glucoside, delphinidin and cyanidin chloride were purchased from Extrasynthese (Genay, France). Ascorbic acid, ellagic acid, hippuric acid, protocatechuic acid, vanillic acid and isovanillic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Breast cancer cells (MDA-MB 231) was purchased from ATCC cell lines (Manassas, VA, USA). Fetal bovine serum, CFSE (Carboxyfluorescein diacetate succinimidyl ester) Cell Proliferation Kit (CellTrace™) and Dulbecco eagle modified culture medium were purchased from GIBCO™ by Life Technologies (Eugene, Oregon, USA). Propidium iodide was purchased from Life Technologies (Eugene, Oregon, USA). Methanol, acetonitrile and formic acid HPLC grade were purchased from Merck Millipore (Darmstadt, Germany).

4.1.2 Grumixama Juice (GJ) Preparation

Commercial frozen fruit was purchased from Bello's farm. Seeds were manually removed, and the juice was prepared with water using a homogenizer and filtered by a blender. Fresh juices were prepared on the experiment day. The phenolic composition of the juice in 100 mL, was 85.19 ± 1.37 mg cyanidin 3-*O*-glucoside, 11.66 ± 0.23 mg total delphinidin eq., 26.74 ± 7.05 mg total quercetin eq., 6.49 ± 0.15 mg free ellagic acid and 471.38 ± 32.99 mg total ellagic acid representative of ETs amount.

4.1.3 Subjects and Study Design

Fifteen healthy volunteers (females) with an average age 27.00±7.48 years, average weight of 58.00 ± 8.45 Kg, average height of 1.60 ± 0.04 meters and with and BMI of 22.27 ± 2.97 Kg/m^2 were recruited for the study. The 15 subjects selected showed acceptable biochemistry parameters (Appendix 2 – Table 1). The samples of only ten subjects were used for bioavailabily study. The inclusion criteria were as follows: (a) no medical history of

cardiovascular, gastrointestinal, hepatic, renal, thyroid or diabetes dysfunctions; (b) no alcohol addiction or smoking (c) does not use vitamins and other supplements; (d) does not use any type of medication that affects digestion and absorption; and not pregnant. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethical Committee of the School of Pharmacy at the University of São Paulo (CAAE nº 17449613.8.0000.0067), and the study was registered with Clinicaltrials.gov (NCT02790658).

The subjects were advised to avoid vegetables and fruits or their derivatives as well as sources of anthocyanins and ETs, such as blackberries, blueberries, cherries, red and purple grapes, strawberries and vegetables for seven days before the study (washout period). The day before and during the experiment day (breakfast, lunch and dinner), the subjects consumed a control diet that consisted of white bread, milk, white cheese, rice or pasta, chicken or fish until the last urine and plasma samples were collected. After 10 h of fasting, the subjects were asked to collect the first urine sample in the morning and ingest grumixama juice (10 mL/Kg body weight). A blood sample was collected before intake and at 15 min, 30 min, 60 min, 120 min, 240 min and 24 h after juice intake. Urine was also collected at the following time periods: 0-1 h, 1-2 h, 2-4 h, 4-6 h, 6-12 h, 12-14 h and the first urine on the morning of the second day (24 h) (**Figure 5**). The plasma samples were acidified with 5% ascorbic acid and the urine with 5% trifluoroacetic acid, respectively, and centrifuged to 2,000 rpm at 4 ºC. Plasma and urine samples were stored at -80 ºC until the analyses.

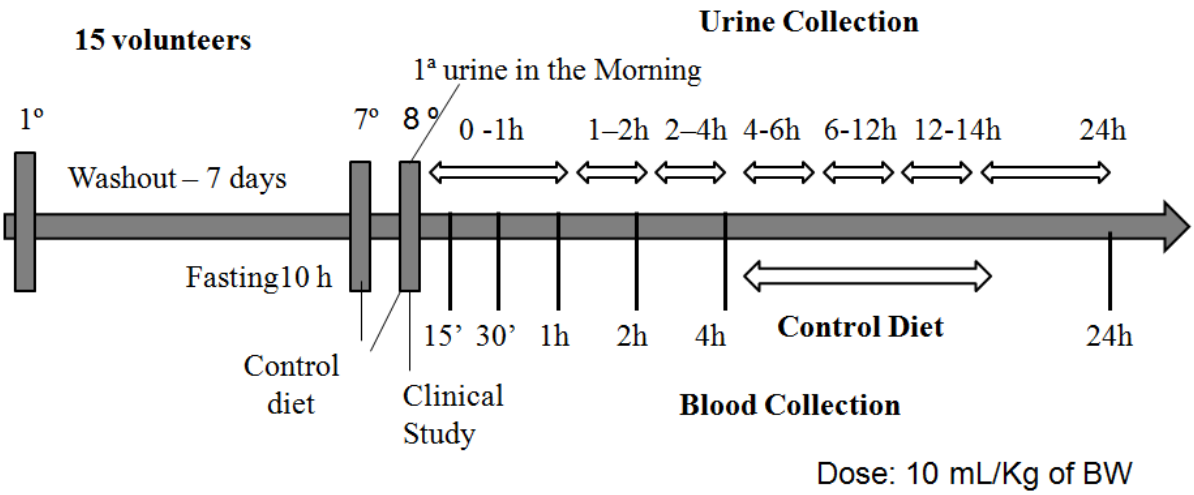

Figure 5- Study design of human protocol.

4.1.4 Anthocyanins, Phenolic Acids and Urolithins Quantification

Solid-phase extraction was used for plasma and urine preparation. Briefly, an aliquot of plasma and urine samples, which was diluted with 10 mM oxalic acid, was loaded onto a C18 column (0.3 g, Supelclean LC-C18alkyl, Supelco, USA) and CC6 polyamide column (1.0 g, Macherey-Nagel Gmbh and Co., Duren, Germany), respectively, that were previously conditioned with methanol and 10 mM oxalic acid. The column was then washed with 10 mM oxalic acid, and the retained compounds were eluted with methanol acidified with 5% TFA. The eluate was reduced in a rotatory evaporator at 40 ºC, reconstituted with methanol acidified with 5% acetic acid and then filtered with a 0.43 μm PTEF filter (Millipore, Bedford, MA). Identification and quantification of exogenous metabolites and anthocyanins were performed by HPLC-DAD, LC-ESI-MS/MS and LC-qTOF-MS.

4.1.5 HPLC-DAD Conditions

The anthocyanins and phenolic compounds in urine and plasma samples were quantified using the liquid chromatograph 1260 Infinity Quaternary LC System (Agilent Technologies, USA, with a quaternary pump and autosampler coupled to a diode array detector (DAD). The column used was a 5 Prodigy ODS3 column (250 x 4.60 mm) (Phenomenex Ltd., United Kingdom) with a flow rate of 1 mL/min and a column temperature of 25 ºC. The mobile phase consisted of the following two solvents: (A) 0.5% formic acid in water and (B) 0.5% formic acid in acetonitrile. The gradient used was as follows: 10% B at beginning, 10% at 5 min, 20% at 15 min, 25% at 25 min, 35% at 33 min, 50% at 38 min, and 90% at 43-44 min and 10% at 45 min. Anthocyanins and other phenolic compounds were detected by monitoring the elution at 525 nm and 270 nm, respectively. The quantification was based on calibration curves using the commercial standards of the C3G, protocatechuic acid (PCA), vanillic acid (VA), isovanillic acid (iVA) and hippuric acid (HA). Urolithin derivatives were quantified as EA. The concentrations were expressed as nmol/L for plasma samples and nmol/mmol and μmol/mmol for creatinine for urine samples. The elimination values were expressed in nmol and μmol using the total volume of urine.

4.1.6 LC-ESI-MS/MS and LC-qTOF-MS Conditions

The chromatography condition was the same used for HPLC-DAD. The anthocyanins and phenolic compounds in urine and plasma samples were identified by LC-ESI-MS/MS using a Prominence Liquid Chromatograph (Shimadzu, Japan) coupled to an ion trap Esquires-LC mass spectrometer (Bruker Daltonics, Billerica, MA) with an electrospray ionization (ESI) interface. After passing through the DAD detector, the flow was changed to 0.2 mL/min for injection into the mass spectrometer. The mass spectrometer conditions were as follows: High-voltage capillary 4000 V and 3500 V for positive and negative mode, respectively, a capillary temperature of 300 °C, and dry gas 6.0 L/min. Urolithins were identified using a Prominence Liquid Chromatograph (Shimadzu, Japan) coupled to microTOF-Q II (Bruker Daltonics, Billerica, MA). The mass spectrometer conditions were as follows: collision cell energy -10.0 eV negative mode, a capillary temperature of 270 °C, capillary 3200 V, and dry gas 6.0 L/min. Both analyses were carried out using a full scan from *m/z* 50 to 1500. The phenolic compounds were identified by comparing their retention times, absorption spectral characteristics and mass spectra, which were obtained by LC-ESI-MS/MS for anthocyanins and phenolic acids and by LC-qTOF-MS for urolithins, with data available in the literature and isolated standards when possible. Co-chromatography was used when possible.

4.1.7 Sample Preparations for Cell Proliferation Assay

The analyzed plasma samples were collected at time 0 before the GJ intake (P0), during 0-2 h (P1), 4 h (P2) and at 24 h (P3) after GJ intake. The analyzed urine samples were collected at time 0 before the GJ intake (U0), during 0-2 h (U1), during 2-4 h (U2) and at 24 h (U3) after GJ intake. Aliquots of urine (8 mL) and plasma (800 μL) samples were passed through CC6 polyamide columns as described above and reduced to dryness on a rotary evaporator and dissolved in DMSO (20 mg/mL, stock solution). Immediately before each experiment, the extract was filtered and dilutions were made in culture medium to achieve the desired final concentrations (200 μg/mL).

4.1.8 Cell Culture

MDA-MB-231 (ATCC, USA) cells were cultured as a monolayer in Dulbecco's modified Eagle's medium (GIBCO, USA) supplemented with FBS (10%, v/v) (GIBCO, USA). The cells were maintained in a humidified incubator chamber with an atmosphere of 5% of CO_2 at 37ºC until logarithmic growth (~80% confluence) was established (3-4 days).

4.1.9 Cell Proliferation Assay

For CFSE staining, the cells were washed with PBS, stained with 5 µM CFSE for the cell proliferation assay and seeded on a 24-well plate ($5x10^{-4}$ cells per well) 24 h before treatment as described by Quah *et al.* (2010). The maximum fluorescence was measured immediately before extract treatment to evaluate the fluorescence of undivided cells. The Cells were further exposed to 200 µg/mL of urine and plasma extract preparations and phenolic compound standards (PCA, VA, HA, C3G) at 2.5 and 25 µg/mL. Then, the cells were incubated at 37ºC under an atmosphere of 5% CO_2 for 48 h. Growth media supplemented with dimethyl sulfoxide (DMSO) and marked with CFSE dye was used as a negative control. Fluorescence was measured with a FACS Verso flow cytometer (BD Biosciences, USA), and ten thousand events were acquired for data analysis. The results were expressed as a percentage of cell proliferation inhibition after setting the percentage of fluorescence decay for the control (DMSO) as 100% proliferation.

4.1.10 Cell Cycle Assay

MDA-MB-231 cells ($5x10^{-4}$ cells per well seeded on a 24-well plate) were treated with extracts as described above, trypsinized and fixed for 30 min on ice in 70% ice-cold ethanol. The cells were incubated for 30 min at 4 ºC, washed with PBS and resuspended in 200 µL of lysis solution (0.1% Triton and 0.1% sodium citrate), containing propidium iodide (10 µg/mL) and RNase (15 µg/mL) (Miranda *et al.*, 2014). After 24 h, the cells were further exposed to 200 µg/mL of urine and plasma extracts and phenolic compound standards (PCA, VA, HA, C3G) at 2.5 µg/mL. Then, the cells were incubated at 37 ºC under an atmosphere of 5% CO_2 for 48 h. Fluorescence was measured with a FACS Verso flow cytometer (BD Biosciences, USA), and ten thousand events were acquired for data analysis. Only the cells in G0/G1, S and G2/M phases were considered to distribute in the cell cycle. The cells in subG0/G1 were

considered to be indicative of apoptosis. The data were analyzed using a FlowJo cell cycle platform (Tree Star, Ashland, OR).

4.1.11 Statistical Methods

Plasma and urine Cmax and the time to peak concentration (tmax) can be observed in the graphics for each volunteer. The area under the curve (AUC) for plasma concentration time (0–24 h) and urine concentration time (0–24 h) was estimated using the linear trapezoidal rule. The results were expressed as the mean ± standard error. Analysis of variance (one-way ANOVA) followed by a t-test was performed using Biostat 5.0. *p*-values< 0.05 was considered significant.

4.2 RESULTS

4.2.1 Plasma and Urine Metabolites

Table 6 shows the anthocyanins and ETs metabolites detected and identified in the urine and plasma samples of the subjects over a 24 h period after a single dose of grumixama juice (GJ). The following three anthocyanins were identified in the urine: C3G, which was the main anthocyanin present in the juice (at *m/z* 449), and the methylated forms of C3G and cyanidin (at *m/z* 463 and *m/z* 301, respectively). In addition, four phenolic acids, which were likely derived from anthocyanin degradation, including PCA (at *m/z* 153), VA, iVA acid (at *m/z* 167), and HA (at *m/z* 178), were detected in both plasma and urine samples.

Furthermore, seven urolithins derived from the metabolism of ETs by gut microbiota were identified in urine (**Table 6**) by comparing the mass spectra obtained by LC-qTOF-MS and literature data (García-Villalba *et al.*, 2013; Espín *et al.*, 2007). Among the urolithins, two isomers of urolithin A glucuronide presented molecular ions ($[M-H]^-$) at *m/z* 403 and MS^2 yielded a characteristic ion at *m/z* 227. One peak of urolithin C presented characteristic $[M-H]^-$ at *m/z* 243 and two isomers of urolithin C methyl ether glucuronide (at *m/z* 433). One peak of urolithin B glucuronide (at *m/z* 387) and urolithin D glucuronide (at *m/z* 435) had characteristic ions at *m/z* 211 and 259, respectively. No evidence of the ET, ellagic acid or other flavonoids was observed in urine or plasma samples.

Table 6 – Phenolic compounds identified in plasma and urine samples of healthy subjects by HPLC-ESI-MS/MS and HPLC-qTOF-MS following intake of a single dose of grumixama juice.

Identified Compounds (Positive mode)	**RT (min)**	**$[M]^+$ (*m/z*)**	**MS^2 (*m/z*)**	**Urine**	**Plasma**
Anthocyanins					
Cyanidin-3-glucoside*	10.6	449	287/ 213	X	-
Methylated cyanidin 3-glucoside	13.4	463	301	X	-
Methylated cyanidin	18.1	301	283	X	-
(Negative mode)	**RT (min)**	**$[M-H^+]^-$ (*m/z*)**	**MS^2 (*m/z*)**	**Urine**	**Plasma**
Phenolic acids					
Protocatechuic acid*	7.8	153	-	X	x
Hippuric acid*	14.5	178	-	X	x
Vanillic acid*	15.3	167	-	X	x
Isovanillic acid*	16.7	167	-	X	x
Urolithins					
Urolithin A glc	19.5	403	227	X	-
Urolithin A glc	20.9	403/807	227	X	-
Urolithin C methyl ether glc	24.7	433	243	X	-
Urolithin C	27.7	243	243	X	-
Urolithin B glc	29.5	387	211	X	-
Urolithin C methyl ether glc	33.2	433	257	X	-
Urolithin D glc	37.6	435	259	X	-

*Compound identity was confirmed with commercial standards. Glc, glucuronide.

Figure 6 showed the proposed route of anthocyanins metabolites and phenolic acids formation and **Figure 7** shows the proposed route of ellagitannins degradation by gut microbiota.

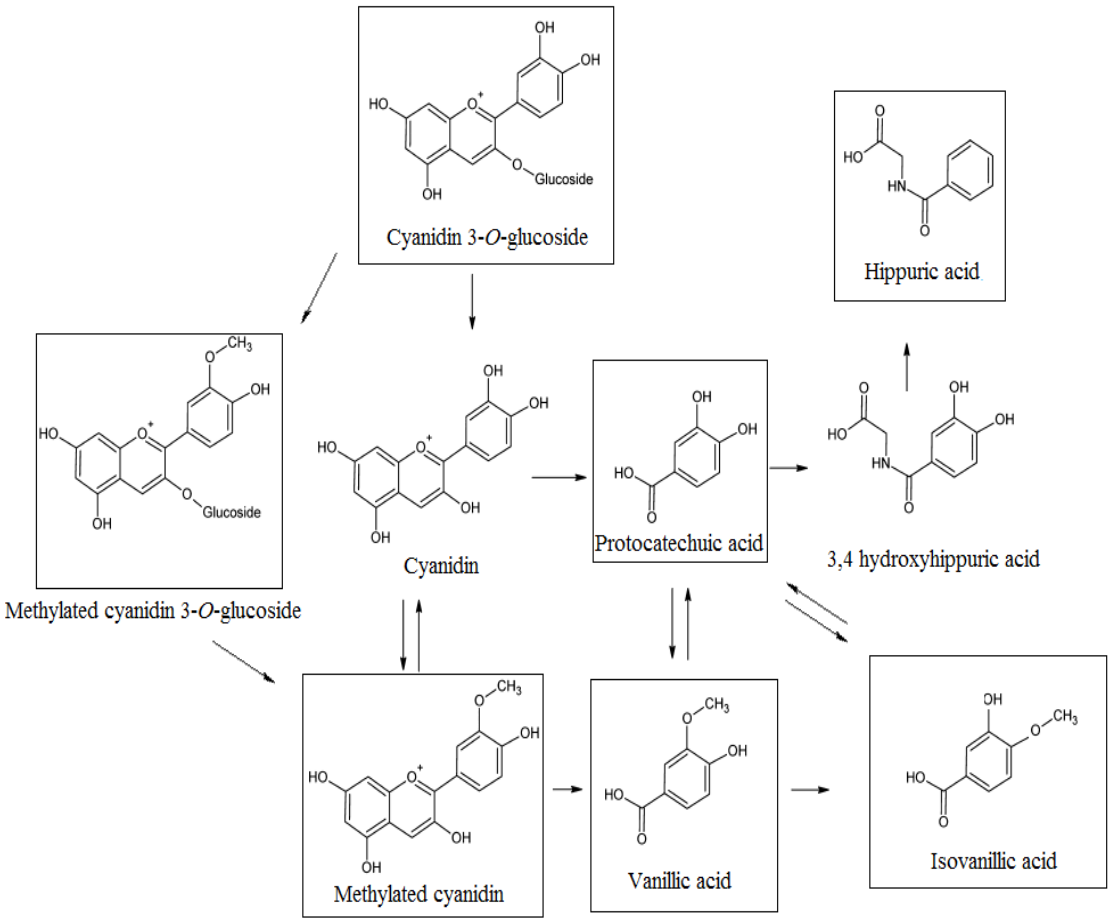

Figure 6 - Proposed route of the chemical and gut microbiota degradation of anthocyanins and Phase II metabolites

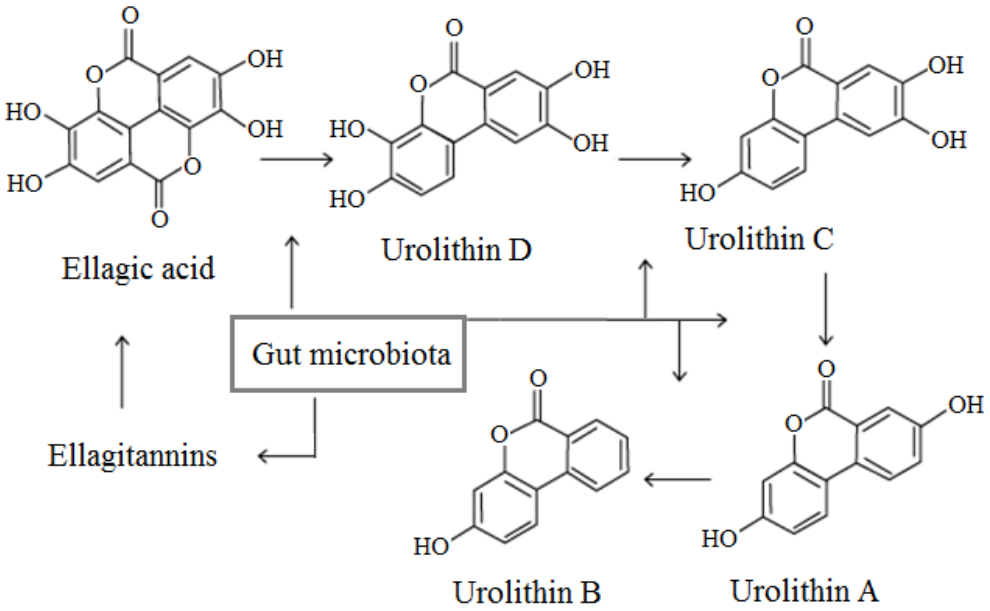

Figure 7 – Proposed route of urolithin formation from ellagic acid and ellagitannins.

4.2.2 Pharmacokinetic Parameters of Anthocyanins and Phenolic Acids in Urine

The pharmacokinetic parameters of anthocyanins and metabolites, including phenolic acids, in the urine samples are listed in **Table 7**, and the time course curves are presented in **Figures 9** and **8**, respectively. According to differences in the pharmacokinetic profiles of anthocyanins metabolites in urine, the subjects were divided into the following two groups based on metabolite excretions: Groups A (n=5 subjects) and B (n=5 subjects) (**Figure 8**).

Table 7 – Pharmacokinetic parameters of metabolites detected in urine of healthy subjects following acute grumixama juice intake.

Metabolites	$C_{máx.}$		$t_{máx.}$ (h)		AUC (µmol/h)	
	Group A	Group B	Group A	Group B	Group A	Group B
Anthocyanin *(nmol/mmol of creatinine)*						
C3G	592±184	322±76	2-4	1-2	1.51±0.57	1.35±0.37
Methylated C3G	159±45	101±37	1-2	2-4	0.54±0.19	0.44±0.15
Methylated cyanidin	172±39	255±122	6-12	12-14	3.10±0.93	2.39±0.91
Phenolic acids *(µmol/ mmol of creatinine)*						
PCA	22.3±7.0	12.1±3.1	1-2	6-12	163±31	112±31
VA	142±45	80.8±10.6	1-2	2-4	1543±407	996±156
IVÃ	18.8±6.2	16.0±2.3	1-2	2-4	260±67	218±41
HÁ	104±27	589±143[a]	6-14	2-4	2459±496	6576±2228[b]
Urolithins *(µmol/mmol of creatinine)*						
Urolithin A	21.3±4.6	258±130[a]	24	12-14	300±90	2691±1696
Urolithin B	8.46±1.79	76.1±19.4[a]	24	24	42.3±13.7	380±152[a]
Urolithin C	20.7±3.4	400±218[a]	4-6	12-14	309±26	3752±2526
Urolithin D	7.02±1.74	50.8±22.5[a]	2-4	24	82.1±17.3	376±143[a]

n=10 subjects; AUC = area under the curve (0-24 h); C_{max}: maximum concentration; t_{max}: time to reach C_{max}. a: $p<0.05$, one-way ANOVA followed by t-test. comparing Cmax of group A and B. b: p= 0.08, one-way ANOVA followed by to t-test comparing Cmax of group A and B. PCA: protocatechuic acid; VA: vanillic acid, iVA: isovanillic acid, HA: hippuric acid, C3G: cyanidin 3-O-glucoside, Urolithin A: two isomers of urolithin A glucuronide; Urolithin B: urolithin B glucuronide, Urolithin C: two isomers of urolithin C methyl ether glucuronide + urolithin C, Urolithin D: urolithin D glucuronide. For urolithin B n=7 subjects. Few AUC values were keep with more than 3 significant figures to keep same unit to all metabolites.

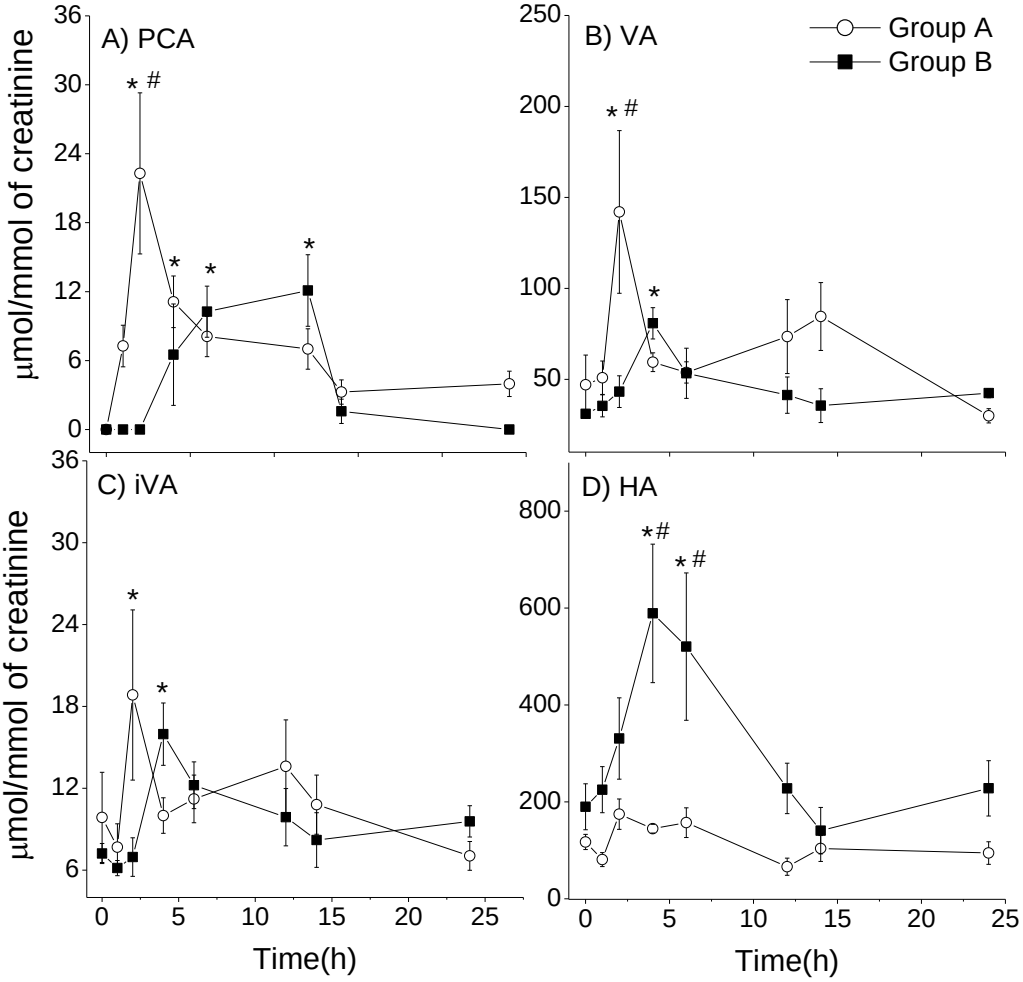

Figure 8 - Time course concentration of phenolic acids identified in urine samples after intake of a single dose of grumixama juice. (A) PCA, protocatechuic acid, (B) VA, vanillic acid, C) iVA, isovanillic acid and (D) HA, hippuric acid. The results were expressed as the mean ± SEM of 10 healthy subjects (group A, n=5; group B, n=5). The results were analyzed with a one-way ANOVA followed by a t-test. *p<0.05 compared to time zero; #p<0.05 compared to the same time collection between groups.

PCA is the main metabolite of C3G degradation. However, its methylated form, VA, was found in higher amounts in urine samples (**Figure 8B**). Group A had a C_{max} of PCA at 1-2 h after GJ intake, which decreased after 4 h, whereas group B had begun to eliminate PCA at 2-4 h after GJ intake, and the excretion increased until 12 h and decreased to the basal level at 24 h (**Figure 8A**). For VA, group A had a C_{max} at 1-2 h and presented a new slight peak at 12-14 h after GJ intake. In contrast, group B had a C_{max} two hours later than group A and excretion decreased after this time (**Figure 8B**). Isovanillic acid followed the same pattern of VA. Hippuric acid was the main phenolic acid excreted through 24 h after GJ intake, and Group B excreted approximately 77% more than Group A (67.35 and 38.12 μmol/mmol of creatinine, respectively) (**Figure 10B**), with a significant difference ($p<0.05$) of three-fold in AUC between groups (**Table 7**).

For the anthocyanins excreted in urine samples, group A showed a more defined C_{max} for C3G and methylated C3G at 1-2 h time after GJ intake, whereas group B had C_{max} two hours later (**Figure 9A** and **9B,** respectively), although there was no significant difference in AUC between the groups (**Table 7**). The methylated form of cyanidin (**Figure 9C**) was excreted later than the other anthocyanins with no well-defined C_{max} through 24 h after GJ intake. The total amount of C3G, methylated C3G and methylated cyanidin, which was excreted throughout 24 h, did not vary significantly between both groups (**Figure 10A**).

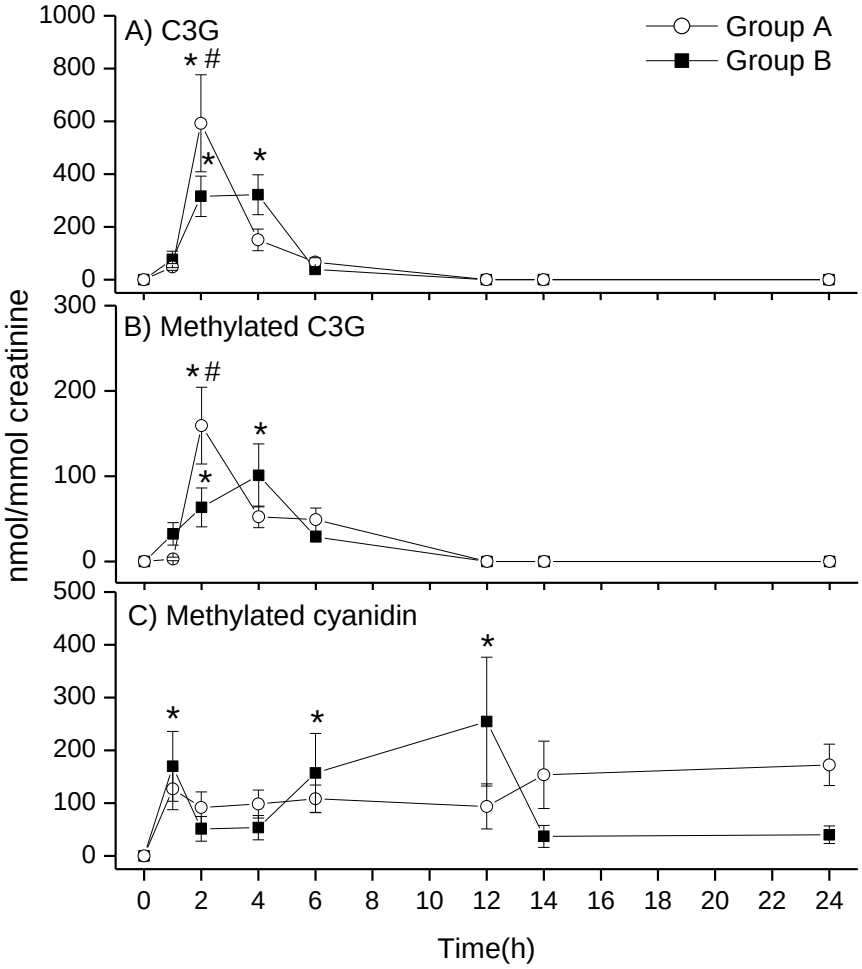

Figure 9 – Time course concentration of anthocyanins identified in urine samples after intake of a single dose of grumixama juice. (A) C3G, Cyanidin 3-O-glucoside, (B) methyled C3G and (C) methyled cyanidin. The results were expressed as the mean ± SEM of 10 healthy subjects (group A, n=5; group B, n=5). The results were analuzed with a One-way ANOVA followed by a t-test. *p<0.05 compared to time zero; #p<0.05 compared to the same time collection between groups.

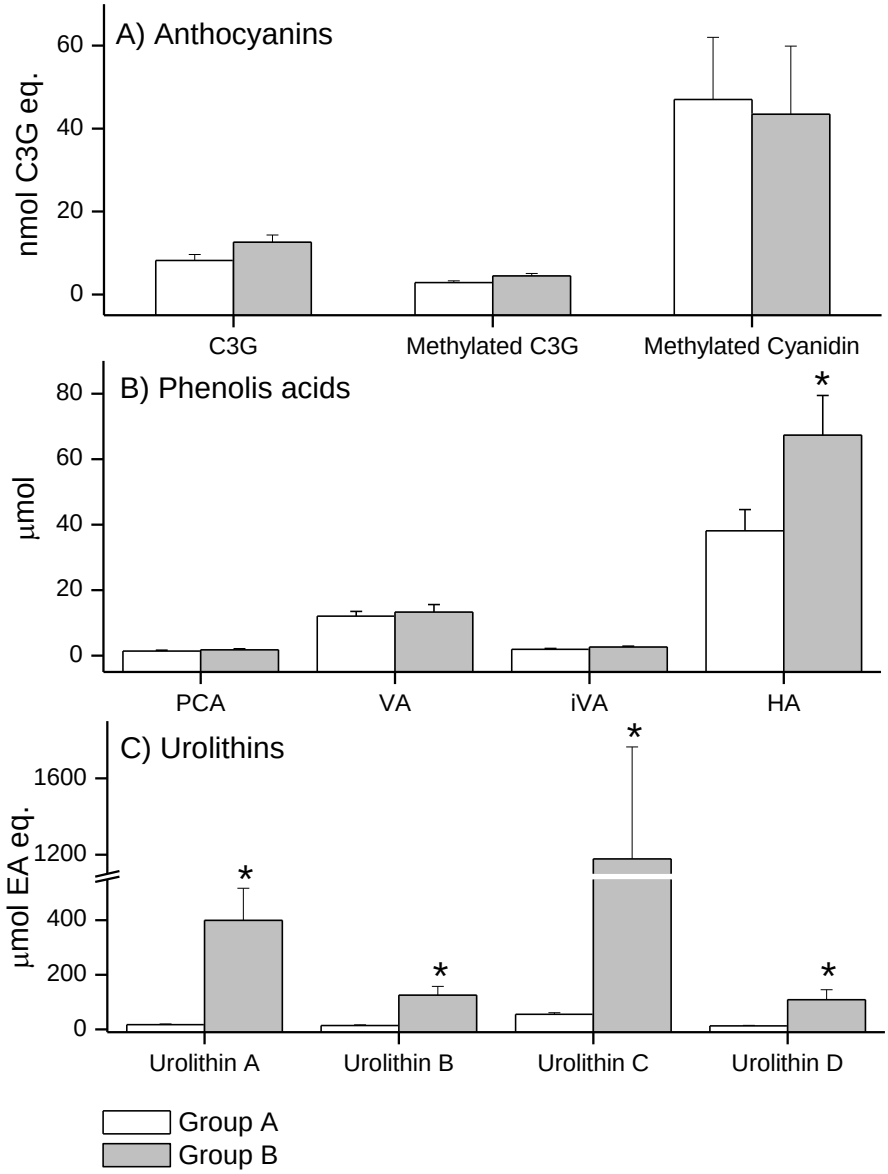

Figure 10 – Total elimination of metabolites in urine samples after intake of a single dose of grumixama juice. (A) anthocyanins, (B) phenolic acids and (C) urolithins. C3G, Cyanidin 3-O-glucoside; PCA, protocatechuic acid; VA, vanilic acid: iVA, isovanilic acid; HA, hippuric acid. The results were expressed as the mean ± SEM of 10 healthy subjects (group A, n=5; group B, n=5). The results were analyzed with a one-way ANOVA followed by a t-test. *$p<0.05$ compared between groups.

Considering all the flavonoid metabolites detected in urine samples after GJ intake, the total phenolic acid and total anthocyanins represented approximately 99.9% and 0.1%, respectively, of the metabolites excreted. Furthermore, PCA, which is a specific metabolite of C3G, represented only 2.6% (group A) and 2.1% (group B) of the total amount of anthocyanin metabolites excreted 24 h after GJ intake.

4.2.3 Pharmacokinetic Parameters of Urolithins in Urine

Urolithins is known to be the ETs metabolites formed by gut microbiota. In the present study, the relative excretion of ETs metabolites in urine samples were evaluated using LC-qTOF-MS. Urolithins were not detected before GJ intake. All subjects showed the ability to produce urolithins, mainly presented as Phase II metabolites of urolithins A, C and D, but only seven subjects were able to produce urolithin B glucuronide (**Table 7** and **Figure 11**).

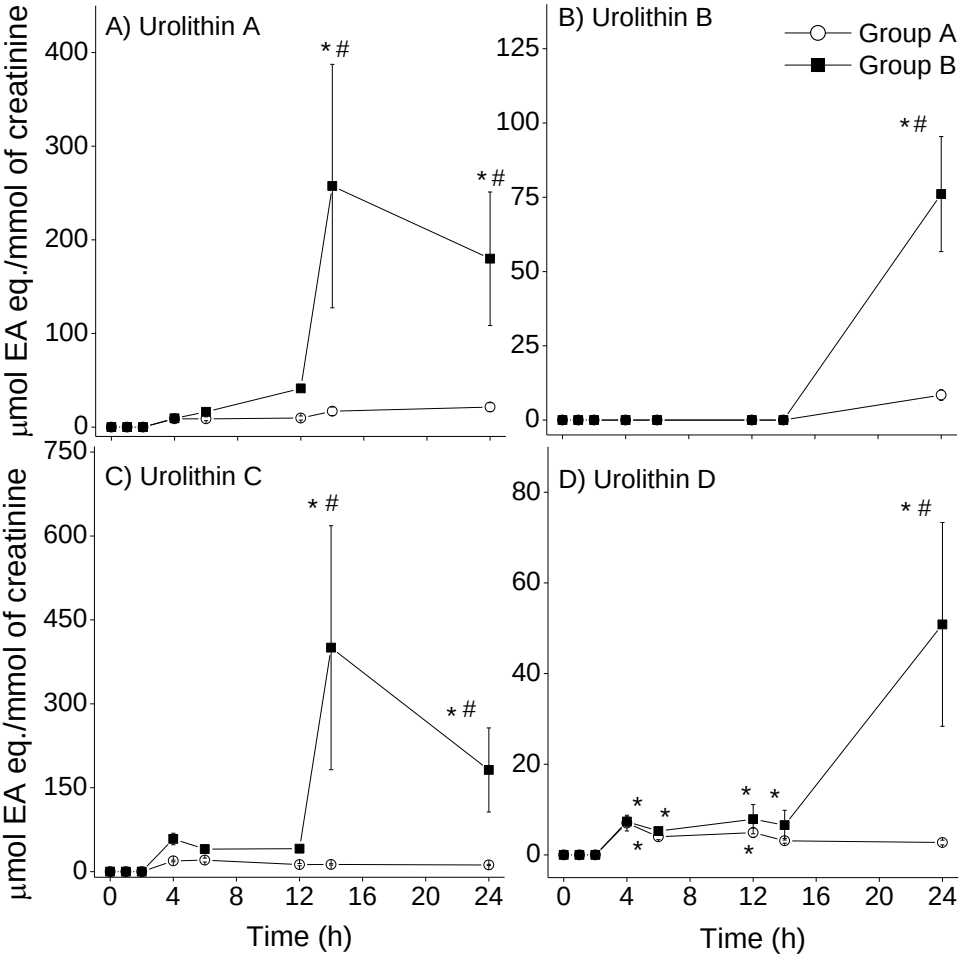

Figure 11 - Time course concentration of urolithins identified in urine samples after intake of a single dose of grumixama juice. A) urolithin A glucuronide, B) urolithin B glucuronide, C) urolithin C methyl ether glucuronide (urolithin C was calculated together) and D) urolithin D glucuronide. The results were expressed as the mean ± SEM of 10 healthy subjects (group A, n=5; group B, n=5). The results were analuzed with a one-way ANOVA followed by a t-test. *p<0.05 compared to time zero; #p<0.05 compared to same time collection between groups.

Similar to phenolic acids, the subjects could be stratified into two groups (Group A and B) of urolithins excretory (n=6 and n=4 subjects, respectively) according to the difference in the pharmacokinetic profile and total elimination. The main urolithins excreted were urolithin C followed by urolithin A. Urolithins beginning excretion 4 h after GJ intake and increasing concentration until 24 h (**Figure 11**). Urolithins were detected after 4 h of GJ intake for both groups. However, while Group A presented a slight increase through 24 h, Group B showed a high increase after 12 h for the urolithins A, B, C and D (**Figure 11**) and ten times fold change in AUC **(Table 7**). In addition, Group B eliminated significant ($p<0.05$) amount of all four urolithins during 24 h than group A (**Figure 10C**).

4.2.4 Pharmacokinetic Parameters of Phenolic Acids in Plasma

All phenolic acids (PCA, VA, IVA and HA) detected in urine was also identified in plasma, but none anthocyanin was detected. These phenolic acids showed a similar time course curve profile (**Figure 12**) compared to urine samples with no well-defined C_{max}. A slight but significantly C_{max} was observed to PCA at 2 and 24 h after GJ intake compared to time zero (before intake) (**Figure 12A**). However, the VA and iVA plasma concentration not varied significantly through 24 h after GJ intake as compared to time 0 (**Figure 12B** and **11C**). **Table 8** shows the pharmacokinetic parameters of metabolites detected in plasma samples.

Table 8 – Pharmacokinetic parameters of metabolites detected in plasma samples of healthy subjects following a single dose of grumixama juice intake.

Metabolites	**$C_{máx.}$** (nmol/mL)	**$t_{máx.}$ (h)**	**AUC (nmol/h)**
Protocatechuic acid	5.14±1.69	24	81.88±33.74
Vanillic acid	11.89±4.32	24	103.81±53.30
Isovanillic acid	7.31±3.41	1	61.34±28.16
Hippuric acid	7.88±3.80	0.25	60.05±24.44

n=10 subjects; AUC: area under the curve (0-24 h); C_{max}: maximum concentration; t_{max}: time to reach

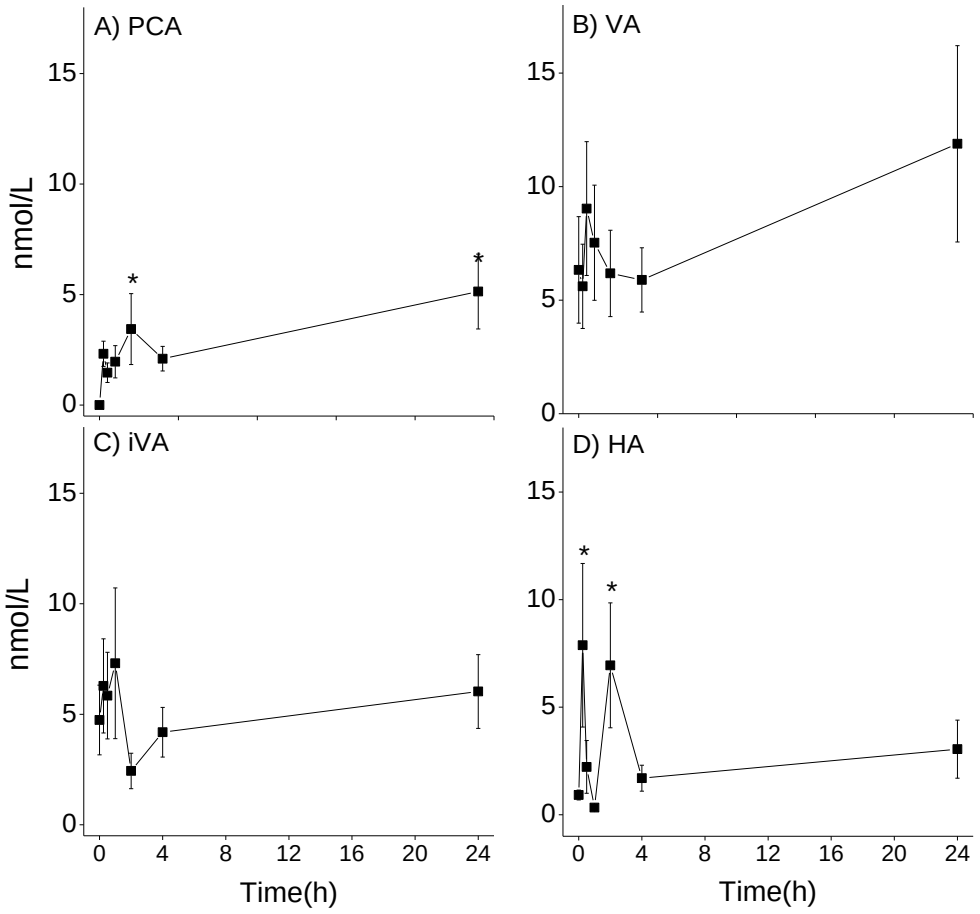

Figure 12 – Time course concentration of phenolic acids identified in plasma samples after intake of a single dose of grumixama juice. (A) APC, protocatechuic acid, (B) VA, vanillic acid, (C) iVA, isovanillic acid and (D) HA, hippuric acid. The results were expressed as the mean ± SEM of 10 healthy subjects. The results were analyzed with a one-way ANOVA followed by a t-test. *$p<0.05$ compared to time zero.

4.2.5 Cell Proliferation Inhibition of Metabolites

Considering that the circulating and excreted metabolites presented some bioactivity, the antiproliferative activity of plasma and urine extracts, which were collected at different time point after GJ intake, were evaluated against MDA-MB 231 human breast cancer cells (**Figure 13**). All urine (U0, U1, U2 and U3) and plasma (P0, P1, P2 and P3) extracts collected before and after GJ intake caused cell proliferation inhibition (at 200 µg/mL) compared to the control (DMSO, $p<0.05$). The highest inhibition was observed for the U2 extract (0-2 h time period), which presented 53% inhibition compared to the control (DMSO, $p<0.05$) and around

twice as much inhibition compared to U0 extract. In contrast, phenolic acids (PCA, AV, iAV and HA) and the anthocyanin C3G, at 2.5 and 25 µg/mL, did not show antiproliferative activity.

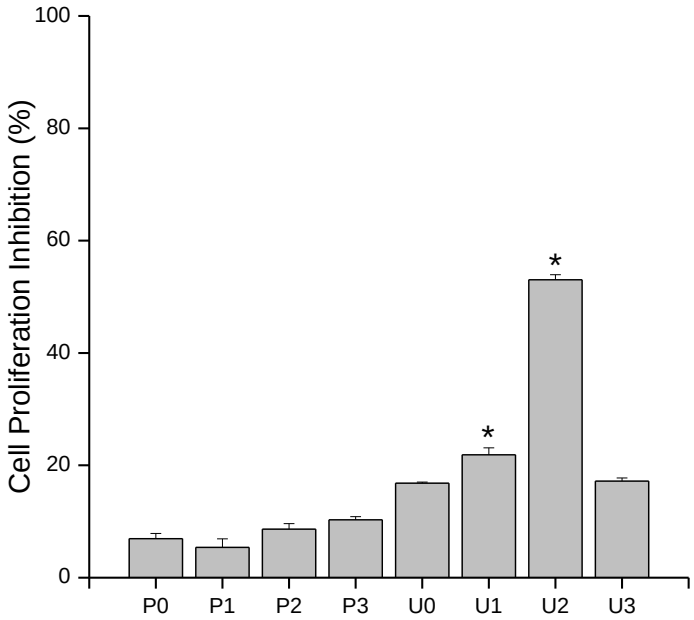

Figure 13 –Inhibition of MDA-MB 231 human breast cancer cells by metabolites extracted from plasma and urine samples of healthy subjects after intake of a single dose of grumixama juice. P corresponded to plasma samples collected at time zero (P0) and a pool of different collection periods after GJ intake; U0, corresponded to urine samples collected at time zero and a pool of different collection periods (see methods). The results were expressed as the mean ± SEM. The results were analyzed with a one-way ANOVA followed by a t-test. *$p<0.05$ compared to time zero, P0 or U0.

4.2.7 Cell Cycle Assay

The U0 and U2 extracts induced significant ($p<0.05$) G2/M cell cycle arrest (27% and 74% increase, respectively) compared to the control (DMSO) (**Figure 14A**). PCA also showed similar results (39% increase) (**Figure 14A**). All urine extracts and C3G induced a significant ($p<0.05$) increase in the subG1 population, which indicated apoptosis (**Figure 14B**). The increase in subG1 population was intense in C3G (86% increase), U0 (272% increase), U1 (152% increase), U2 (454% increase) and U3 (100% increase) compared to the control (DMSO).

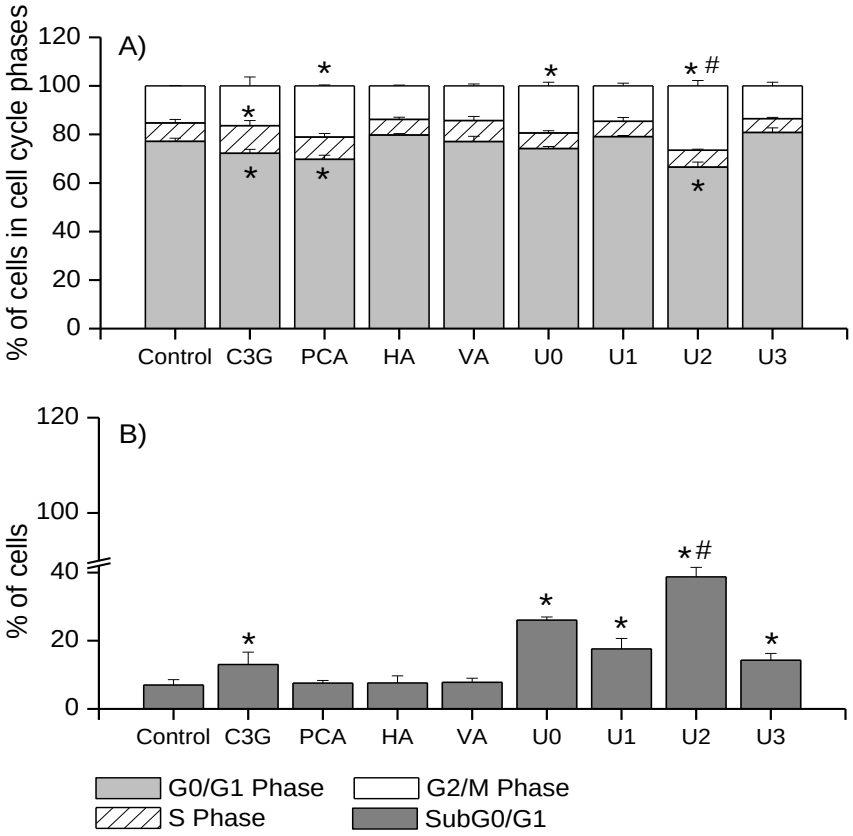

Figure 14 – Cell cycle analysis of MDA-MB 231 human breast cancer cells treated with metabolites extracted from urine samples of healthy subjects following intake of a single dose of grumixama juice. PCA, protocatechuic acid (2.5 µg/mL); VA, vanilic acid (2.5 µg/mL); iVA, isovanilic acid (2.5 µg/mL); HA, hippuric acid (2.5 µg/mL); C3G, cyanidin 3-*O*-glucoside (2.5 µg/mL); U, corresponds to urine samples collected at time zero and a pool of different collection periods (see methods). (A) Percentage of cells in cell cycle phases (B) Percentage of cells in SubG0/G1 related to the total number of cells. The results were expressed as the mean ± SEM. The results were analyzed with a one-way ANOVA followed by a t-test. *$p<0.05$ compared to DMSO control, #$p<0.05$ compared to time zero, U0.

4.3 DISCUSSION

Purple grumixama fruit is a good source of anthocyanins and ETs (TEIXEIRA *et al.*, 2015), which have been shown to play an important role in enhancing health (DEL RIO *et al.*, 2013, FANG, 2014; KASIMSETTY *et al.*, 2010). However, the main compounds found in circulation and excreted were their metabolites, including phenolic acids and urolithins, which are mainly associated with gut microbiota metabolism (DEL RIO *et al.*, 2013, SELMA *et al.*, 2014).

According to pharmacokinetic parameters and time course curves, the study subjects could be divided into two groups of phenolic acid excretions, with one group that has two peaks of AV and iVA, and another group with a higher excretion of HA 24 h after GJ intake. Anthocyanins, such as C3G, could be degraded to phenolic acids spontaneously in the neutral pH of the small intestine and circulation to form PCA, which could be further methylated by phase II enzymes to VA and iVA or by gut metabolism (VITAGLIONE *et al.*, 2007; Kay *et al.*, 2009; WILLIAMSON AND CLIFFORD, 2010; FERRARS *et al.*, 2014). The chemical instability of C3G on physiological pH could explain the early peak concentration of PCA, VA and iVA, which coincided with C3G in urine. In addition, a second peak concentration of VA and iVA (at 12-14 h) observed in Group A and an increased concentration of HA (Group B) could be due to the metabolism of unabsorbed anthocyanin by gut microbiota.

The effect of gut microbiota is relevant for producing PCA because HA can be formed by attaching the glycine moieties to form 3-hydroxyhippuric acid or 4-hydroxyhippuric acid and hippuric acid by the loss of two hydroxyl groups from the aromatic ring (OLIVEIRA *et al.*, 2011). In addition, 99.9% of the total metabolites excreted were phenolic acids, especially HA, considered an important biomarker of polyphenol intake (KRUPP *et al.*, 2012), including C3G (CZANK *et al.*, 2013; FERRARS *et al.*, 2014).

Ellagitannins (ETs) are the main phenolic compounds present in GJ (471.38 ± 32.99 mg total ellagic acid/100 mL), especially the monomers ET castalagin/vescalagin (TEIXEIRA *et al.*, 2015). All subjects were able to metabolize ET after GJ intake to form urolithins, which are the main catabolites from gut microbiota and are excreted mainly as phase II metabolites. All urolithin forms (A, B, C and D) were excreted in urine samples. Theses urolithins were excreted in higher amounts for group B 24 h after GJ intake compared to group A. The urolithins (A, C and D) began to appear in urine over 4 h after GJ intake, which was earlier compared to other studies that found urolithins over 7 h after intake of ET source (GONZALEZ-BARRIO *et al.*, 2010; LUDWIG *et al.*, 2015). The earlier production of

urolithins could be due to gut microbiota metabolism in the small intestine and the presence of free EA in the juice (ESPÍN *et al.*, 2007). Furthermore, urolithin B glucuronide, which is mainly produced in the distal parts of the intestine (ESPÍN *et al.*, 2007), was detected only 24 h after GJ intake. In the present study, all subjects were urolithin producers, but one group was more capable of producing urolithins than the other, which could indicate a different gut microbiota composition.

Few bacterial groups were associated with the production of urolithins, such as *C. coccoides spp*., *E. rectal spp*., some species of *Ruminococcus* genus (GARCÍA-VILLALBA *et al.*, 2013) and *Gordonibacter spp*. (SELMA *et al.*, 2014).

Anthocyanins and ETs are related to several mechanisms of cancer chemoprevention (KASIMSETTY *et al.*, 2010; GONZALEZ-BARRIO *et al.*, 2010; FANG, 2014; FERNANDES *et al.*, 2013; LIU *et al.*, 2013; FURLANETTO *et al.*, 2012; GONZÁLEZ-SARRÍAS *et al.*, 2014). The extracts of urine obtained after GJ intake inhibited MDA-MB 231breast cancer cells proliferation, and the urine extract collected during 2-4 h time point (U2) presented the highest inhibition. At this time point, the main metabolite that was identified was HA, but neither isolated HA nor the other phenolic acids showed proliferation inhibition in breast cancer cells. Beyond the phenolic acids, anthocyanins and urolithins were also detected at this time point after GJ intake. The urolithins (aglycone or Phase II metabolites) also showed proliferation inhibition in colon cancer cells (Caco-2 and HT-29) (KASIMSETTY *et al.*, 2010; GONZÁLEZ-SARRÍAS *et al.*, 2014), which performed better than isolated ETs and phenolic acids for intestinal (HT-29) and breast (BT-549) cancer cells (KASIMSETTY *et al.*, 2010). The C3G were previously related to proliferation inhibition in MDA-MB 231 cells and were related to apoptosis (LI at al., 2009, LIU *et al.*, 2013). Therefore, the cell proliferation inhibition observed in U2 extract could be due to a synergistic effect among phenolic acids, anthocyanins and urolithins.

The inhibition observed with the U2 extract could be promoted by G2/M cell cycle arrest, which is indicative of apoptosis and was demonstrated by the increase in sub-G0/G1 populations. Flavonoids, such as quercetin, also induced G2/M cell cycle arrest and led to inhibition of COX-2 expression and cyclin D1, which were essential processes for cell cycle progression (LI *et al.*, 2011; GÓMEZ-ALONSO, 2012; PARK *et al.*, 2012).

In conclusion, the subjects could be separated into two different pharmacokinetic profiles of anthocyanins and ETs metabolite excretion. One group showed better excretion of phenolic acids and the other showed better excretion of urolithins. This study also showed that GJ intake provided a high amount of ETs metabolites, which were excreted in urine at an

earlier time than the times from previous studies. In addition, the tested urine extracts showed inhibition of MDA-MB-231 human breast cancer cells proliferation, and this effect could be due to synergy among anthocyanins and ETs metabolites.

5 PART 3: CHANGES IN URINE METABOLOMIC PROFILE AFTER SINGLE DOSE OF PURPLE GRUMIXAMA JUICE INTAKE

5.1 MATERIAL AND METHODS

Urine and plasma samples were collected after single dose of purple grumixama juice as describe in the study design (item 4.1.3).

5.1.1 Chemicals

Benzoic, fumaric, citric, cis-aconitic, malonic, oxalic, tartaric and glyoxylic acids, beta-alanine, L-histidine, L-phenylalanine, L-tyrosine, 4-hydroxyphenylacetic acid, glutamic acid, DL-alanine-2,3,3,3-d4 and methyl chloroformate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and chloroform HPLC grade were purchased from Merck Millipore (Darmstadt, Germany).

5.1.2 Sample Preparation

Urine samples collected before (T0) and after GJ intake (0-1 h, 1-2 h, 2-4 h and 24 h) were acidified with 5% ascorbic acid and centrifuged at 2,000 rpm and 4 ºC and stored at –80ºC until analyses. U0 indicated the urine samples obtained before GJ intake, and after GJ intake, the samples were designated as U1 (0-1 h), U2 (1-2 h), U3 (2-4 h) and U4 (24 h). The urine samples were centrifuged, and the supernatant was aliquoted in tubes. DL-alanine-2,3,3,3-d4 (10 mM) was added to each tube as an internal standard. The samples were derivatised using the modified methyl chloroformate alkylation procedure detailed by Smart et al. (2010)[17–19]. All analyses were conducted in triplicate. The same derivatisation procedure was performed for all of the following standards: benzoic acid, fumaric acid, citric acid, isocitric acid, cis-aconitic acid, succinic acid, malonic acid, oxalic acid, tartaric acid, glyoxylic acid, beta-alanine, L-histidine, L-phenylalanine, L-tyrosine, 4-hydroxyphenylacetic acid and hippuric acid.

5.1.3 GC-MS Conditions

GC-MS analysis was performed using an Agilent 5975C INERT XL EI/CI gas chromatograph (Agilent, MA, USA) equipped with an autosampler injector and coupled with a single quadrupole mass detector. Compounds were separated in a capillary column HP-5MS (30 m×0.250 mm×0.250 μm film thickness) (Agilent Technologies). The column oven temperature program was as follows: temperature maintained at 45 ºC for 2 min, increased to 180 °C at 9 °C/min, held at 180 °C for 5 min, increased to 220 °C at 40 °C/min, and held at 220 ºC for 5 min. The oven temperature was then increased from 220 °C to 240 °C at 40 °C/min, held at 240 ºC for 11.5 min, and increased from 240 °C to 280 °C at 40 °C/min. Lastly, the temperature was maintained at 280 ºC for 10 min, with 1 min of post run at 45 °C. The total runtime was 51 min. Helium was used as the carrier gas with a constant flow of 0.71 mL/min. The injections were made in the splitless mode with an injection volume of 1 μL, and the injector temperature was set to 290 °C. The interface temperature was maintained at 250 °C. The quadrupole mass spectrometer was operated in the electron impact (EI) mode at 70 eV, and the source temperature was set to 250 °C. The analysis was carried out using a full scan in the 38-550 m/z range.

5.1.4 Data Processing and Identification

The GC-MS raw data were deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) (online software distributed by the National Institute of Standards and Technology, USA - http://www.amdis.net/). The peak identities were obtained, considering a match factor up to 70%, retention time with a window of 2 min compared with library compounds and match factor penalties in the very strong level and low threshold (automatic values). AMDIS was combined with R-based software to PLS-DA and OPLS-DA analysis by MetaboAnalyst 3.0. The data were normalized using an internal standard to reduce errors in the sample preparation and derivatisation steps, and the data were also normalised by creatinine values considering volume variations in each urine samples. Analyses were carried out partly using ‘R’ platform version 2.15.0 (http://www.r-project.org/) and also using MetaboAnalyst 3.0 (http://mirror.metaboanalyst.ca). In MetaboAnalyst 3.0, the matrix data were log transformed (log2) to improve data display with no additional normalisation performed using MetaboAnalyst 3.0.

5.1.5 GC-MS Quantification

After univariate and multivariate statistical analysis, the main metabolites identified using the library database were confirmed and quantified via commercial standards. For quantification, the data were normalised using internal standards and creatinine values. The results are expressed as μmol/μmol of creatinine. The calibration curves were also normalised using the internal standard DL-alanine-2,3,3,3-d4. The calibration curves provided strongly linear correlations for benzoic acid (R^2>0.99), fumaric acid (R^2>0.99), citric acid (R^2>0.99), cis-aconitic acid (R^2=0.99), malonic acid (R^2>0.99), oxalic acid (R^2=0.99), tartaric acid (R^2=0.99), glyoxylic acid (R^2>0.99), beta-alanine (R^2>0.99), L-histidine (R^2>0.98), L-phenylalanine (R^2>0.99), L-tyrosine (R^2>0.99) and 4-hydroxyphenylacetic acid (R^2>0.99).

5.1.6 Antioxidant Capacity Assay

Plasma antioxidant capacity was measured using the ORAC method (oxygen radical absorbance capacity) for all time points collected before (P0) and after GJ intake (P1, P2, P3, P4, P5 and P6, at 15 min, 30 min, 1 h, 2 h and 24 h, respectively). The antioxidant capacity was expressed as mmol of Trolox equivalent/mL of plasma.

5.1.7 Multivariate Analysis Methods

The normalized peak intensity was used to performer the Partial Least Squares Discriminant Analysis (PLS-DA) and the Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Cross validation (Accuracy, R2, Q2) and permutation tests were carried out to validate the PLS-DA. To validate the OPLS-DA, permutation tests (n=100) were carried out to check the consistency of the model and to calculate Q2 and R2Y. Each subject was removed from the matrix data one at a time, and the OPLS-DA model was rebuilt after each removal, and the permutation tests (n=100) were recalculated (Q2 and R2Y) for each new model. The identified compounds that mostly contributed to separation of time points (potential biomarkers) were selected between 0.5< p (corr) <-0.3 in the OPLS-DA subplot and submitted to a pathway analysis using MetaboAnalyst 3.0.

5.1.8 Univariate Analysis Methods

Linear correlation analyses were also carried out using MetaboAnalyst 3.0 for each data matrix to verify whether all variables (identified and unknown metabolites) had different contributions to the graphic projection, and also to verify the correlations among the metabolites. T-tests were also performed using MetaboAnalyst 3.0 for each data matrix to verify significant variations when comparing each time point collected after juice intake with time 0 (before juice intake) (p-values<0.05). One-way ANOVA followed by Tukey's test (p-values<0.05) for antioxidant capacity analysis in plasma and one-way ANOVA followed by the t test (p-values<0.05) were performed after standard quantification using Biostat 5.0.

5.1.9 Pathway Analysis

The metabolites that varied significantly (t test, p<0.05 and 0.5< p (corr) <-0.3 in the OPLS-DA subplot) were submitted to a pathway analysis by MetaboAnalyst 3.0. These analyses provided graphics with the assessment of pathway impact through a hypergeometric test by topological analysis based on the centrality measures of a metabolite in a given metabolic network (XIA, 2010).

5.2 RESULTS

5.2.1 Metabolites Identified by GC-MS

A total of 114 metabolites were detected and found abundant in urine samples (U0, U1, U2, U3 and U4), including 17 amino acids, 47 organic acids, 7 other classes of compounds and 43 unknown compounds.

5.2.2 Multivariate analysis

Looking for maximize the difference between time collection points, an OPLS-DA was performed to better expose separation and consider the variation that was not assessed by PLS-DA (WORLEY and POWERS, 2013). **Figure 15A** and **15B** showed the OPLS-DA model for U0 (before juice intake) compared to U2 (1-2 h) after GJ intake. The separation observed in permutation test (n=100) showed R^2Y and Q^2 values of 0.97 and 0.94,

respectively. The method were rebuilt by removal each time one subject from the matrix data and the permutation tests (n=100) were repeated each time, obtained 0.94 ±0.01 and 0.97±0.01 as Q^2 and R^2Y values, respectively.

Following the criteria described above, the 25 identified metabolites responsible for the time point separation and influenced by the acute intake were as follows: nicotinamide, oxalic acid, hexanoic acid, malonic acid, dodecane, lactic acid, tridecane, nicotinic acid, octanoic acid, glyoxylic acid, pyroglutamic acid, pentadecane, azelaic acid, heptadecane, L-phenylalanine, ornithine, lysine, L-histidine, trans-11-octadecenoic acid, L-tyrosine, nonacosane, beta-alanine, threonine, asparagine and suberic acid.

Furthermore, the separation observed between urine U0 and U3 by permutation test (n=100) showed R^2Y and Q^2 values of 0.98 and 0.93, respectively (**Figure 15C** and **15D**). The method was rebuilt by removing one subject from the data matrix at a time, and the permutation tests (n=100) were repeated in each instance with Q^2 and R^2Y values of 0.94±0.01 and 0.97±0.01, respectively. The main metabolites that influenced the separation were nicotinamide, oxalic acid, hexanoic acid, dodecane, octanoic acid, glyoxylic acid, pentadecane, suberic acid, azelaic acid, heptadecane, ornithine, lysine, histidine and tyrosine.

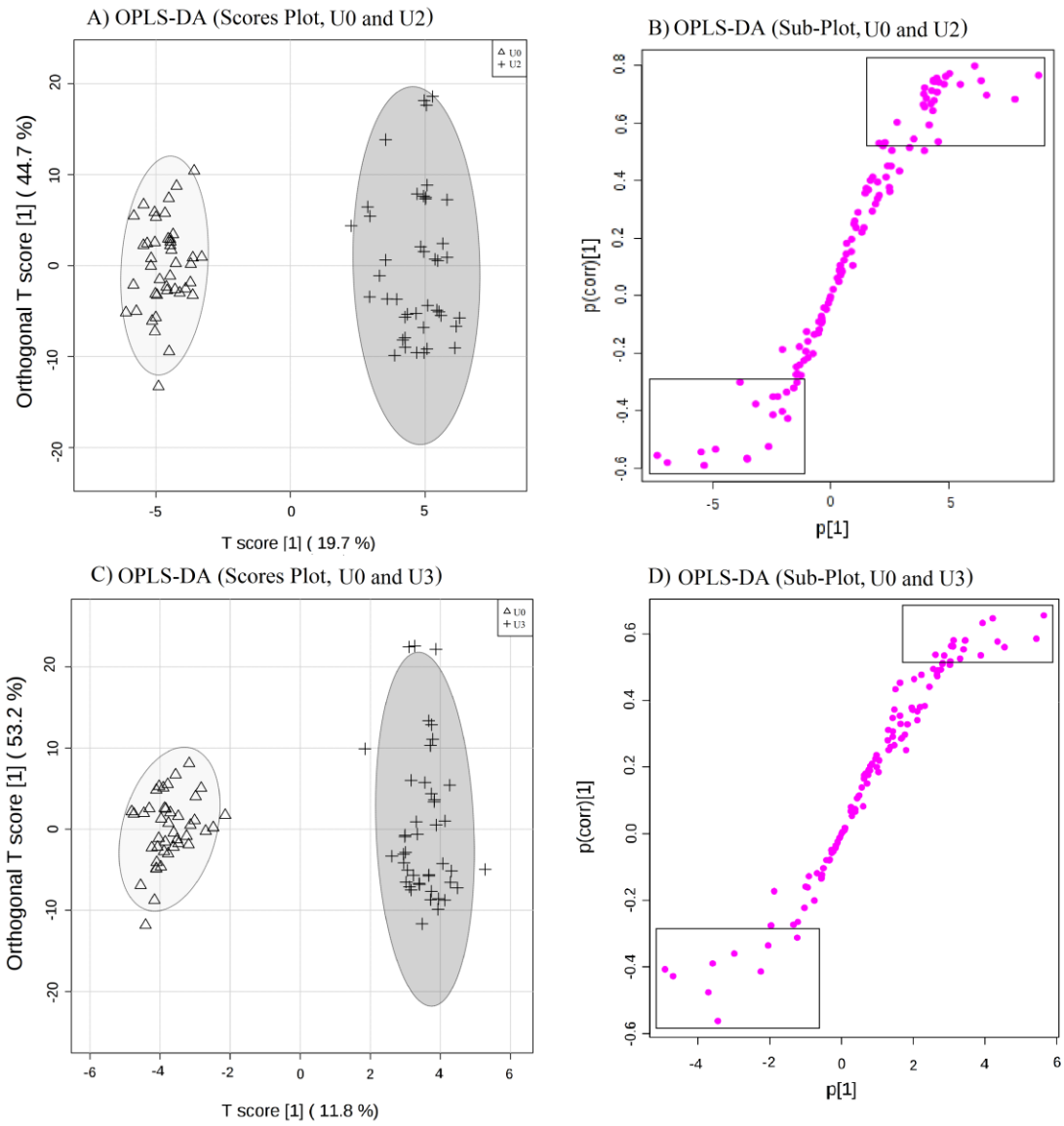

Figure 15 - OPLS-DA graphics of metabolites identified following the acute intake of purple grumixama juice. A) scores plot of OPLS-DA model for urine samples collected at time 0 (U0) and at 1-2 h (U2) after juice intake, B) subplot for OPLS-DA of U0 and U2, C) scores plot of OPLS-DA model of urine samples collected at time 0 (U0) and at 2-4 h (U3) after juice intake, D) subplot for OPLS-DA of U0 and U3 after juice intake.

5.2.3 Univariate Analysis

A univariate analysis (t test) was performed in order to verify the variation between U0 and U2, and U0 and U3 (Appendix 4 - Table 3). Following this analysis, in addition to the metabolites already responsible for the time point collection separation as identified by

OPLS-DA analysis, other metabolites showed significant differences ($p<0.05$) (77 and 64 metabolites, respectively).

Among the metabolites identified, 43 were common for both comparisons and are as follows: glyoxylic acid, heptadecane, pentadecane, dodecane, nicotinamide, tridecane, hexanoic acid, nonacosane, oxalic acid, azelaic acid, ornithine, octanoic acid, lysine, histidine, suberic acid, cis-aconitic acid, lactic acid, nicotinic acid, tyrosine, trans-11-octadecenoic acid, vanillic acid, pyroglutamic acid, anthranilic acid, 2-hydroxybutyric acid, malonic acid, 2-isopropylmalic acid, beta-alanine, tartaric acid, 2-hydroxyisobutyric acid, NADP/NADPH, itaconic acid, threonine, glutaric acid, asparagine, phenylalanine, adipic acid, 4-hydroxyphenylacetic acid, benzoic acid, citric acid, 2-aminoadipic acid, fumaric acid, 2-aminobutyric acid, and leucine. Furthermore, suberic acid, pimelic acid, hippuric acid and asparagine also showed variations (p-values<0.05) between U0 and U3. The metabolites that varied significantly were submitted for pathway analysis.

5.2.4 Pathway Analysis

The urine metabolome may be considered a good reflection of the metabolism, although changes to the metabolome do not imply metabolic pathway regulation. Considering all of the metabolites that changed after a single dose of the polyphenol-rich juice, a good correlation and high impact was observed among some metabolites that could be associated with three metabolic pathways (**Figure 16**)) as follows: glyoxylate and dicarboxylate metabolism, phenylalanine metabolism and beta-alanine metabolism. A brief description of each metabolic pathway and the metabolites that were significantly changed after GJ intake is shown in **Figure 17**. **Table 9** was showed the information about the metabolites quantified for these 4 pathways.

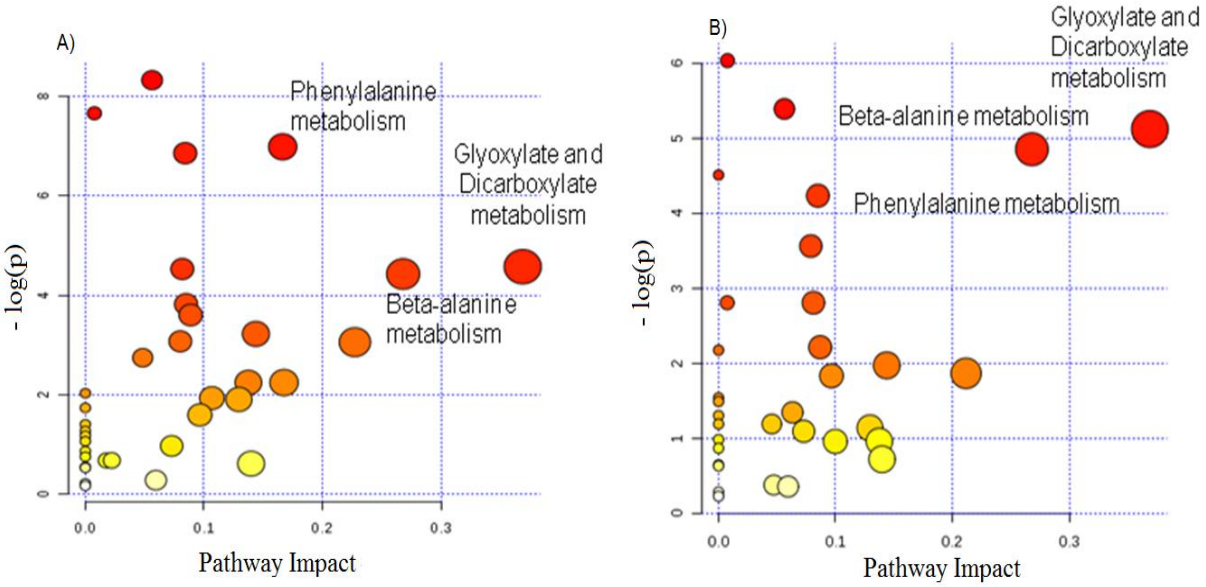

Figure 16 - Pathway analysis map view for main metabolites identified comparing urine at (A) time 0 to urine at 1-2 h after GJ intake and comparing urine at time 0 to urine at 2-4 h after GJ intake.

The metabolites that showed changes in the urine samples and were associated with glyoxylate and dicarboxylate metabolism were fumaric acid, tartaric acid, citric acid, isocitric acid, glyoxylic acid, *cis*-aconitic acid and oxalic acid (**Figure 17B**). The metabolites associated with phenylalanine metabolism were benzoic acid, hippuric acid, L-tyrosine, L-phenylalanine, 4-hydroxyphenylacetic acid and fumaric acid (**Figure 17A**). In addition, beta-alanine, malonic acid and L-histidine were associated with beta-alanine metabolism (**Figure 17C**).

Table 9 – Metabolites identifiedby GCMS in urine samples and regulates after acute intake of purple grumixama juice.

Metabolite Identified	RT	RI	CAS
Oxalic acid	5.753	59	144-62-7
Malonic acid	6.618	101	141-82-2
Fumaric acid	8.323	113	110-17-8
Succinic acid	8.505	115	110-15-6
Benzoic acid	9.63	105	65-85-0
Glyoxylic acid	10.259	75	298-12-4
beta-Alanine	11.643	88	107-95-9
cis-Aconitic acid	14.981	153	585-84-2
Citric acid	14.989	143	77-92-9
4-Hydroxyphenylacetic acid	18.125	121	156-38-7
Tartaric acid	18.494	59	87-69-4
Hippuric acid	18.765	105	495-69-2
Phenylalanine	19.056	162	63-91-2
Isocitric acid	19.133	129	320-77-4
Histidine	25.199	139	71-00-1
Tyrosine	27.375	236	60-18-4

RT: retention time; RI: reference íon after MFC derivatization; CAS: Chemical Abstract Service registry number.

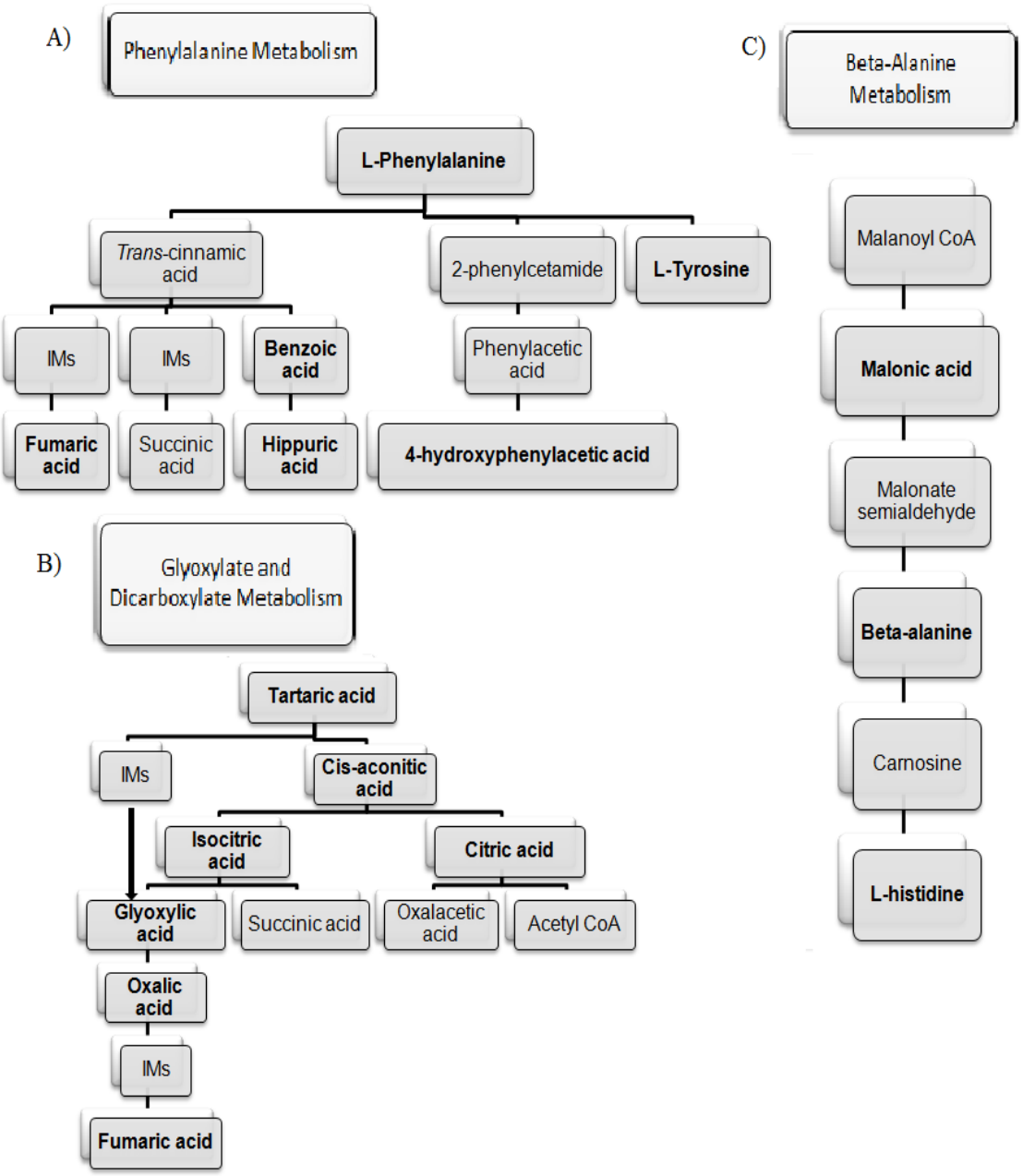

Figure 17 – Proposed pathways influenced by single dose of purple grumixama juice intake. A) Phenylalanine metabolism; B) Glyoxylate and dicarboxylate metabolism and C) Beta-alanine metabolism (IMs: Intermediate metabolites).

Other related metabolites that did not vary between these two comparisons (U0 x U2 and U0 x U3) were also quantified to better understand the influence of the GJ intake on metabolism (**Figure 18, 19** and **20**). Most of these were also found to vary significantly

among plasma samples but in lower number, and the PLS-DA analysis did not provide an evident separation group.

5.2.4.1 Phenylalanine Metabolism

Following the correlation test applied between U0 and U2, L-phenylalanine was decreased and strongly correlated with L-tyrosine (R^2=0.87) and 4-hydroxyphenylacetic acid (R^2 = 0.79) with a moderate correlation among the phenolic acids located in this pathway ($0.30<R^2<0.7$). These findings indicated the possible influence of exogenous metabolites from the GJ, such as hippuric acid and benzoic acids (**Figure 18**).

Comparing the amount of each compound, L-phenylalanine decreased 31% and 19% in the U1 and U2 samples, respectively, compared with the U0 sample, with L-tyrosine also decreasing after GJ intake, which was most obvious within the first 4 h after GJ intake (48%, 57% and 48% at U1, U2, U3, respectively). The 4-hydroxyphenylacetic acid level decreased in the U1 (29%) and U2 (17%) samples compared with that of U0 (**Figure 18**). Otherwise, benzoic acid (40 and 60%) and hippuric acid (65 and 144%) levels increased in U2 and U3, respectively, and decreased to basal levels at U4. The fumaric acid level increased in U2 (54%) and decreased after this time point collection.

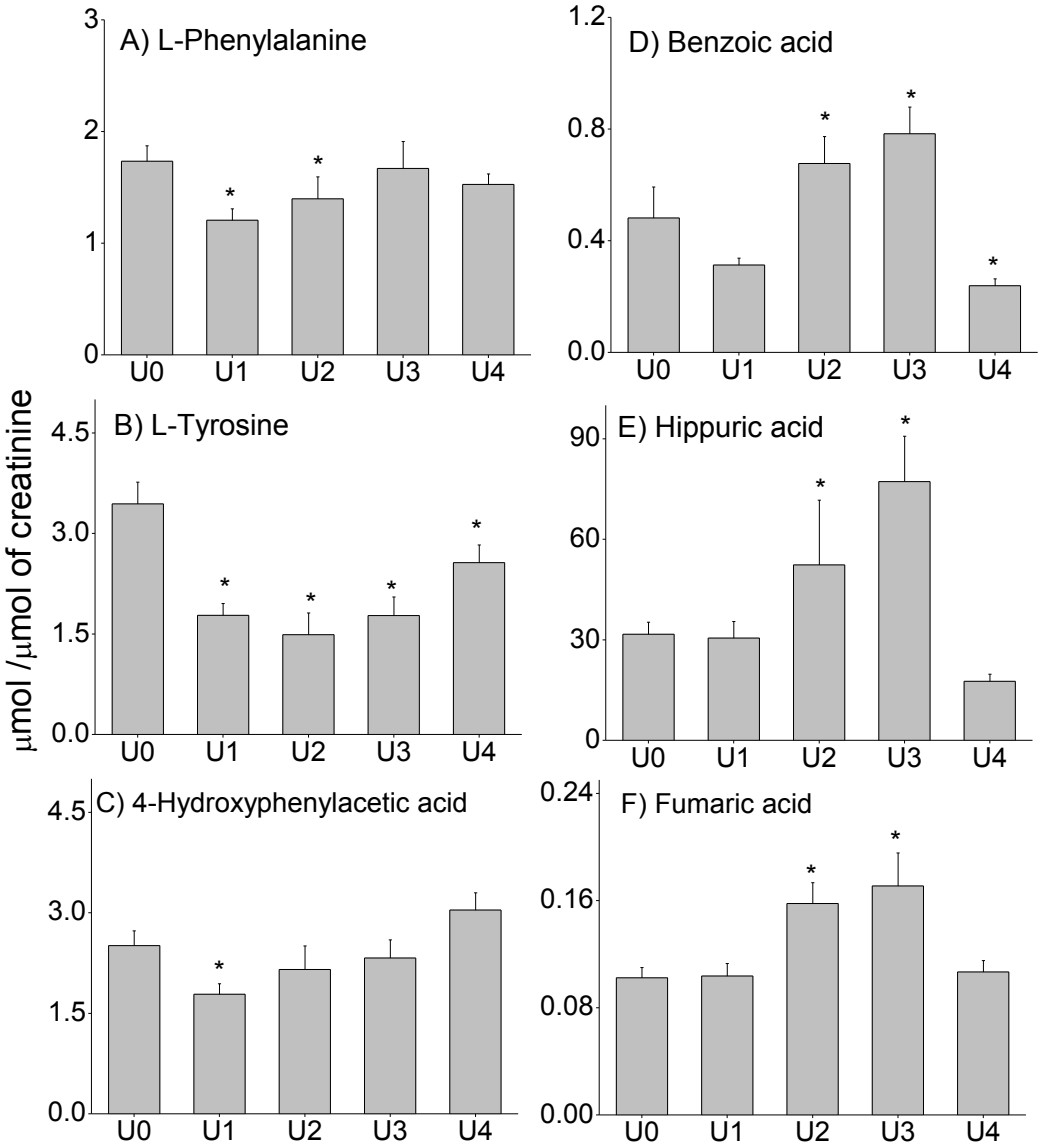

Figure 18 – Time course concentration of metabolites in urine samples changed by by single dose of purple grumixama juice intake and located in phenylalanine metabolism A) L-Phenylalanine, B) L-Tyrosine, C) 4-Hydroxyphenilacetic acid, D) Benzoic acid, E) Hippuric acid and F) Fumaric acid. Results expressed as mean ± standard errors, *p-values<0.05, Anova one way following t test comparing time 0 with each consecutive sample collection point.

5.2.4.2 Glyoxylate and Dicarboxylate Metabolism

The metabolites associated with glyoxylate and dicarboxylate metabolism increased in the urine after GJ intake (**Figure 19**). Following the correlation test applied between the U0 and U2 samples, a strong correlation was observed between the following acids: citric acid

and cis-aconitic acid (R^2=0.88), isocitric acid and cis-aconitic acid (R^2= 0.82) and cis-aconitc acid and tartaric acid (R^2=0.80). Furthermore, glyoxylic acid showed a strong correlation with oxalic acid (R^2=0.86) but a moderate to weak correlation ($0.30<R^2<0.7$) with other acids located in glyoxylate and dicarboxylate metabolism. However, isocitric acid and glyoxylic acid did not show a correlation (R^2 = 0.13), which suggested that the increase in glyoxylic acid can be related to other intermediate metabolites (IMs), such as S-malate, S-malyl CoA, 2-hydroxyl-3-oxopropanoate, which are not identified here (**Figure 17B**). Similar results were observed between U0 and U3.

Tartaric acid was significantly increased in the U2 sample (381% increase) and decreased thereafter. Cis-aconitic acid was increased during 1-4 h (U2 and U3) after GJ intake (42 and 71%, respectively), whereas glyoxylic acid and oxalic acid were increased throughout the time period after GJ intake, with the greatest values observed in U2 after GJ intake (774% and 472%, respectively) and at U3 (375% and 313%, respectively).

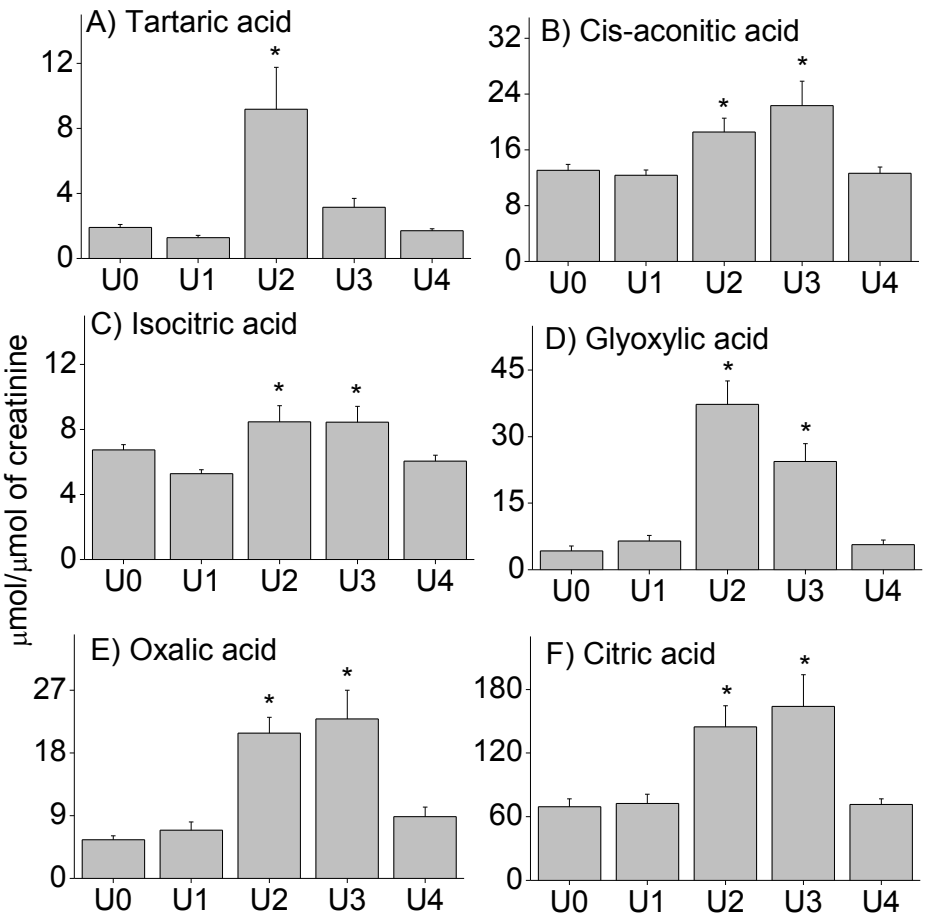

Figure 19 – Time course concentration of metabolites in urine samples changed by by single dose of purple grumixama juice intake and located in glyoxylate and dicarboxylate metabolism. A) Tartaric acid, B) Cis-aconitic acid, C) Citric acid, D) Isocitric acid, E) Succinic acid, F) Glyoxylic acid and G) Oxalic acid. Results expressed as mean ± standard errors, *p-values<0.05, Anova one way following t test comparing time 0 with each consecutive sample collection point (Fumaric acid is also present in this this pathway, but it was already showed in **Figure 18F**).

5.2.4.3 Beta-Alanine Metabolism

Following the correlation test applied for the comparison between U0 and U2, a moderate correlation was observed between beta-alanine and L-histidine (R^2 = 0.54), and a weak correlation was observed between malonic acid and beta-alanine (R^2 = 0.43) (**Figure 20).**

Malonic acid increased during 1-4 h (U2 and U3) after GJ intake (109% and 215%, respectively). Beta-alanine and L-histidine decreased throughout the time course, with the greatest decrease in U1 (81% down) for beta-alanine and in U2 for L-histidine (74% down). However, L-histidine started to increase in U3 and did not vary between U4 and U0, whereas beta-alanine was still low in U4.

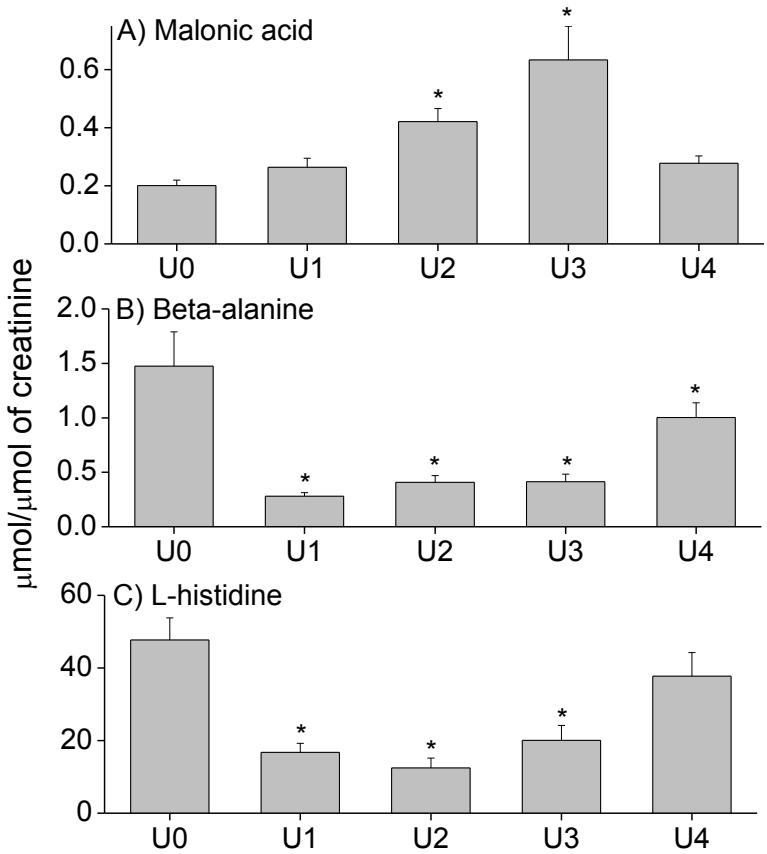

Figure 20 – Time course concentration of metabolites in urine samples changed by by single dose of purple grumixama juice intake and located in beta-alanine metabolism. A) Malonic acid, B) Beta-alanine and C) L-histidine. Results expressed as mean ± standard errors, *p-values<0.05, Anova one way following t test comparing to time 0.

5.2.5 Plasma Antioxidant Capacity

The antioxidant capacity of plasma samples increased after GJ intake and had a maximum peak of activity at 2 h (P4). Antioxidant levels decreased thereafter but remained higher ($p<0.05$) than those before intake until 24 h after ingestion (P6) (**Figure 21**).

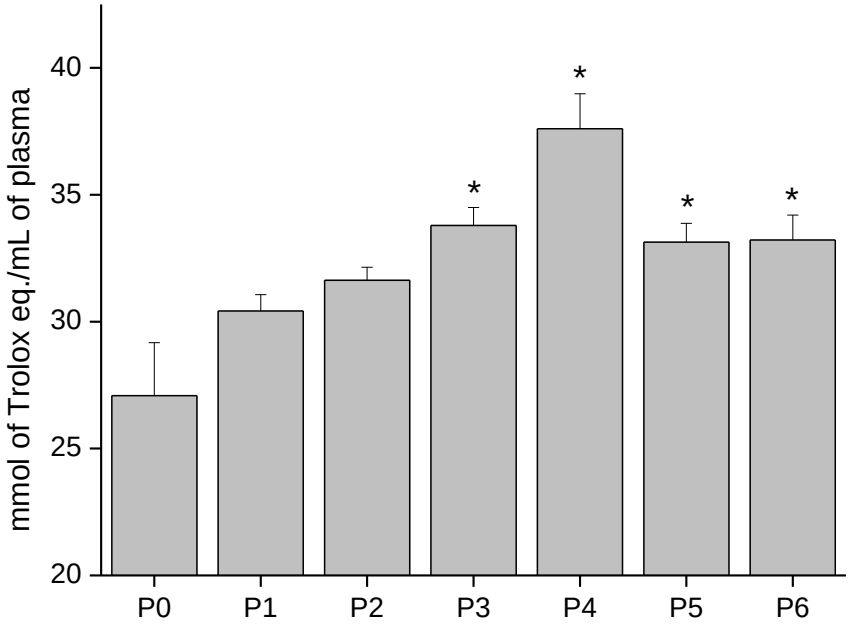

Figure 21 - Antioxidant capacity of plasma samples after intake of grumixama juice evaluate by ORAC assay. Results expressed as mean ± standard errors of 10 healthy subjects, *$p<0.05$, Anova one way following Tukey test, comparing to time 0.

5.3 DISCUSSION

The metabolome can be generated through a targeted or untargeted analysis driven by specific and nonspecific sample extraction and preparation processes, respectively (MANACH *et al.*, 2009; SCALBERT *et al.*, 2014). A targeted metabolomic approach following acute intake of phenolic compounds was applied to identify phenolic exposure biomarkers, such as exogenous metabolites from food sources, and also endogenous metabolites (LLORAC *et al.*, 2014). In our study, some exogenous metabolites was identified after GJ intake, such as hippuric acid, which was previously described as a potential biomarker of fruit and vegetable intake (SCALBERt *et al.*, 2014, KRUPP *et al.*, 2012). Other metabolites found here were not as specific but were characteristic of phenolic exposure, such

as 4-hydroxyphenylacetic acid and benzoic acid, that can result from gut microbiota metabolisation under flavonoids and vanillic acid, which can be obtained from further phase II metabolisation (BOTO-ORDÓÑEZ *et al.*, 2013, FERREAS *et al.*, 2014). Otherwise, these phenolic acids can also be part of the endogenous metabolome. Furthermore, the targeted metabolome could be applied to identify the possible influence of food components intake on metabolic pathway, which can be influenced by endogenous metabolome profile changes.

Furthermore, the targeted metabolome could be applied to identify the possible influence of food components intake on metabolic pathway, which can be influenced by endogenous metabolome profile changes (SCALBERT *et al.*, 2014).

In urine, 114 metabolites were considered abundant and identified by the library data according to the mass spectra profile. Sixteen metabolites were confirmed by commercial standards and correlated with three metabolic pathways. The metabolites of these three pathways were influenced by GJ intake as demonstrated by the strong correlation between the metabolites from specific pathway specificity and the high number of metabolites from a specific metabolism and topographic position, which was reflected in the urine metabolome.

In this way, three metabolites were altered after GJ intake. One metabolite was beta-alanine, which is specific to the beta-alanine pathway, and the two other metabolites were closely related to this metabolism. Furthermore, six metabolites were influenced by GJ intake and located in the glyoxylate and dicarboxylate metabolic pathways, which showed a high impact in pathway analysis. Among these metabolites, glyoxylic acid showed high specificity to this pathway, whereas the other six carboxylic acids can also be located in the Kreb´s cycle due to the strong linear correlation among these molecules. In addition, phenylalanine metabolism presents a specific metabolite (L-phenylanine) and five more related metabolites.

L-tyrosine was decreased after GJ intake and showed a strong correlation with phenylalanine. On the other hand, fumaric, benzoic and hippuric acids were increased with a moderate correlation. In this case, the increase observed with benzoic acid and to hippuric acid occurred mainly during 1-4 h after GJ intake and was associated with phenolic compound metabolites from GJ. The reduction of L-tyrosine through a food intervention with a source of anthocyanins and ellagitannins was also described by Jacobs (2012) and showed a possible effect on amino acid metabolism.

The GJ intake possibly influenced glyoxylate and dicarboxylate metabolism mainly during the first 4 hours after GJ ingestion, as observed by the highest increase in glyoxylic acid amount (over 700%) that returned to basal levels after 24 hours (U4). Additionally, the strong correlation between glyoxylic acid and oxalic acid and the moderate correlation with

fumaric acid indicated a significant influence on the tricarboxylic acid cycle, as both carboxylic acids are intermediates in this pathway. In addition, four other intermediates of the tricarboxylic acid cycle, citric acid, cis-aconitic acid, tartaric acid and malonic acid, were also increased after GJ intake, with a strong correlation among almost all of these metabolites. The influence of polyphenols under energetic metabolism has also been shown in other studies where the polyphenols from coffee improved mitochondrial fat metabolism.

Beta-alanine and L-histidine decreased in urine at the first hour after intake and continued to decrease for 24 h, which may reflect some influence on beta alanine metabolism. Concurrently, malonic acid, an important precursor of both beta alanine and L-histidine and also an intermediate of the tricarboxylic acid cycle, was increased. L-histidine is a precursor of histamine, an immunomodulatory compound with extensive effects on immunologic cells formed by the loss of a carboxyl group from L-histidine by L-histidine decarboxylase (O'MAHONY, 2011).

Anthocyanins and ellagitannins are both phenolic compounds present in the purple grumixama fruit (TEIXEIRA *et al.*, 2015) and increase the antioxidant capacity of the plasma (CLIFFORD and BROWN, 2006). Compared with the plasma sample before GJ intake (P0), the plasma antioxidant capacity increased in the plasma at the first 2 h (P4) after GJ intake and decreased thereafter but remained higher until 24 h (P6). The increase in plasma antioxidant capacity could be due to the increase in exogenous metabolites from GJ intake, such as phenolic compounds, as shown by several studies after an acute intake of polyphenol-rich diet (CLIFFORD and BROWN, 2006; DIÁS-RUBIO *et al*., 2015).

In conclusion, the endogenous metabolome was influenced by acute intake of a polyphenol-rich juice, as observed by changes in several metabolites related to energy metabolism. However, the hypothesis of pathways interaction should be explored in further studies to elucidate the biological significance on health promotion because the urine metabolome reflects only some aspects of cell metabolism and not pathway regulation.

6 PART 4: BIOLOGICAL ACTIVITY OF POLYPHENOL-ENRICHED EXTRACTS OF THE PURPLE AND YELLOW GRUMIXAMA IN ANIMAL MODEL OF OBESITY AND INSULIN RESISTANCE

This study was done in collaboration with Université Laval with financial support by CAPES DFAIT (Project number 009/2013), as a part of the project entitled "*Procurar terapias alternativas contra o desenvolvimento da disfunção dos adipócitos associada à obesidade*" coordinated by William Festuccia (ICB-USP) and André Marette (Department of Medicine, Université Laval).

6.1 **MATERIAL AND METHODS**

6.1.1 Chemicals

Catechin, quercetin, hippuric acid, hydroxyhippuric acid, hydroxybenzoic acid, hydroxyphenylacetic acid, benzoic acid, ellagic acid, coumaric acid, ferulic acid, caffeic acid were purchased from Sigma-Aldrich (St. Louis, MO). Liquid chromatography grade solvents acetone, methanol, and acetonitrile were purchased from EMD Millipore Chemicals (Billerica, MA).

6.1.2 Grumixama Extracts

The edible part of purple and yellow grumixama fruits were extracted with ethanol 70% acidified with 0.2 % of acetic acid and filtered. The extracts were further dried using a rotaevaporator at 40 °C and ressupended in filtered water to a concentration of 50 mg of extract per mL. The phenolic composition of purple and yellow grumixama extracts was showed in **Table 10**.

Table 10 - Phenolic composition of purple and yellow grumixama extracts.

Polyphenols	Purple Grumixama Extract		Yellow Grumixama Extract	
	Content (mg/100g DW)	Daily intake (mg/kg BW)	Content (mg/100g DW)	Daily intake (mg/kg BW)
Flavonols[1]	71.82±2.02	0.14±0.00	132.11±2.89	0.26±0.01
Anthocyanins[2]	401.81±11.15	0.80±0.02	ND	-
Catechine	284.34±12.91	0.57±0.03	264.79±3.34	0.53±0.01
Ellagic acid	20.12±0.63	0.04±0.00	54.81±2.00	0.11±0.00
Total ellagic acid	572.02±17.19	1.14±0.03	939.91±13.00	1.88±0.03

1: mostly quercetin 3-glucoside calculated as quercetin aglycone; 2: mostly cyaniding 3-*0*-glucoside; ND: not detected; DW: dry weight; BW: body weight.

6.1.3 Animals and Study Protocols

Seven-week-old male C57BL/6J mice (n=48, Jackson, USA) were separated one animal per cage in the animal facility of the Quebec Heart and Lung Institute. Animals were kept in a controlled environment (12 h daylight cycle, lights off at 18:00) with food and water *ad libitum*. Before started, the animals spent 2 weeks of acclimation on a normal chow diet (Teklad 2018, Harlan). During protocol, mice were fed either a normal chow (control) or a high-fat and high-sucrose (HFHS) diets containing 65% lipids, 15% proteins and 20% carbohydrates (Appendix 4). Animals were randomly divided into four groups (n=12), assigned as follow (**Figure 22**):

1. **Group A** received normal chow diet and water once-daily by gavage;
2. **Group B** received HFHS diet and water once-daily by gavage;
3. **Group C** received HFHS diet and purple grumixama extract (200 mg/kg of body weight) once-daily by gavage;
4. **Group D** received HFHS diet and yellow grumixama extract (200 mg /kg of body weight) once-daily by gavage.

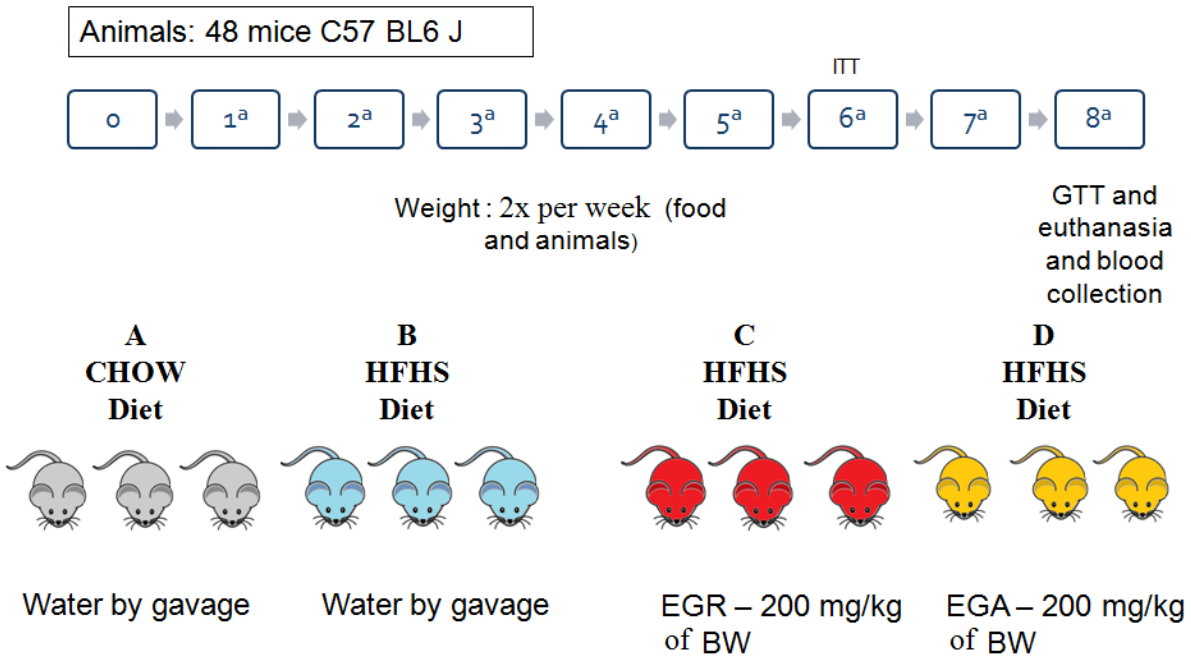

Figure 22 – Study design of animal protocol during 8 weeks of treatment (GTT: glucose tolerance test, ITT: insulin tolerance test, HFHS: high fat and high sugar, BW: body weight)

Body weight gain and food intake were assessed twice a week. After 8 weeks, animals were anesthetized using a chambers saturated with isoflurane and then euthanized by cardiac puncture. Blood was drawn in EDTA-treated and without anticoagulant tubes and immediately centrifuged to obtain plasma and serum, respectively. Liver and adipose tissues (EWAT, IWAT and RPWAT as epididymal, inguinal and retroperitoneal adipose tissues) were also collected and immediately frozen under liquid nitrogen. All samples were stored at -70 °C until analysis. All procedures were previously approved by the Laval University Animal Ethics Committee (2012129-3).

6.1.4 Glucose Homeostasis

Insuline tolerance test (ITT) was performed at the 6th week of protocol. Animals were 6-h fasted and blood sample was taken from the saphenous vein. Additional blood sample were taken at 5, 10, 15, 20, 25, 30 and 60 min after an intraperitoneal injection of insulin (0.75 UI/kg body weight) to glucose and insulin measure.

Oral glucose tolerance test (OGTT) was performed at 8th week of protocol. Mice were 6-h fasted and blood sample was taken from the saphenous vein. Additional blood samples were collected at 15, 30, 60, 90 and 120 min after single dose of glucose by gavage (1 g/kg

body weight). The glucose measures for ITT and OGTT were performed using Accu-Check glucometer (Bayer) and results were expressed as mM. Insulin were analysed using an ultrasensitive ELISA kit (Alpco, Salem, USA) and expressed as ng/mL of plasma.

6.1.5 Serum Lipid Profile and Liver Triglyceride Contents

Liver triglyceride content was assessed after chloroform–methanol extraction and quantified using a commercial kit (Randox Laboratories, Crumlin,UK), and the results were expressed as μmol/g of tissue. Total cholesterol, triglycerides (TG) and HDL in serum were assessed using commercial kit (Labtest, MG, Brazil), and results were expressed as mM. The non-HDL cholesterol was calculated by the difference between total cholesterol concentration and HDL concentration.

6.1.6 Plasma Lipopolysaccharides (LPS) Analysis

The plasma LPS was assessed by mouse lipopolysaccharides ELISA Kit (Mybiosource, California, USA). The results were expressed as ng/mL of plasma.

6.1.7 Plasma Phenolic Compounds Extraction

Phenolic compounds extraction was performed according to a previously described method (Dudonne *et al.*, 2014). Waters OASIS HLB μelution plates (Milford, MA) were preconditioned using 250 μL of methanol and 250 μL of aqueous acetic acid 0.2%. Plasma samples (10 to 60 μL) were loaded to each well, washed with 200 μL of ultrapure water and 200 μL of the aqueous acetic acid 0.2%, and the retained phenolic compounds were then eluted with 75 μL of acetone/ultrapure water/acetic acid solution (70/29.5/0.5, v/v/v). The eluted solutions were directly injected in UHPLC-MS/MS.

6.1.8 UHPLC-MS/MS Conditions

The identification and quantification of phenolic acids, urolithins and flavonoids were performed by UHPLC MS/MS using a Waters Aquity (Waters Corp, MA, USA), at 30 °C, using an Agilent Plus C18 column (2.1 mm × 100 mm, 1.8 μm) (Santa Clara, CA). A flow rate of 0.4 mL/min was used with a mobile phase consisting of aqueous acetic acid 0.2% and

acetonitrile (solvent A and B, respectively) following the gradient elution: 0−8 min, 5−50% B; 8−9.10 min, 50−90% B; 9.10−10 min, 90% B; 10−10.10 min, 90−5% B; 10.10−13 min, 5% B.

The MS/MS analyses were carried out in negative mode using the electrospray source parameters as follows: electrospray capillary voltage was 3.01 kV, source temperature was 150 °C, desolvation temperature at 400 °C, and cone and desolvation gas flows were 80 L/h and 800 L/h, respectively. The analysis was performed using a full scan from *m/z* 100 to 1000.

6.1.9 RT^2-PCR Array Gene Expression for Fatty Liver

RNA was isolated using an RNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. RNA quality was determined using the ratio 260/280 nm and was reverse transcribed using a cDNA conversion kit for pre-amplification RT^2 PreAMP Primer Mix (Qiagen, Germany). The cDNA obtained were storage at -80 °C until RT^2-PCR Array Gene Expression analysis with a panels of 84 genes related to fatty liver (Qiagen, Germany) (Appendix 4 – Panel genes list). The cDNA was used on the real-time RT^2 Profiler PCR Array (Qiagen, Germany) in combination with RT^2 SYBR® Green qPCR Mastermix. PCRs amplifications were performed using an Applied Biosystems 7500 Real-Time PCR Systems (Thermo-Fisher, MA, USA). The data were expressed as relative gene expression (2^ (-ΔΔCt)), considering group B as a control group and the normalization using geometric means for five houskeepins (Actb, B2m, Gapdh, Gusb, Hsp90ab1).

6.1.10 Statistical Analysis

The area under the curve (AUC) for ITT, OGTT and insulin was estimated using the linear trapezoidal rule. The results were expressed as the mean ± standard error (SE). Analysis of variance (one-way ANOVA) followed by a Tukey test., $p \leq 0.05$ was considered significant or, when non-parametric data, One-way Mann-Whitney (Wilcoxon) were performed using Biostat 5.0.

6.2 RESULTS

6.2.1 Body Weight and Glucose Homeostasis

The body weight and the total energy intake did not change significantly among groups B, C and D during the 8 weeks of protocols (**Figure 23**). Otherwise, Group C had a lower weight gain ($p=0.05$) as compare to Group B. In the OGTT, it was not observed significant difference ($p<0.05$) during each time points between Groups B and C and Groups B and D (**Figure 24A**). However, a significant decrease in AUC was observed to both extracts as compared to Group B (**Figure 24B**), with no difference in insulin levels (**Figure 24E**). Furthermore, ITT had not showed difference among groups (**Figure 24C** and **24F**).

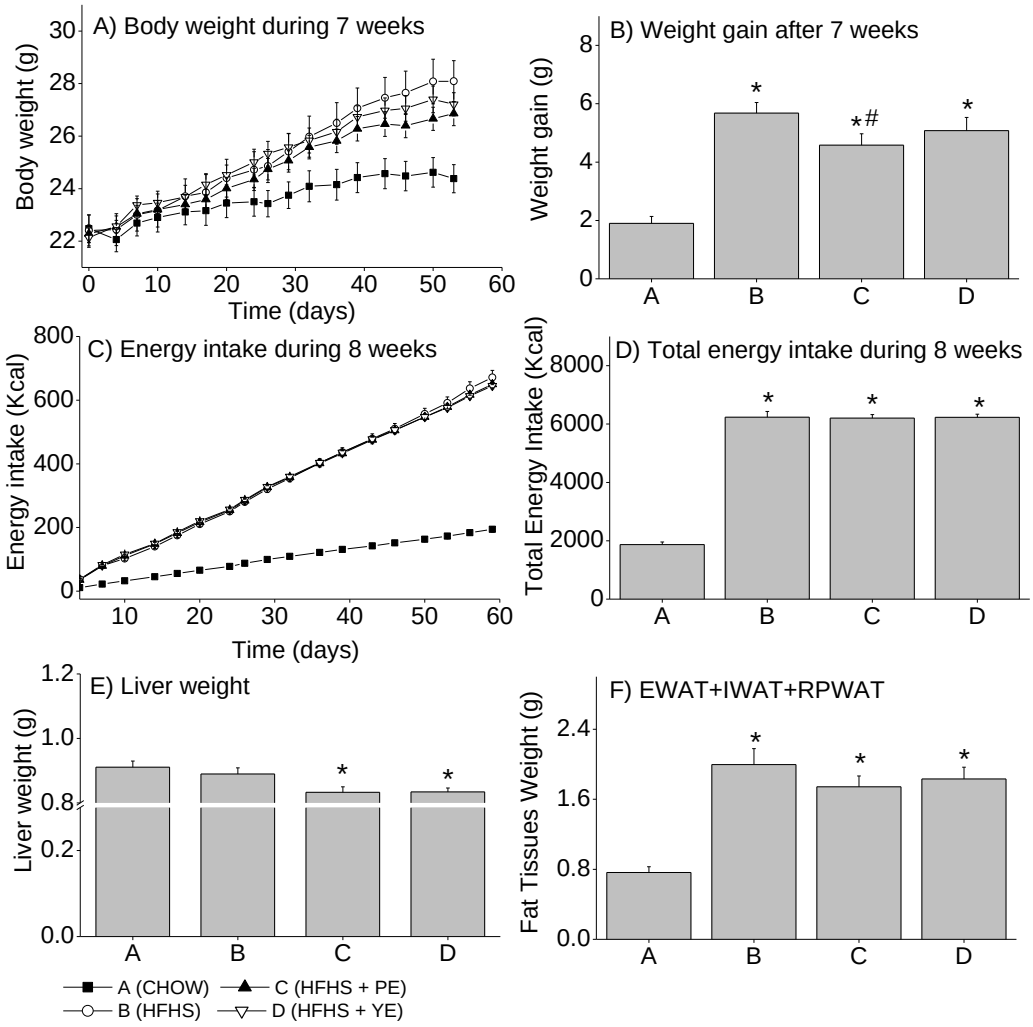

Figure 23 - Body weight, tissue weight and energy intake in C57BL/6J mice given normal diet and high-fat and high-sucrose diet, daily administered with polyphenols-enriched extracts of the purple and yellow grumixama, for 8 weeks. A) Body weight during 7 weeks, B) weight gain after 7 weeks, C) energy intake during 8 weeks and D) total energy intake after 8 weeks of protocol. Data are means ± standard error mean. One-way ANOVA followed by Tukey test. *$p<0.05$ compared to control chow diet (group A) to groups B, C and D, #: $p<0.05$ compared to HFHS diet (group B) to groups C and D (n=10-12 mice).

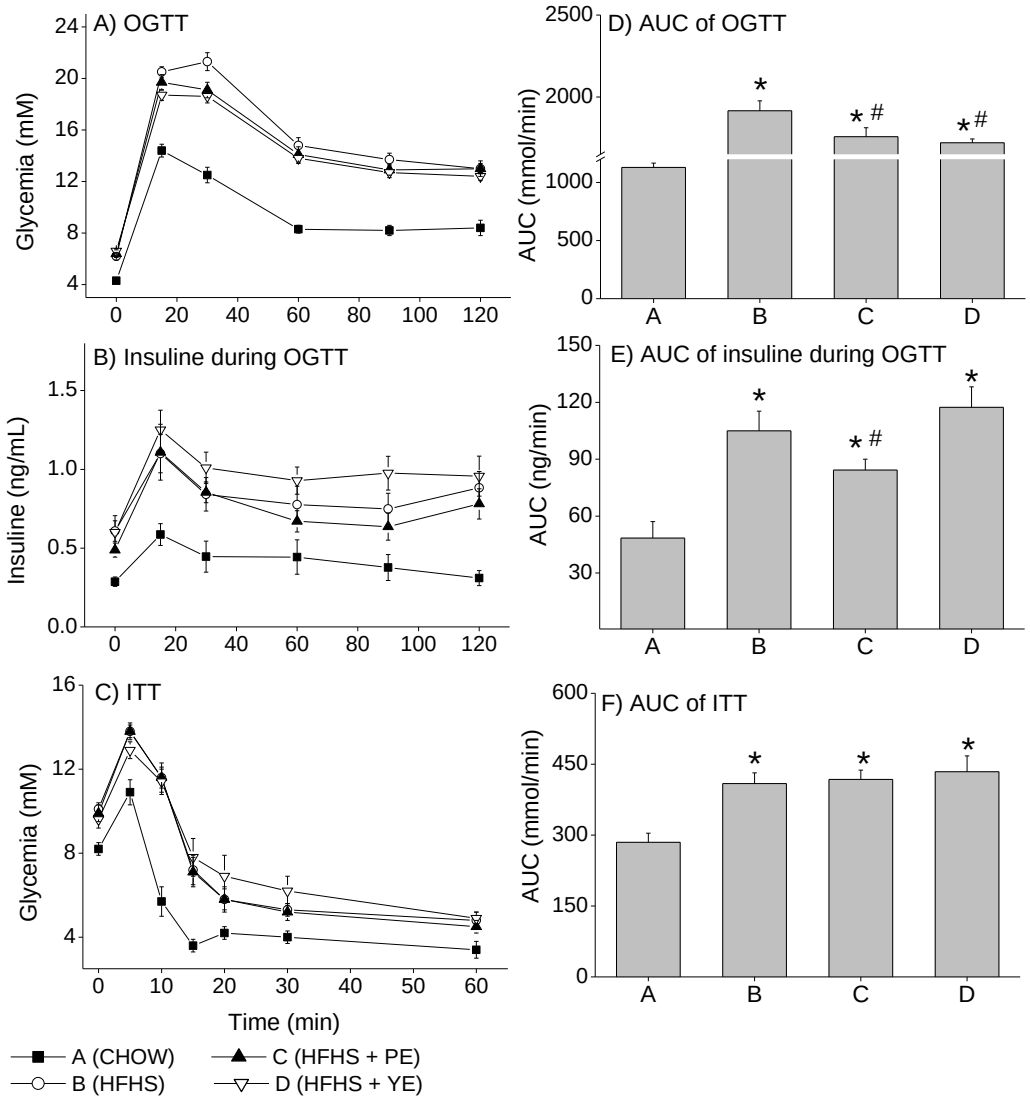

Figure 24 - Plasma and insulin level during an OGTT, and ITT in C57BL/6J mice given normal diet and high-fat and high-sucrose diet, daily administered with polyphenols-enriched extracts of the purple and yellow grumixama, for 8 weeks.A) OGTT, B) insulin during OGTT, C) ITT, D) Area under curve of OGTT, E) Area under curve of insulin during OGTT, F) Area under curve of ITT. Data are means ± standard error mean. One-way Mann-Whitney (Wilcoxon). *p<0.05 compared groups B, C and to control chow diet (group A), #p<0.05 compared group B to groups C and D. (n= 9-12 mice)

6.2.2 Triglyceride, Cholesterol and LPS Contents

Blood triglyceride, total chloresterol and HDL concentrations had not showed significant difference among Groups B, C and D (**Figures 25C, 25D and 25E**). However, Group D presented a 50% increase in non-HDL cholesterol as compared to group HFHS (Group B) (**Figure 25F**). A decrease in liver triglyceride content (20% less) was observed in Group C as compared to Group B ($p<0.05$) (**Figure 25A**). In addition, plasma LPS content was found lower in both groups treated with purple and yellow grumixama extracts (20% and 23% decrease for Groups C and D, respectively) as compared to Group B (**Figure 25B**).

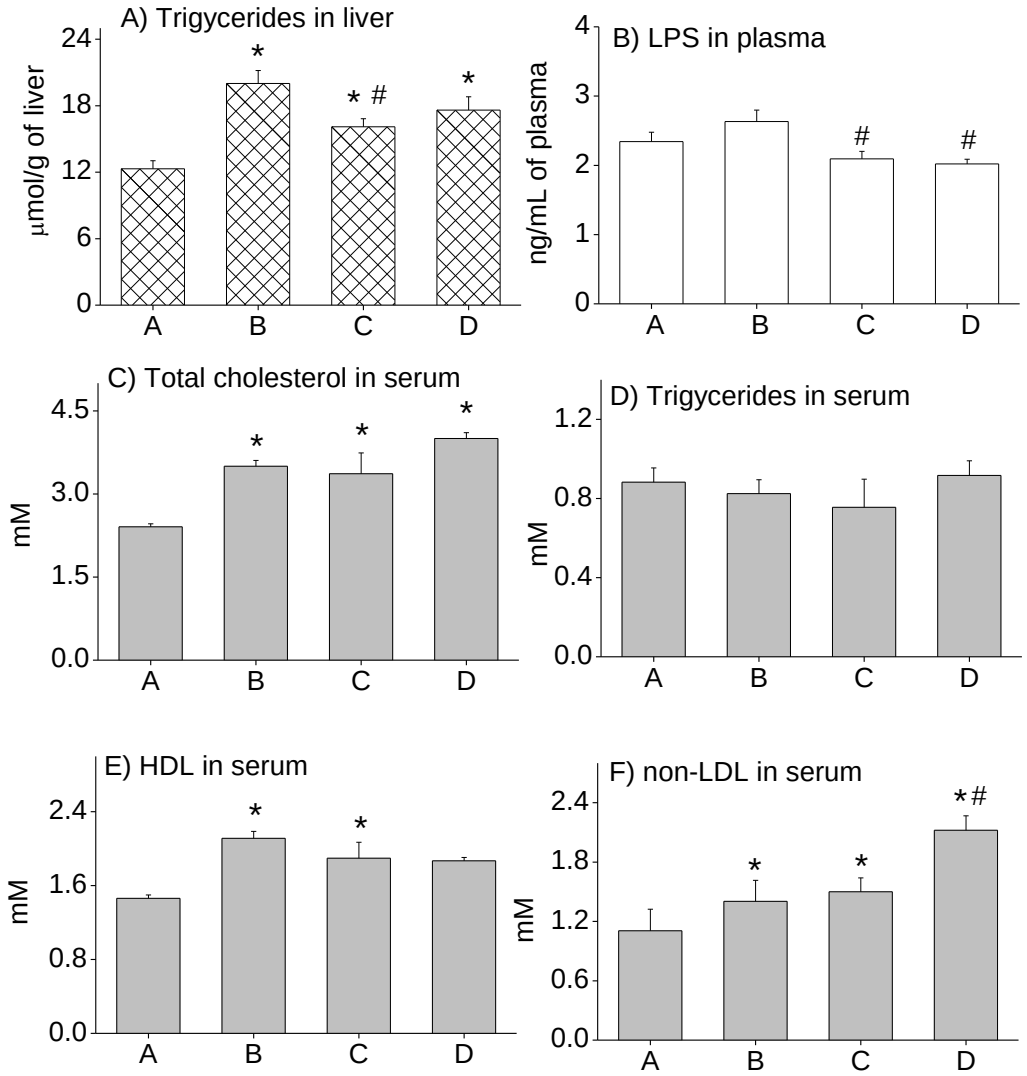

Figure 25 – Lipid contents assessed in liver, plasma and in serum in C57BL/6J mice given normal diet and high-fat and high-sucrose diet, daily administered with polyphenols-enriched extracts of the purple and yellow grumixama, for 8 weeks. (A) triglycerides in liver, (B) LPS (Lipopolysaccaride) in plasma and (C) total cholesterol, (D) triglycerides, (E) HDL and (F) non-LDL in serum. Data are means ± standard error mean. One-way Anova followed by Tukey test comparing with group A to groups B, C and D (**p*-values <0.05) and comparing to group B to groups C and D (#*p*-values <0.05). (n= 7-11 mice).

6.2.3 Bioavailability of Polyphenols

Eight ellagitannins metabolites (Phase II metabolism) were identified in mice plasma for both groups treated with grumixama extracts (Group C and D), before and through 60 min after the last gavage. Among them ellagic acid glucuronide, dimethyl ellagic acid glucuronide (**Figure 26A**), urolhitins A (three isomers), B and D sulphated and urolhitin C methyl ether sulphate (**Figure 26B**). Total Urolithin A presented the highest content in both groups treated with the grumixama extracts (Group C and D), varying from 250 to 350 μg/mL.

Catechins metabolites, presented as Phase II metabolites, had an increase through 60 min after the last extract gavage on both groups, reaching a C_{max} between 15-30 min (**Figure 27A**). Quercetin derivates, also presented as Phase-II metabolites, were detected before and not change concentration after the last gavage (**Figure 28B**). Furthermore, eight phenolic acids were detected in plasma before the last extract gavage on both groups (Group C and D), but only coumaric acid presented an increase in concentration, reaching a C_{max} at 15 min (**Figure 24F**). Among the phenolic acids, 3-hydroxybenzoic acid and benzoic acid presented the highest plasma concentrations (**Figure 24C and 24D**, respectively). The urolithins and flavonoids metabolites (catechin and quercetin) were not detected on the group that received only the HFHS diet (Group B).

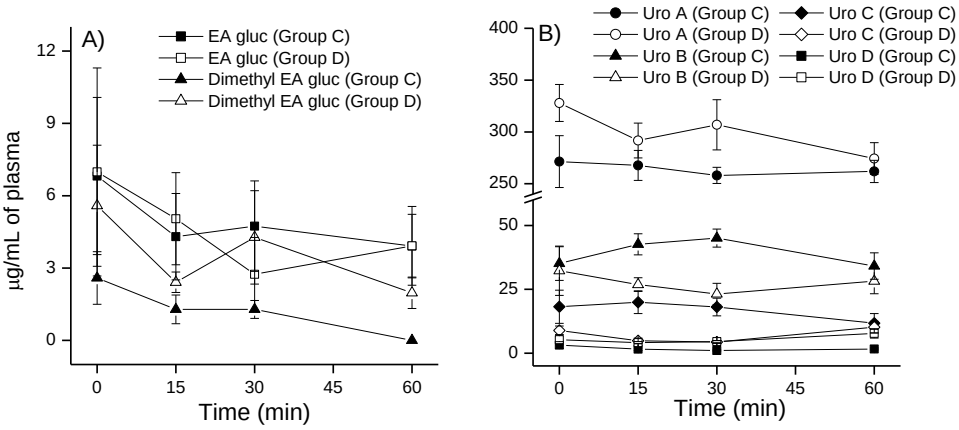

Figure 26 – Time course concentration of ellagitannins metabolites circulating in plasma of in C57BL/6J mice given normal diet and high-fat and high-sucrose diet, daily administered with polyphenols-enriched extracts of the purple and yellow grumixama, for 8 weeks. (A) ellagic acid glucuronide (EA gluc) and dimethyl ellagic acid glucuronide (dimethyl EA gluc); (B) urolithin A sulphate isomers (Uro A), urolithin B sulphate (Uro B), urolithin C methyl ether sulphate (Uro C) and urolithin D sulphate (Uro D). Data are means ± standar error mean (n= 10 mice).

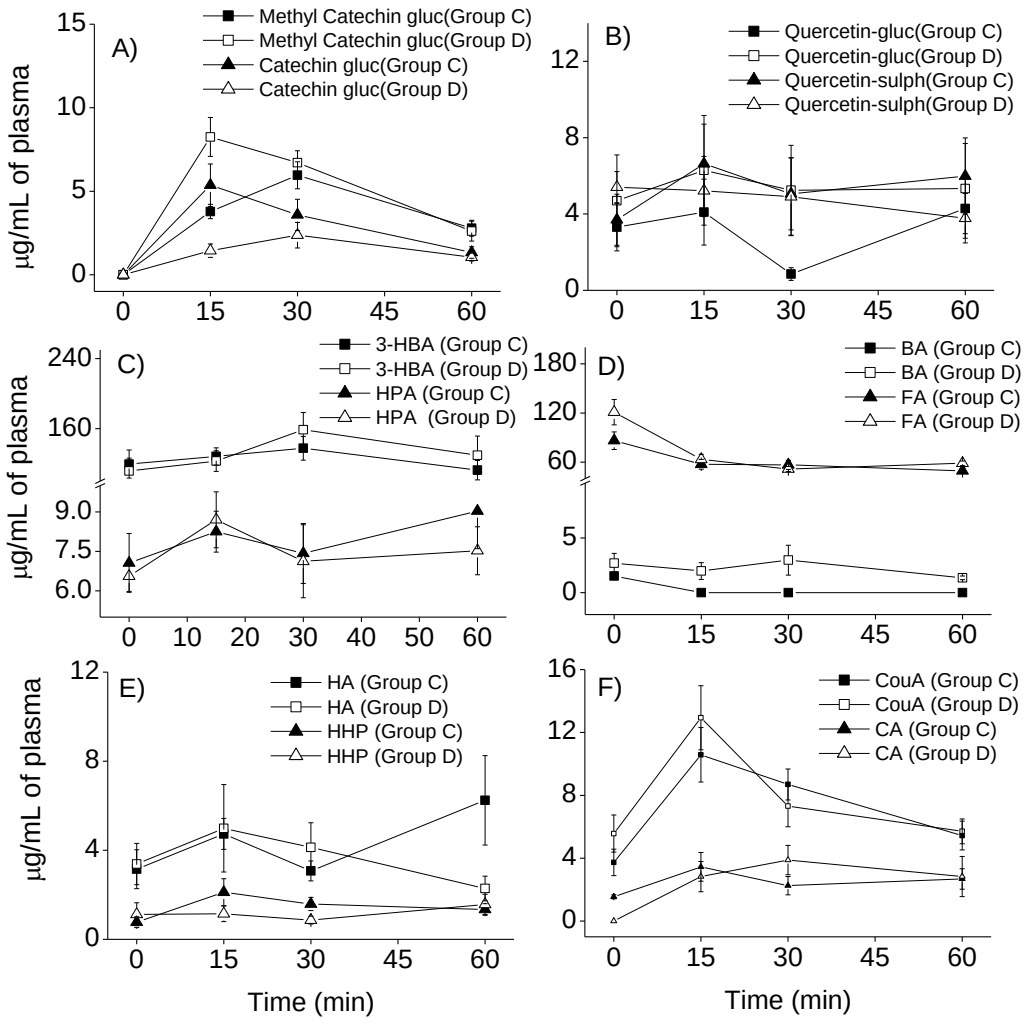

Figure 27 – Time course concentration of flavonoids metabolites and phenolic acids circulating in plasma of in C57BL/6J mice given normal diet and high-fat and high-sucrose diet, daily administered with polyphenols-enriched extracts of the purple and yellow grumixama, for 8 weeks.A) methyl catechin glucuronide (methyl catechin gluc) and catechin glucuronide (catechin gluc); B) quercetin glucuronide (querecetin-gluc) and quercetin sulphate (quercetin sulp); C) 3-hydroxybenzoic acid (3-HBA) and hydroxyphenylacetic acid (HPA); D) ferulic acid (FA) and benzoic acid (BA); E) hippuric acid (HA) and hydroxyhippuric acid(HHA) and F) cumaric acid (CouA) and caffeic acid (CA). Data are means ± standar error. (n= 10 mice).

6.2.4 Gene Expression

Among the 84 genes presents in the panels, five genes showed significant difference ($p<0.05$) in gene expression influenced by both grumixama extracts as compared to Group B (**Figure 28**). Four genes had been related with colesterol metabolism as follow Acox1, ApoB, Cyp7a1 and Pparγ (**Figures 28A, 28B, 28F and 28I**). The Acox1 and ApoB genes were over-expressed up to once as compared to Group B. The Socs3 was under-expressed in group C and D, comparing to B. The Cyp7a1 was over-expressed ($p<0.05$) in all HFHS Groups (Group B, C and D) as compared to Group A (**Figure 28F**), and Pparγ and Serpine genes was under-expressed in group C and Group D, respectively, as comparing to Group B.

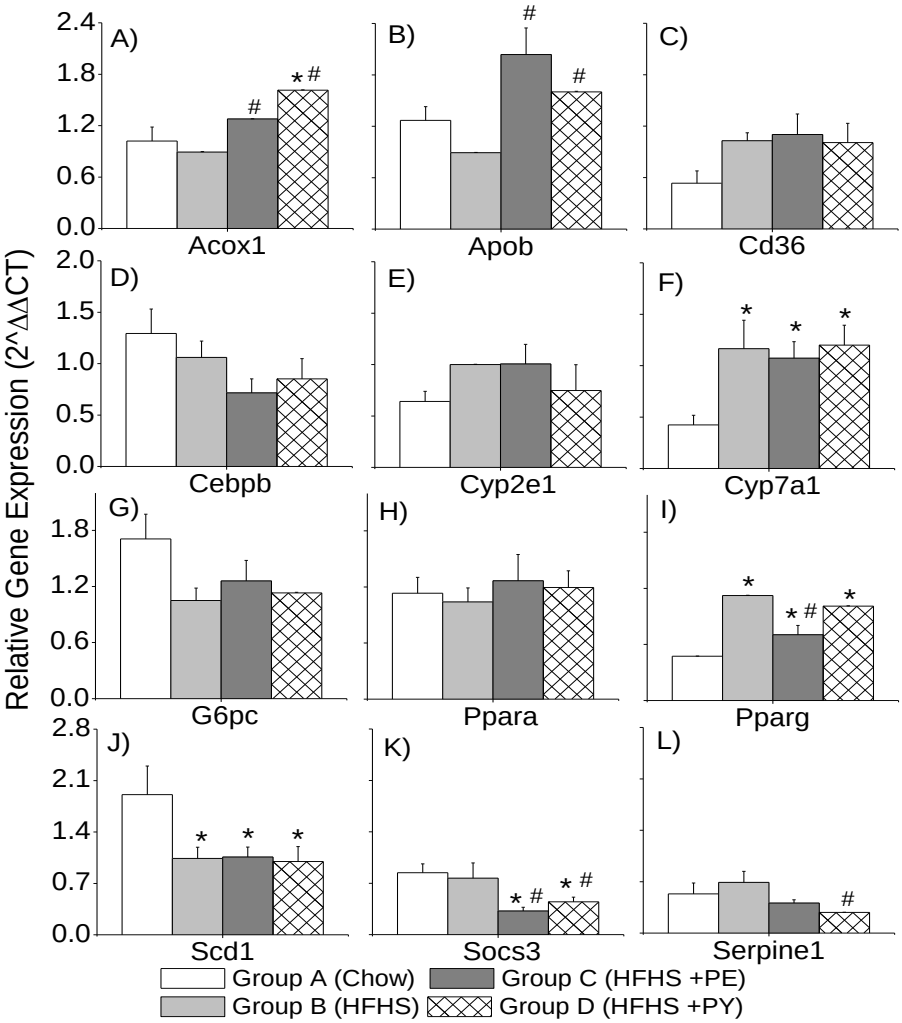

Figure 28 – Relative gene expression in liver of C57BL/6 mice given normal diet and high-fat and high-sucrose diet, daily administered with polyphenols-enriched extracts of the purple and yellow grumixama, for 8 weeks.A) Acyl-Coenzyme A oxidase 1, palmitoyl ; B) Apolipoprotein B; C) CD36 antigen; D) CCAAT/enhancer binding protein (C/EBP), beta; E) Cytochrome P450, family 2, subfamily e, polypeptide 1; F) Cytochrome P450, family 7, subfamily a, polypeptide 1; G) Glucose-6-phosphatase, catalytic; H) Peroxisome proliferator activated receptor alpha) I) Peroxisome proliferator activated receptor gamma; J) Stearoyl-Coenzyme A desaturase 1; K) Suppressor of cytokine signaling 3; L) Serine (or cysteine) peptidase inhibitor, clade E, member 1. Data are means ± standar error. One-way ANOVA followed by Tukey test comparing with group A to groups B, C and D (**p*-values <0.05) and comparing group B to groups C and D (#*p*-values <0.05). (n= 4-6 mice).

6.3 DISCUSSION

The HFHS diet increased the glycemia and plasma insulin level resulting in insulin resistance as comparing to normal chow diet. However, the daily administration of the purple and yellow grumixama extracts together with the HFHS diet, through 8 weeks, improve the insulin sensitivity, but not avoid the insulin resistance.

The biochemical parameters indicated that grumixama extracts modulate lipid metabolism in different way. Although total serum cholesterol values were not changed between HFHS groups, an increase in non-HDL cholesterol was observed to the Group D treated with yellow grumixama extract. On other hand, group treated with purple grumixama extract showed lower accumulation of triglycerides (20% less) in liver.

The HFHS diet over expressed some genes related to lipid metabolism in liver, such as Pparγ and Cyp7A1, the last one encoded the enzyme cholesterol 7α-hydroxylase, which catalyzes the initial step in cholesterol catabolism and bile acid synthesis (PULLINGER *et al*., 2002; AOKI *et al.*, 2015). Pparγ, under-expressed in Group C and over-expressed on Group B, is an important transcriptional regulator of adipogenesis and plays an important role in the process of lipid storage, mainly expressed in adipocyte and also in insulin responsive tissues, such as liver and muscle (SALOMONE *et al.*, 2016). The over expression of Pparγ in adipose tissues is usually associated to improve insulin resistance, probably, because it promotes the relocalization and storage of fat in adipose tissue, protecting peripheral tissues from lipotoxicity and through it reduce systemic inflammation (BERLANGA *et al.*, 2014). However, Pparγ in liver is usually finding over-expressed in obese subjects, which promotes the storage of free fatty acids in liver and leading to steatosis (BERLANGA *et al.* 2014). In this way, the purple grumixama extract promoted an under-expression of Pparγ , which can be associated to the lower accumulation of liver triglycerides in Group C as compared to mice treated only with HFHS diet, although the exactly mechanism of action still unclear.

The lower accumulation of liver triglycerides observed on Group C can also be associated to the over-expression of Apob, gene responsible for encoded the apolipoprotein B that transport cholesterol and triglycerides to tissues (BERLANGA *et al.* 2014). However, the serum total cholesterol had been maintained in high levels, in all HFHS groups, that was not consisted with the over expression of Cyp7a1, responsible for the cholesterol degradation to bile acid (PULLINGER *et al.*, 2002).

Besides these genes, the over-expression of Acox1 was observed for both groups that received grumixama extracts (Groups C and D), which could indicate the enhancement of

peroxisomal fatty acid β-oxidation, which can promote hypotriglyceridemic activity (SHIMODA *et al.*, 2009). Similar result was observed in the same HFHS model where animals received walnuts, an ellagitannins-rich source, during 2 months (SHIMODA, 2009). In this way, the lower concentration on liver triglycerides found in our work could be due to the improvement on triglycerides transport from liver to peripherical tissues and also an increase in peroxissomal β-oxidation.

The under-expression of Socs3 (Supressor of cytokine signaling 3 protein) can be related to insulin sensitivity improvement. The increase on Socs3 expression has been associated to insulin resistance, as a result of the insulin signaling inhibition by binding insulin receptor, targeting IRSs (Insulin receptor substrates) proteins for proteasomal degradation, among other mechanisms (SACHITHANANDAN *et al.*, 2010).

The improvement of liver and blood biochemical parameters observed on groups that received grumixama extracts as compared to that received only HFHS diet, could be due to the presence of metabolites from the daily grumixama extracts intake. Ellagitannins and flavonoids were the major phenolic compounds identified in both grumixamas varieties. The ellagitannins contents and profile were found similar for both varieties (TEIXEIRA *et al.*, 2015), but the flavonoids profile were different been the purple and yellow fruits rich in cyanidin 3-*O*-glucoside and quercetin, respectively (TEIXEIRA *et al.*, 2015; FLORES *et al.*, 2013). The urolithins and phenolic acids, detected in high amount circulating in Group C and D, were the catabolites of ellagitannins and flavonoids by gut microbiota, respectively (GONÇALEZ-BARRIO *et al.*, 2010), that explain why even 28 h these catabolites were still found circulating in high amount. Also, the acute dose of extracts on the last experiment day showed that other metabolites, such as catechin phase II metabolites, could had a role on the biological effects, even presenting a short half-life time. In this way, the biological effects observed in animals treated with both grumixama extracts could be a result of the constant and high amount of the circulating gut microbiota catabolites.

LPS is an endotoxins derived from gut microbiota that can promote an abnormal Toll-like receptors activation, leading to inflammatory responses, contributing to the insulin resistance development. Obesity has been associated to increase on intestinal permeability, which leads to high levels of LPS in plasma and tissues promoting insulin resistance (TEIXEIRA *et al.*, 2012). The reduction on plasma LPS content observed in groups treated with purple and yellow grumixama extracts (Group C and D) as compared to group only feed with HFHS diet could be associated to an improvement of LPS permeability through

intestines. Thus, the decrease in plasma LPS can also have impact on insulin sensivity improvement.

As conclusions, both extracts improved insulin sensibility, and the purple extract showed an evident protection against non-alcoholic fatty liver disease. These results could be associated by the circulating flavonoids and ellagitannins metabolites.

7 CONCLUSION

The grumixama showed to be a good source of flavonoids and ellagitannins and the interaction among exogenous and endogenous metabolites following an acute intake can be related to changes in amino acids and energetic metabolisms.

In addition, the polyphenols metabolites had also demonstrated potencial antiproliferativy activity for human breast cancer cells, as well as induced protection against non-alchoolic fatty liver disease in animal model.

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APPENDIX 1

Supplementary Material for Part 1

Flesh of purple grumixama (*Eugenia brasilienses* Lam.)

POSITIVE MODE

Figure 1- Delphinidin Hexoside **(Compound 1)**

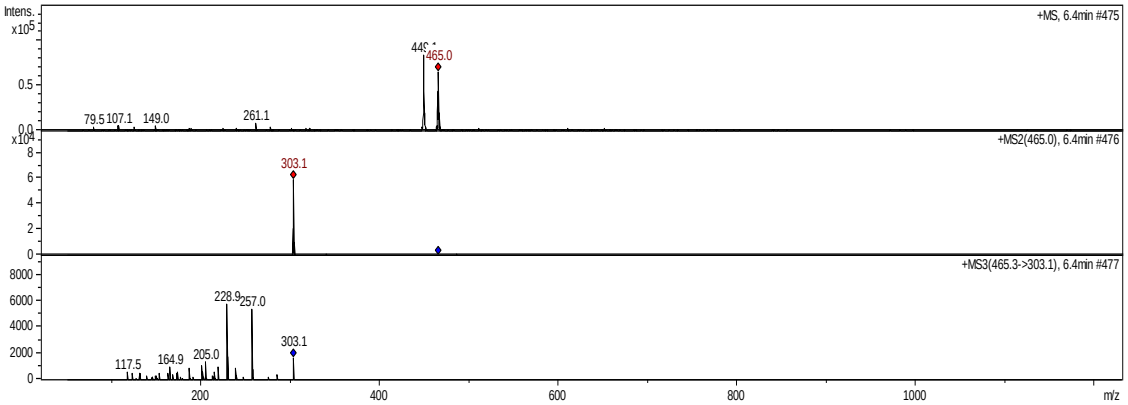


Figure 2 - Cyanidin-3-galactoside **(Compound 2)**

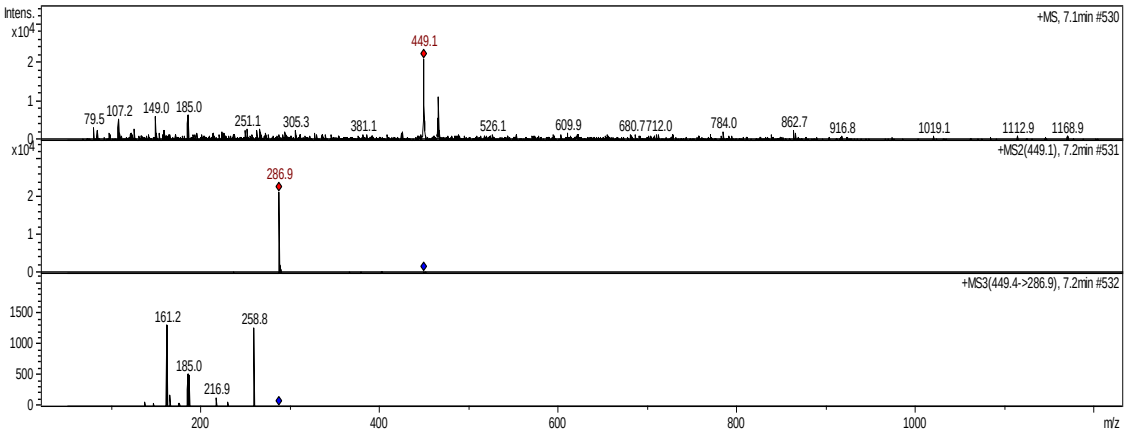
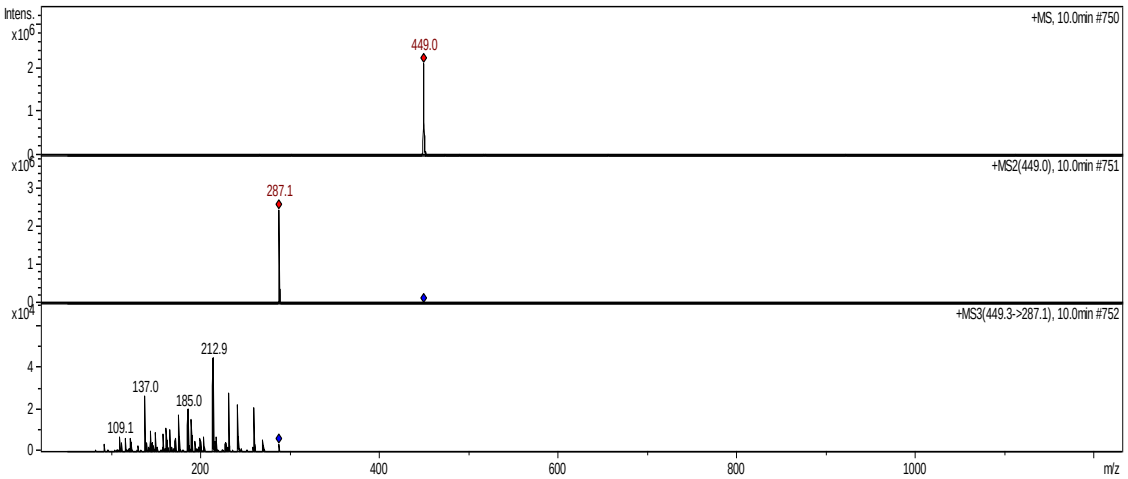


Figure 3 – Cyanidin 3-*O*-glicoside **(Compound 3)**



APPENDIX 1 - Continuation

Figure 4 - Cyanidin-3-xyloside/arabinoside **(Compound 4)**

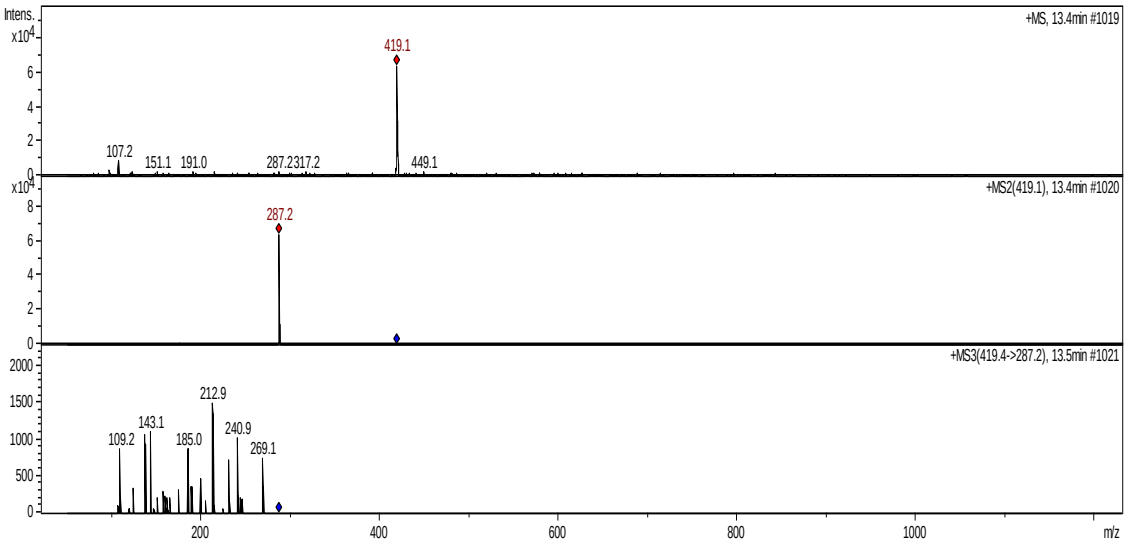
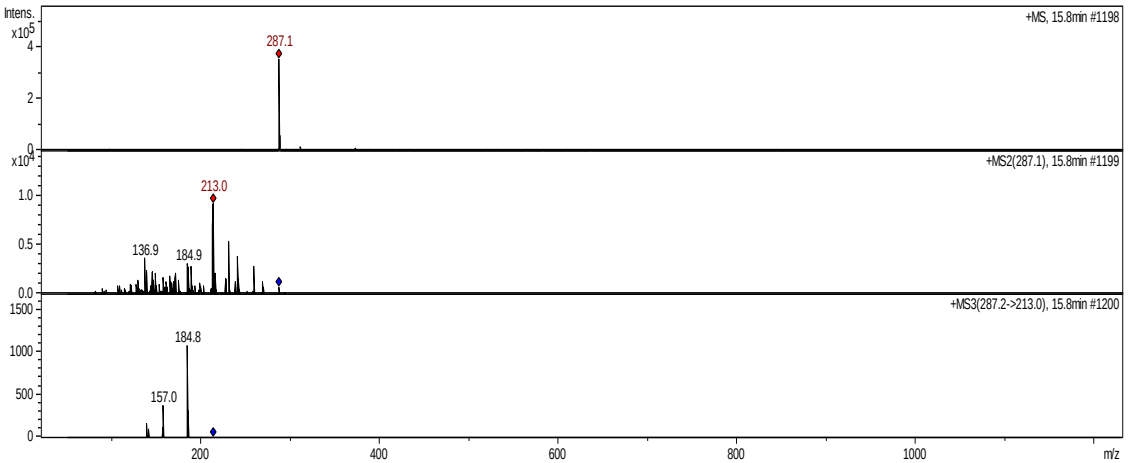
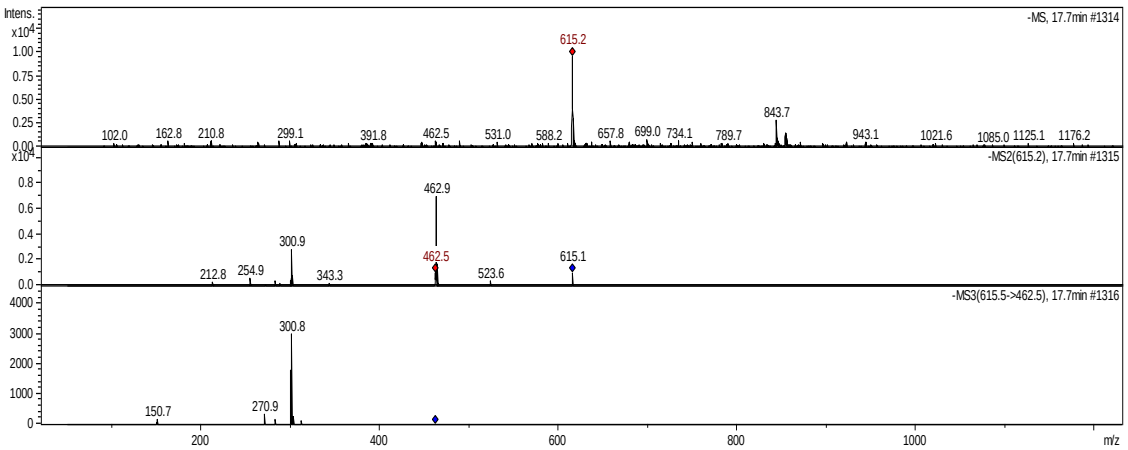


Figure 5 - Aglycone Cyanidin **(Compound 5)**



NEGATIVE MODE

Figure 6 - Ellagic acid galloyl hexoside **(Compound 6)**



APPENDIX 1 - Continuation

Figure 7 - Free Ellagic acid **(Compound 7)**

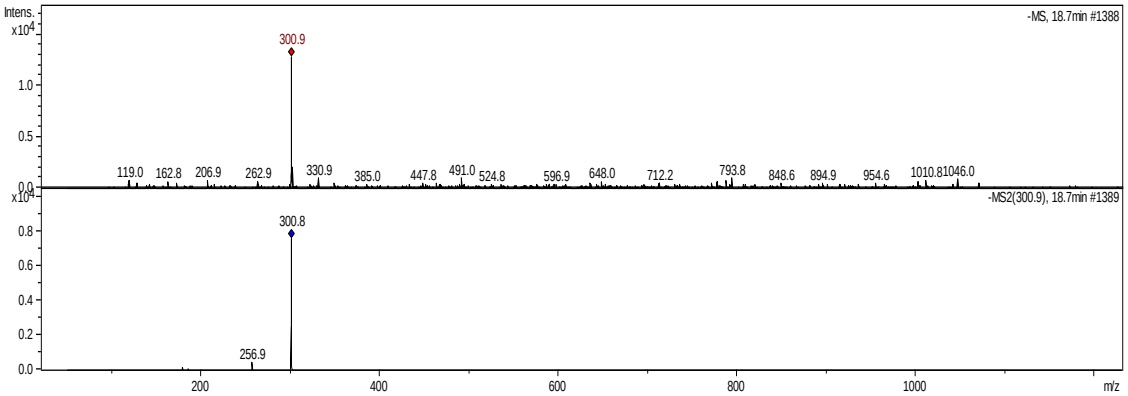


Figure 8 - Quercetin-3-glucoside **(Compound 8)**

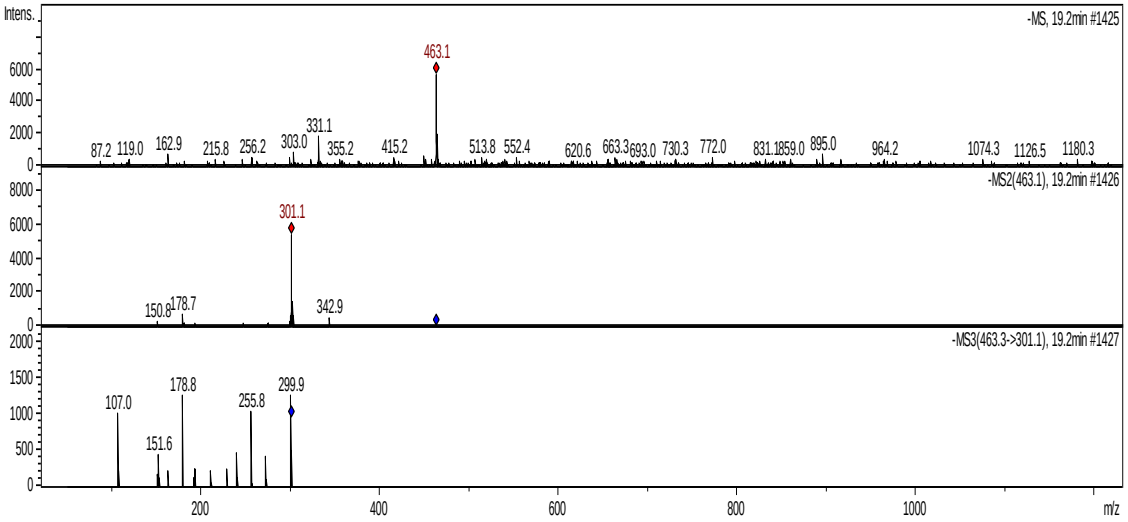
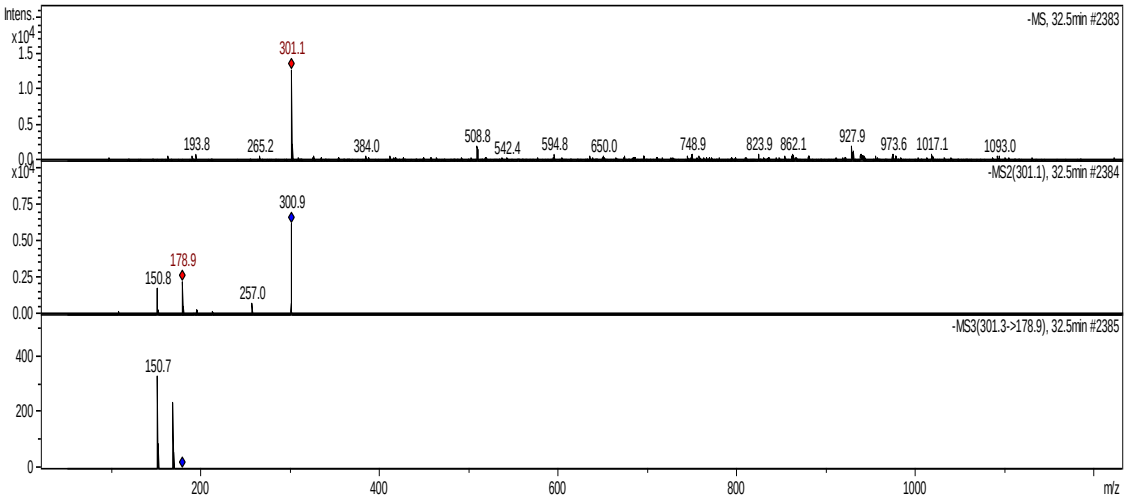


Figure 9 - Aglycone Quercetin **(Compound 9)**



APPENDIX 1 - Continuation

Seed of purple grumixama and yellow (*Eugenia bresilienses* Lam.)

Figure 10 - HHDP-galloyl glucose **(Compound 17)**

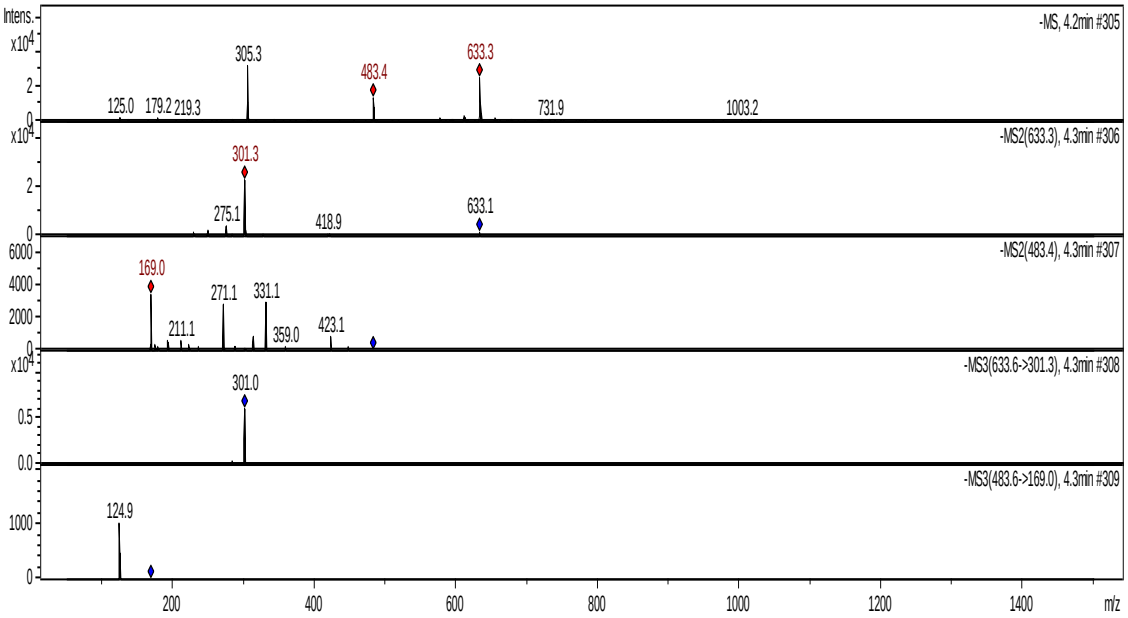
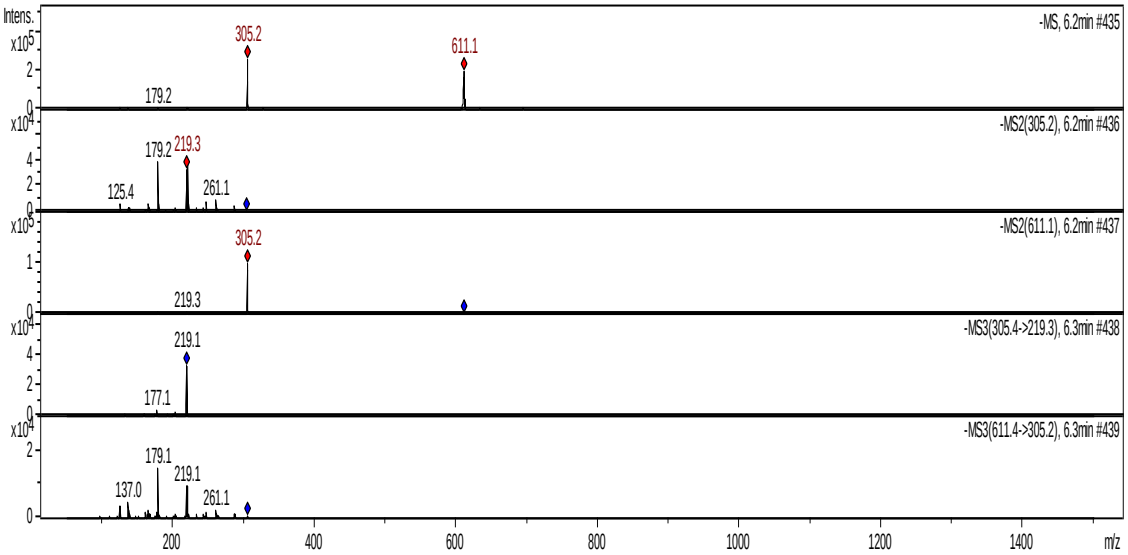


Figure 11 - Galocatechin ionization dimer **(Compound 10)**



APPENDIX 1 - Continuation

Figure 12 - HHDP-galloyl glucose **(Compound 18)**

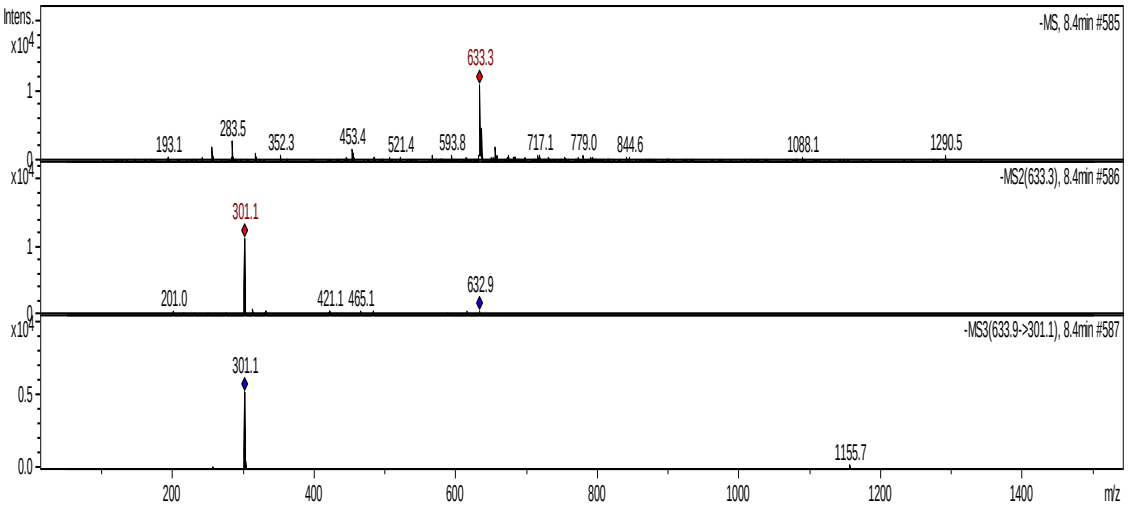


Figure 13 - (epi)catechin(epi)catechin **(Compound 19)**

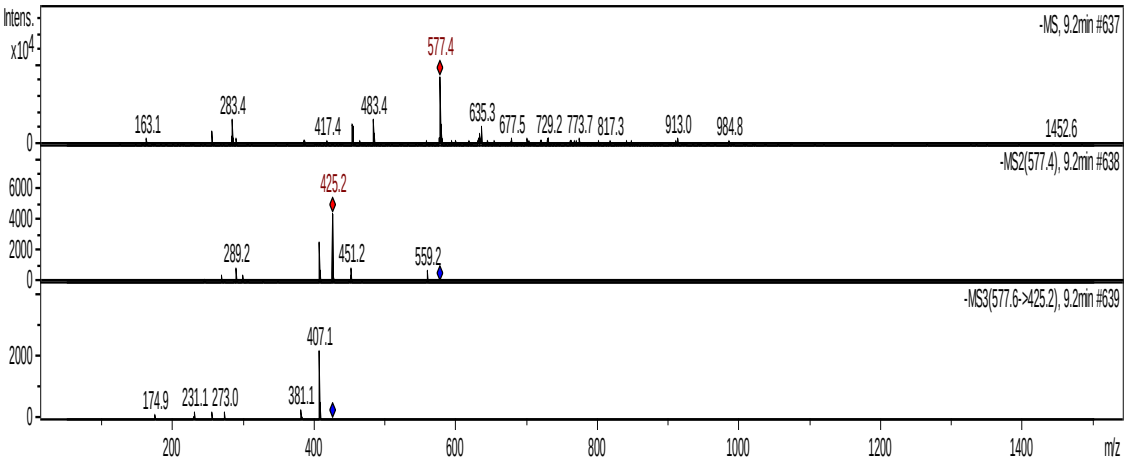
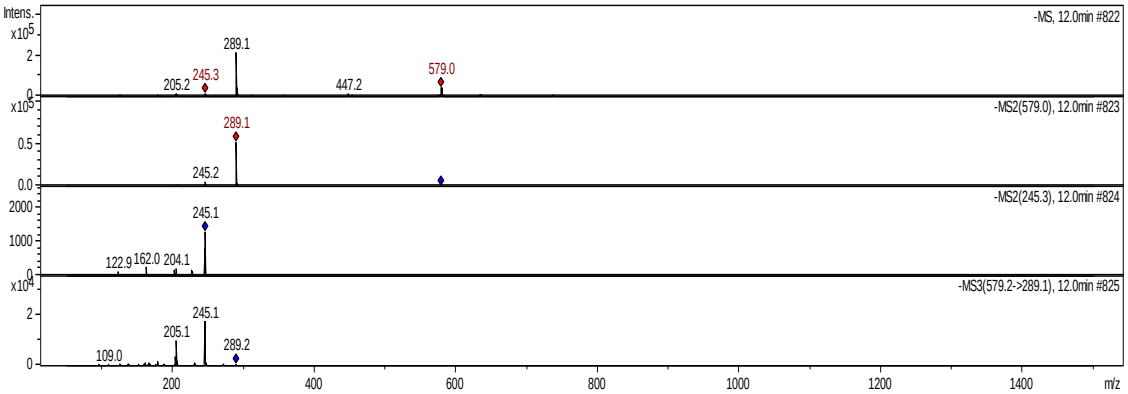


Figure 14 - Catechin/Dímer of Ionization **(Compound 12)**



APPENDIX 1 - Continuation

Figure 15 - Galocatechin gallate **(Compound 20)**

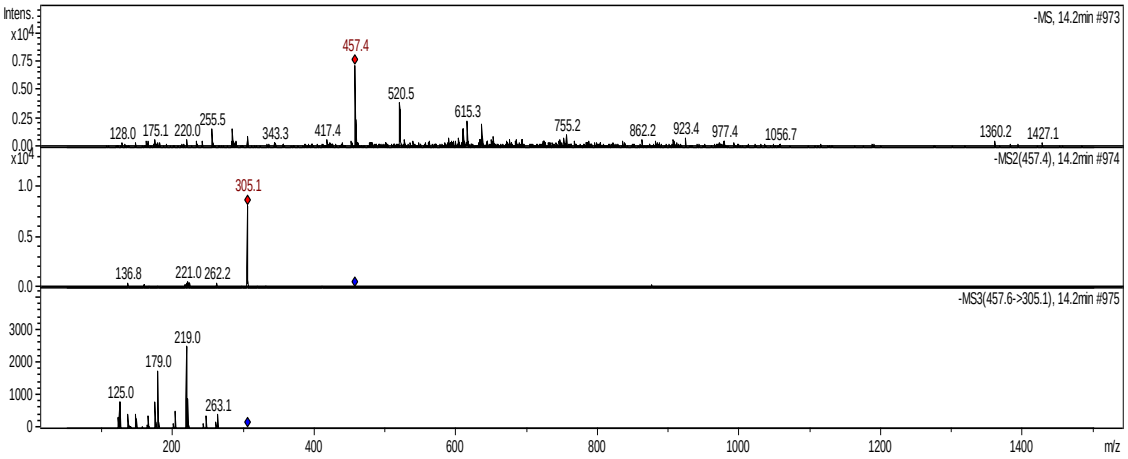
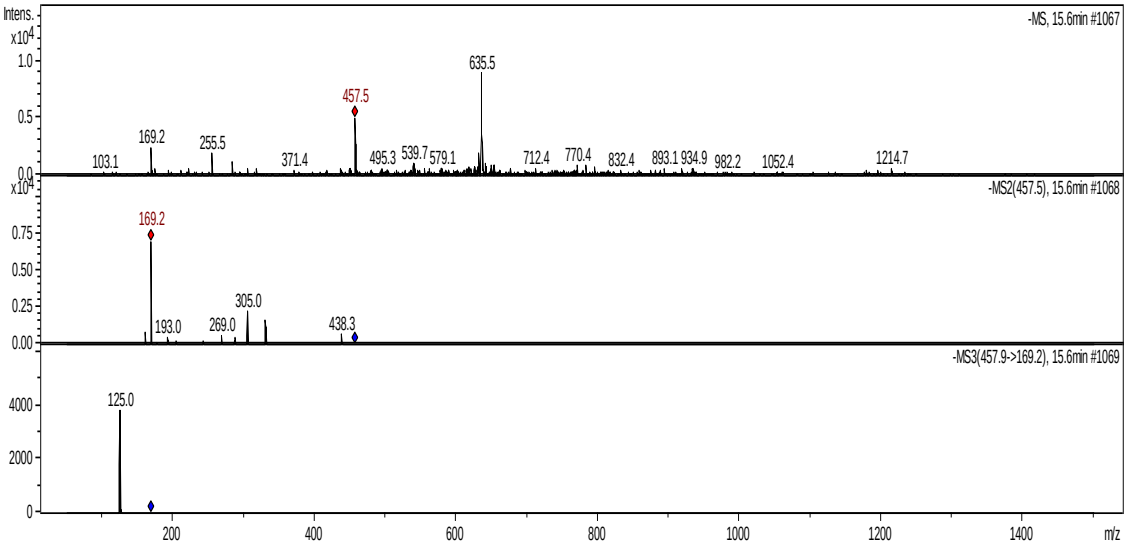


Figure 16 - Epigallocatechin gallate* **(Compound 21)**



APPENDIX 1 - Continuation

Figure 17 - Myrecetin Galloyl Hexoside **(Compound 22)**

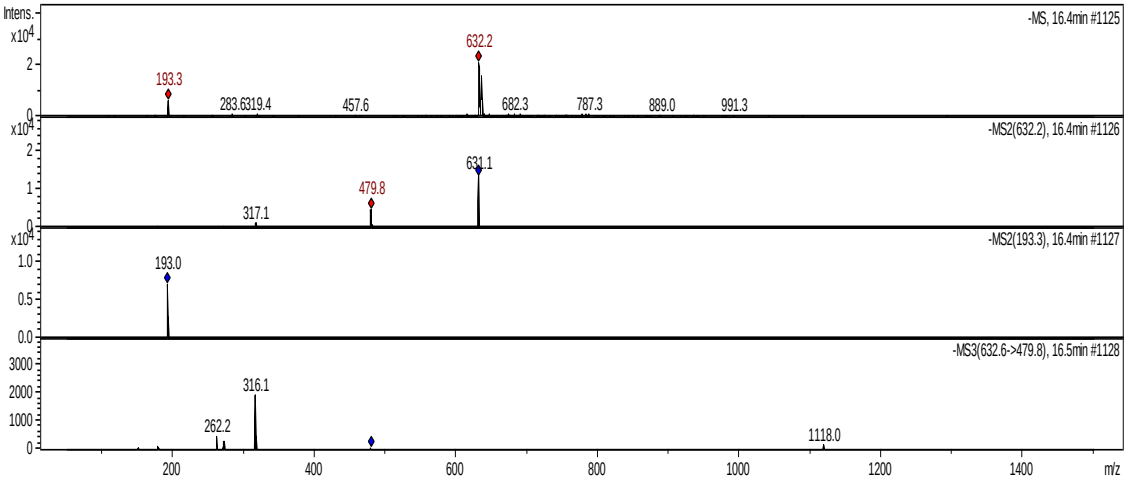


Figure 18 - Ellagic acid dipentoside **(Compound 23)**

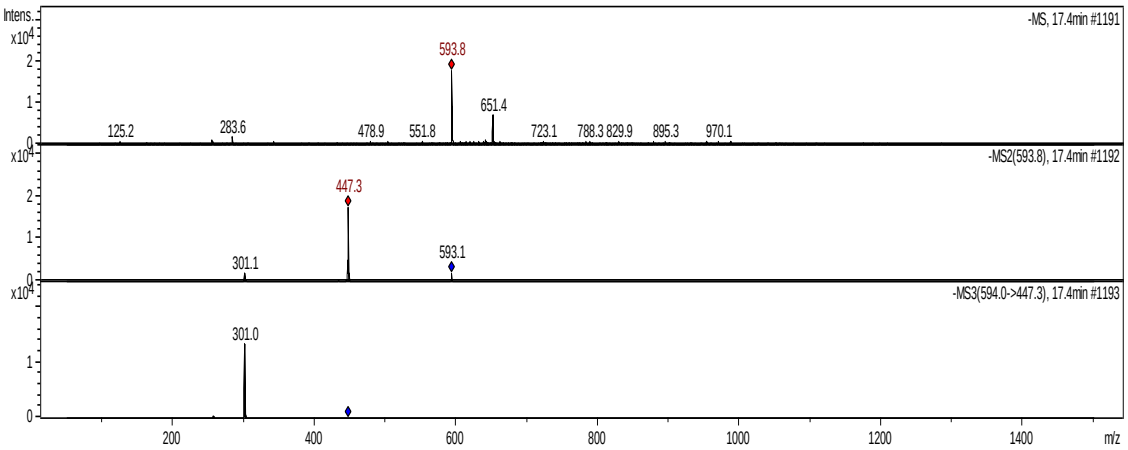
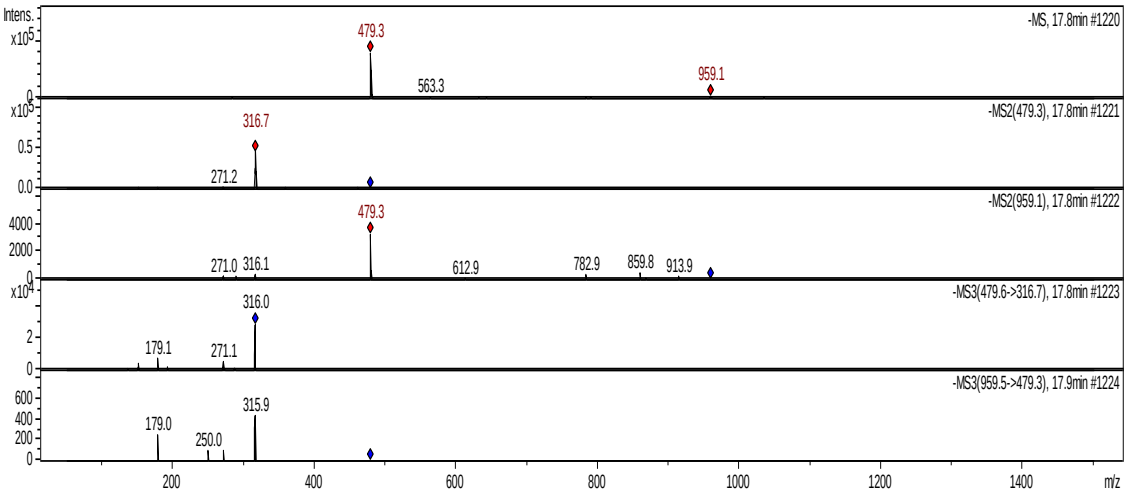


Figure 19 - Dimer of Ionization of Myrecetin hexoside **(Compound 24)**



APPENDIX 1 - Continuation

Figure 20 - Ellagic acid galloyl hexoside **(Compound 6)**

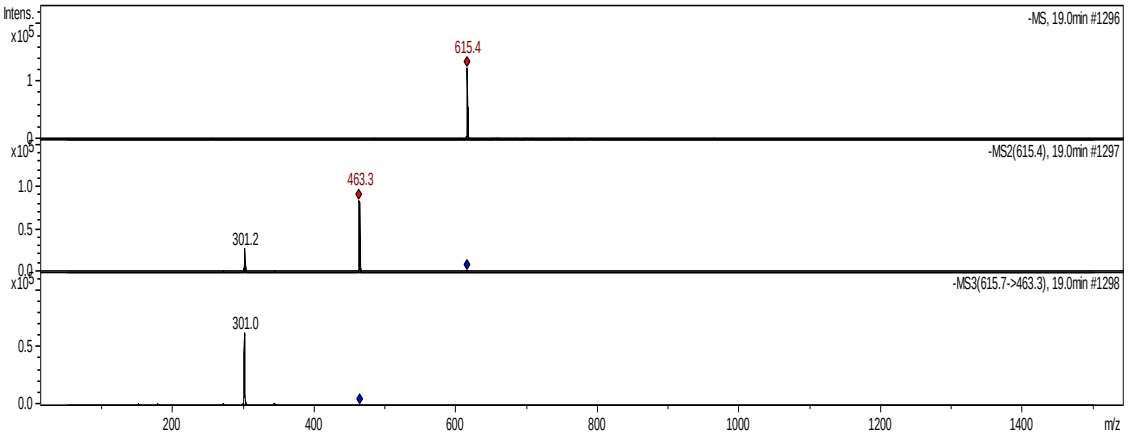


Figure 21 - Ellagic acid Hexosideo **(Compound 25)**

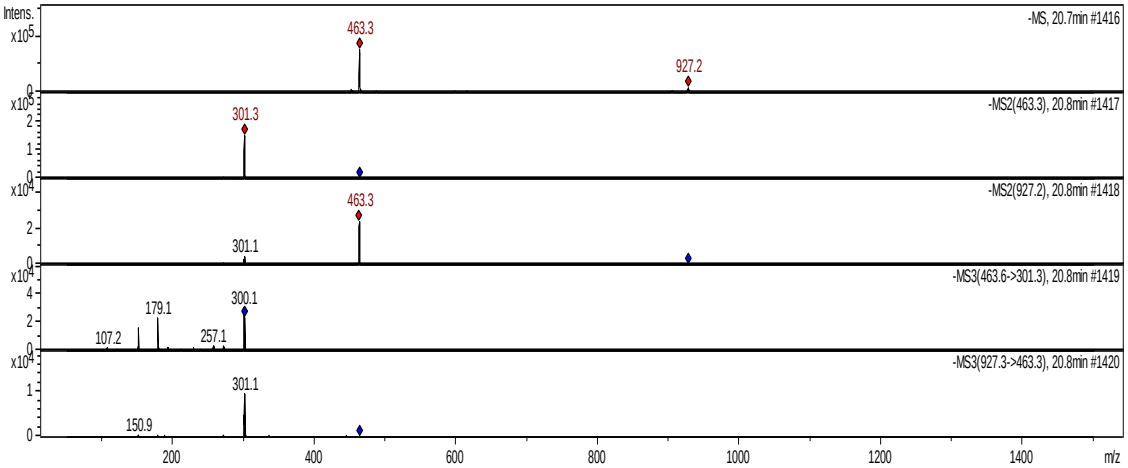
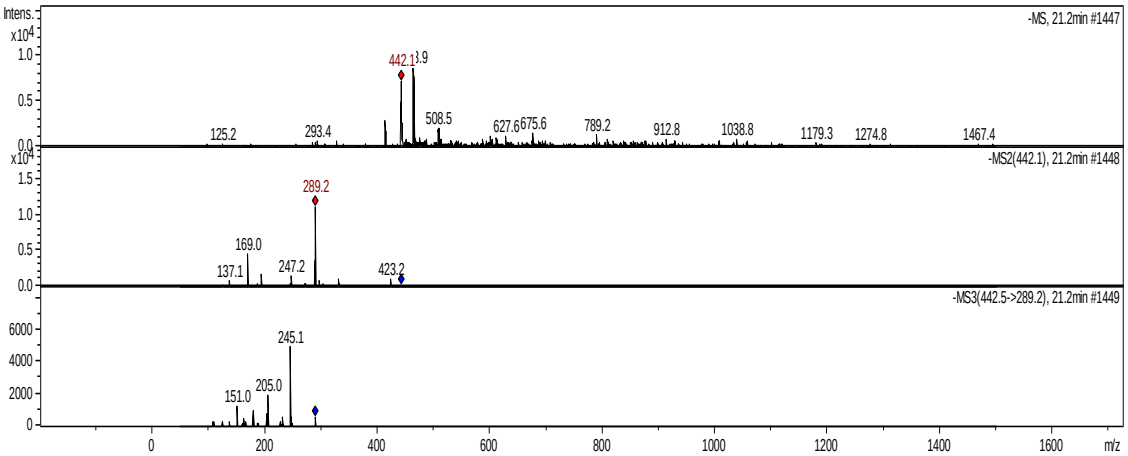


Figure 22 - (−)-Epicatechina gallate* **(Compound 26)**



APPENDIX 1 - Continuation

Figure 23 - Ellagic acetyl rhaminoside/Dímer of Ionization **(Compound 27)**

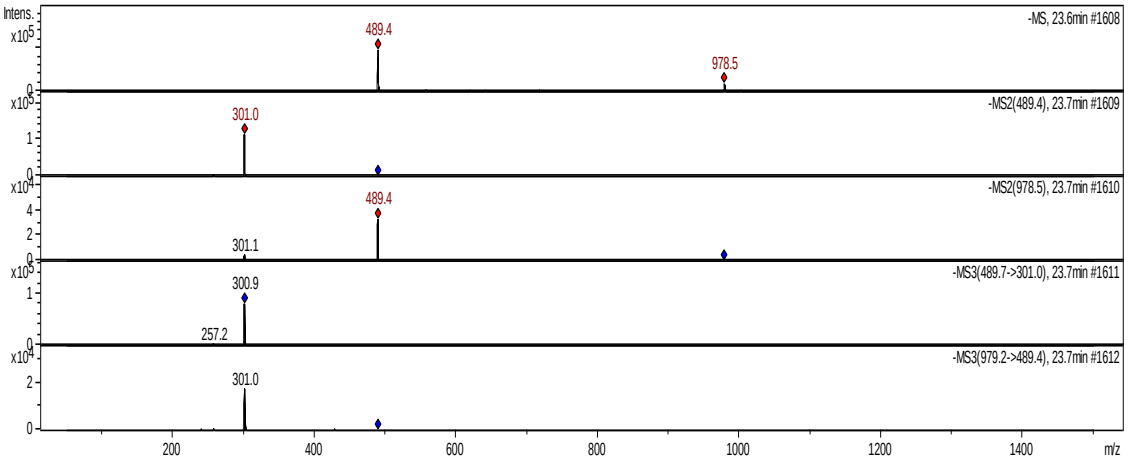
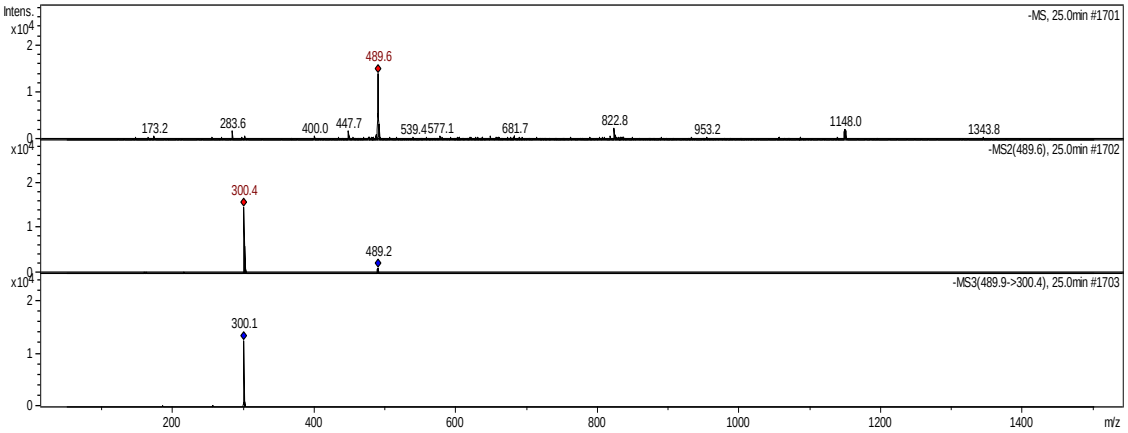


Figure 24 - Ellagic acetyl rhaminoside Dímer **(Compound 28)**



APPENDIX 2

Supplementary Material for Part 2

Identifications by LC-ESI – MS

Anthocyanins in Urine

Positive mode

Figure 1 - Cyanidin 3-*O*-glucoside m/z 449

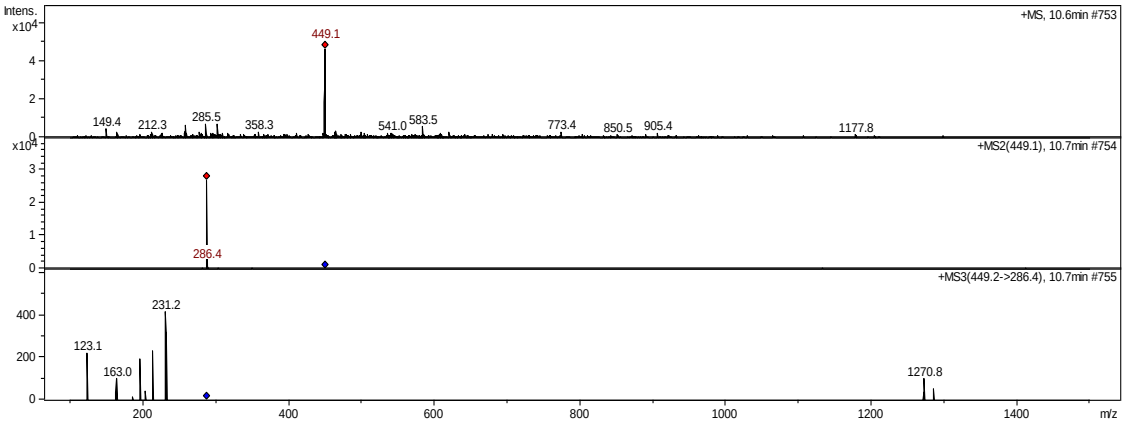
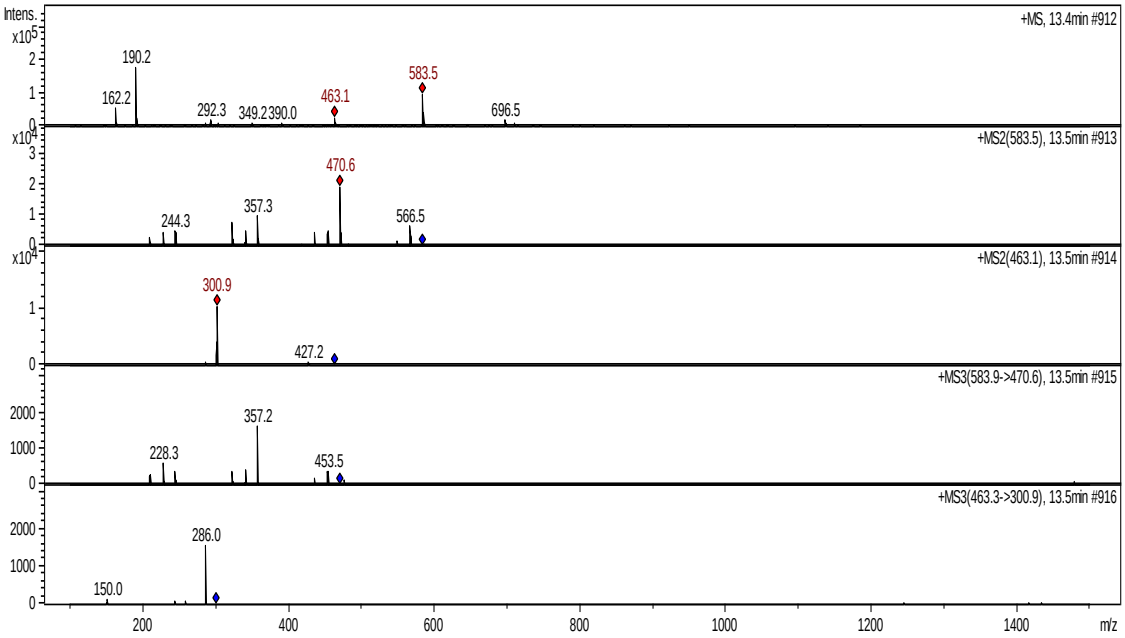
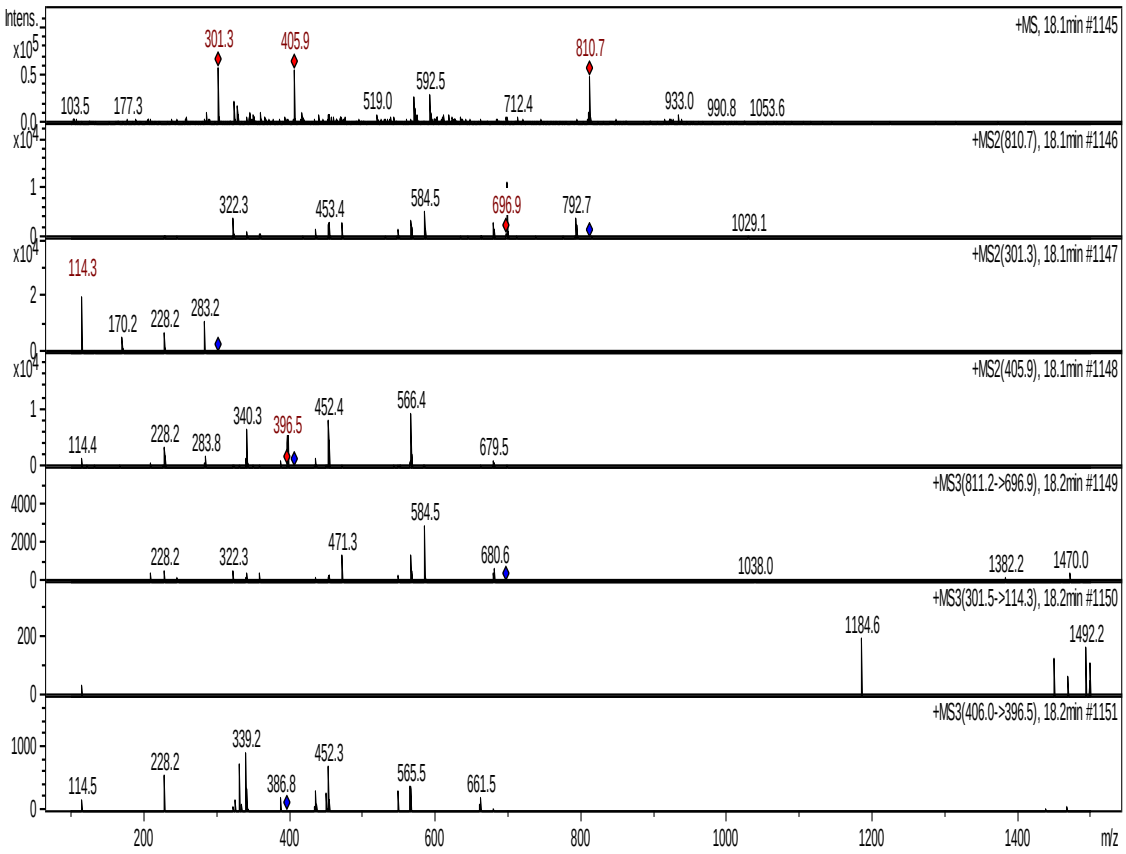


Figure 2 - Methylated cyanidin 3-glucoside m/z 463



APPENDIX 2 - Continuation

Figure 3 - Methylated cyanidin m/z 301



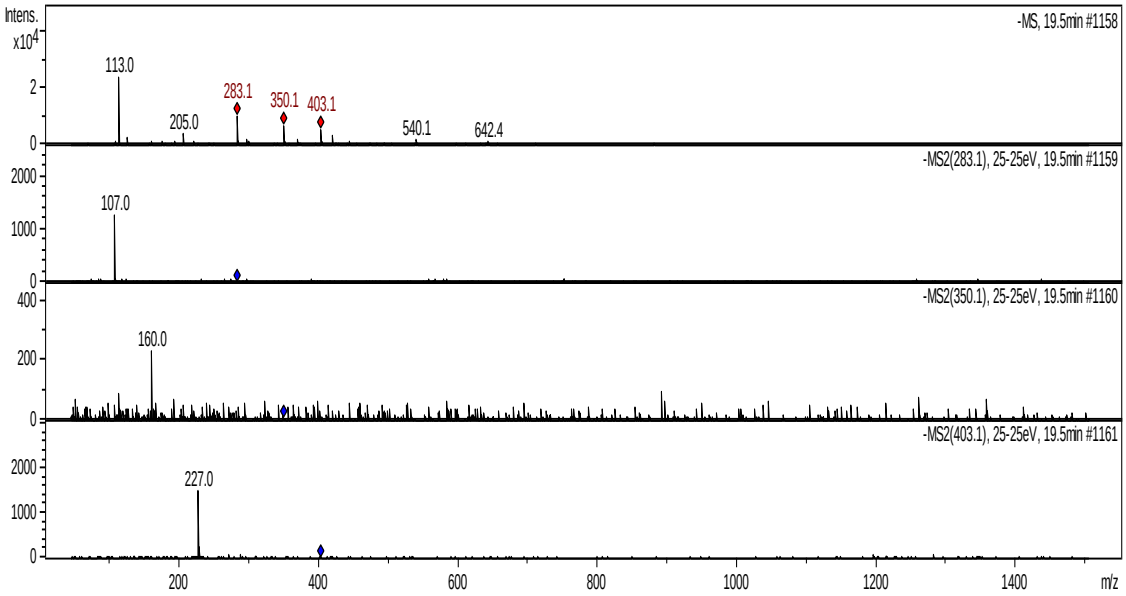
APPENDIX 2 - Continuation

Urolithins and Metabolites Identified in Urine

Negative mode

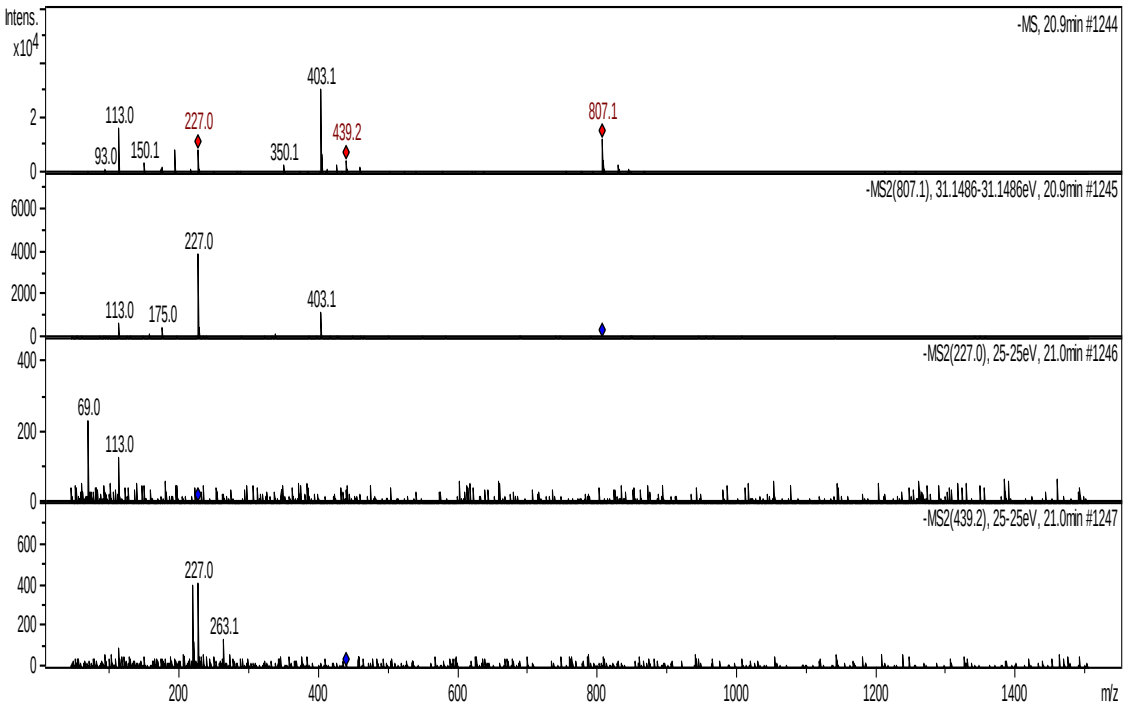
Identification of urolithins was done by comparing mass fragmentation profile from literature.

Figure 4 - Urolithin A glucuronide



APPENDIX 2 - Continuation

Figure 5 - Urolithin A glucuronide



APPENDIX 2 - Continuation

Figure 6 - Urolithin C methyl ether glucuronide

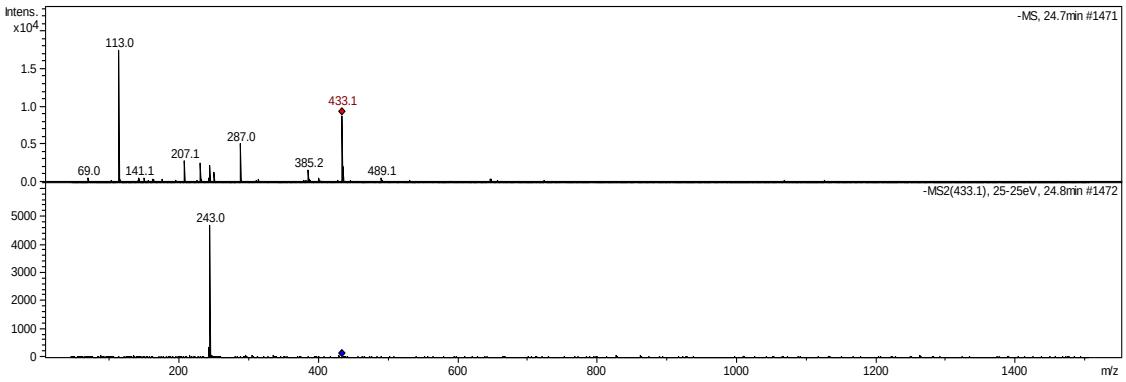


Figure 7 - Urolithin C

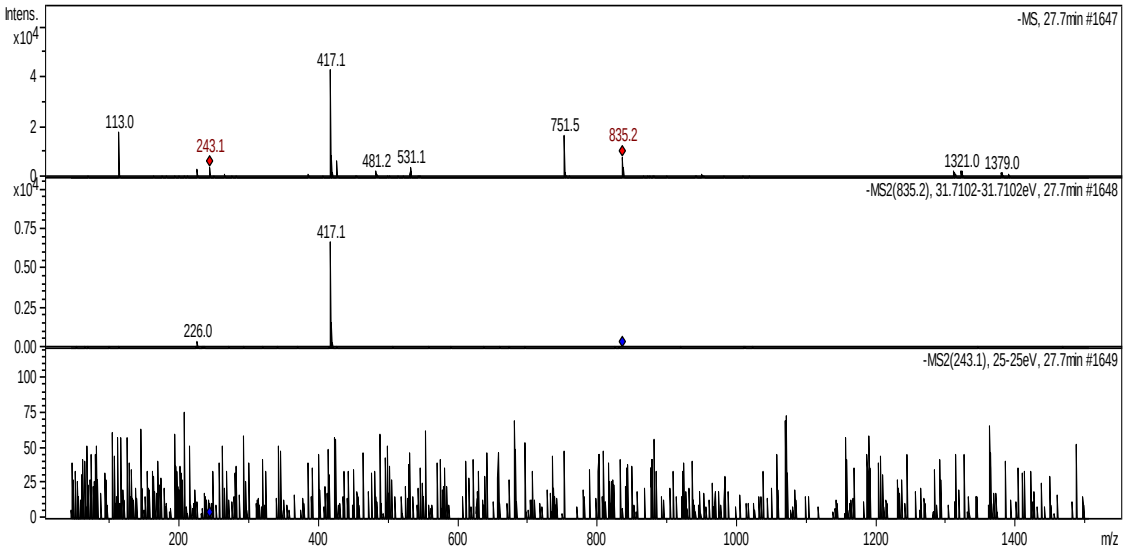
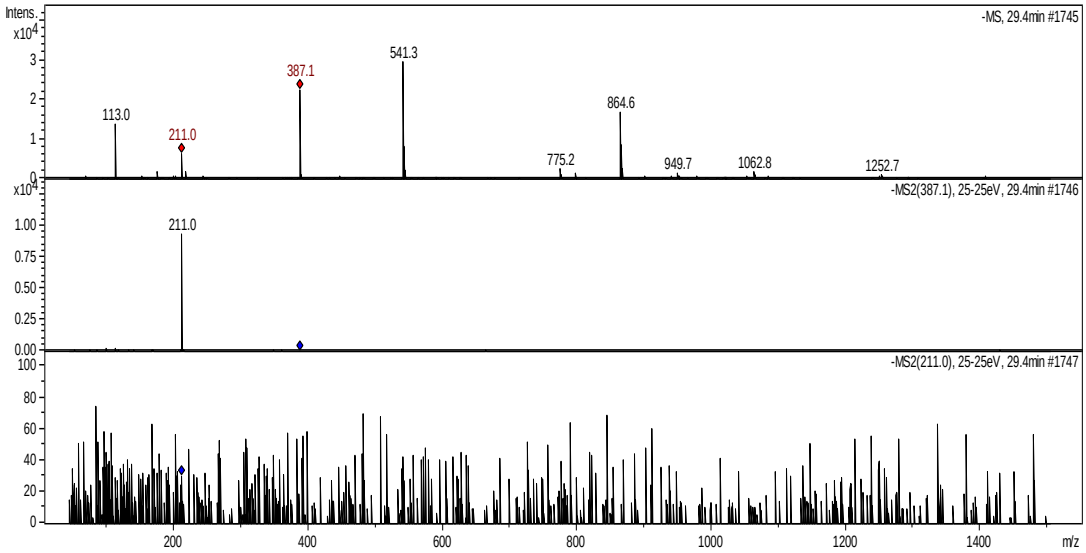


Figure 8 - Urolithin B glucuronide



APPENDIX 2 - Continuation

Figure 9 - Urolithin C methyl ether glucuronide

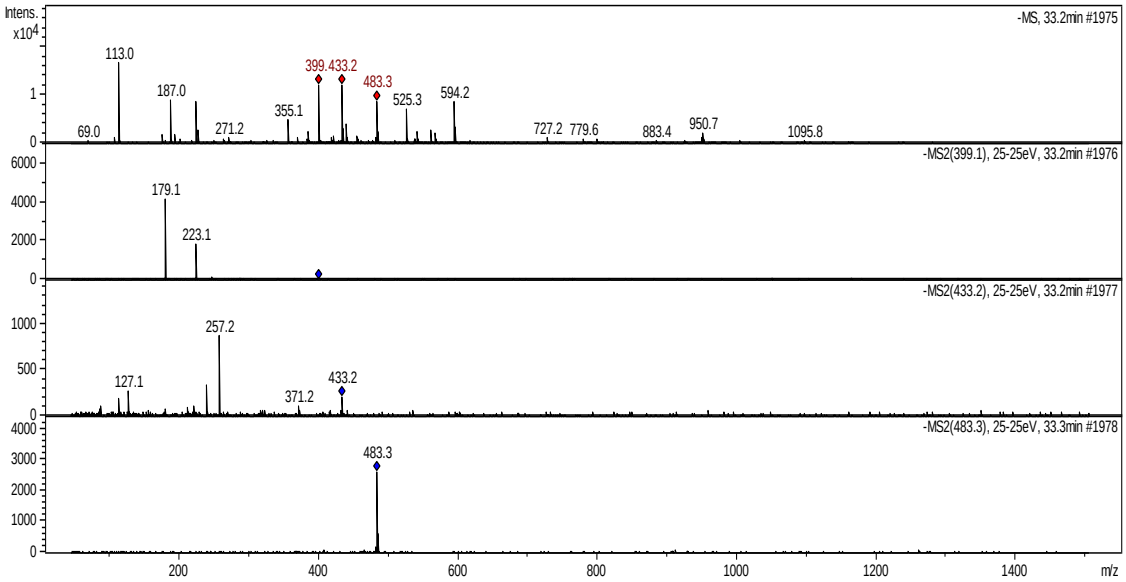
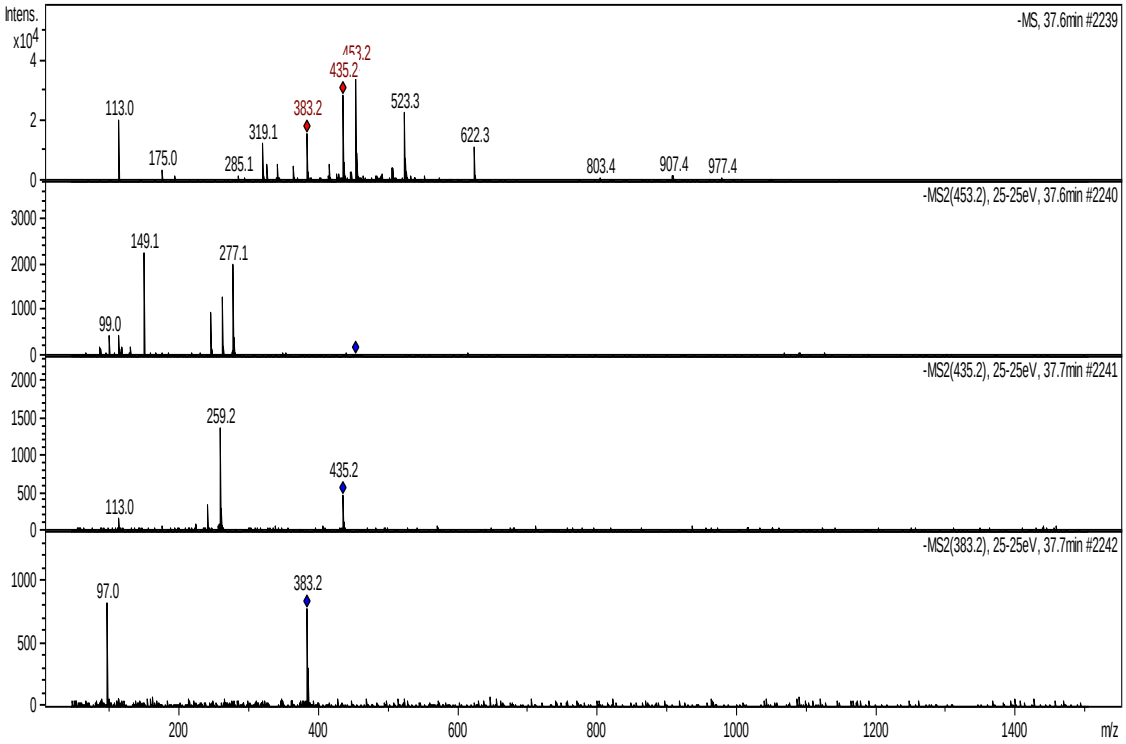


Figure 10 - Urolithin D glucuronide



APPENDIX 2 - Continuation

Table 1 - Biochemistry parameters results in plasma samples before grumixama juice intake by subjects.

Parameters	Values	Acceptable values
Glycemia (mg/dL)	93.04 ± 5.05	<100
Total Cholesterol (mg/dL)	190.08 ± 21.27	<239
Triglycerides (mg/dL)	110.92 ± 40.97	<200
HDL (mg/dL)	56.19 ± 13.22	35 - 85
LDL (mg/mL)	108.50 ± 23.77	<159
Creatinina (mg/dL)	0.76 ± 0.08	0.4 - 1.3
AST (U/L)	16.94 ± 4.13	13 - 35
ALT (U/L)	20.83 ± 2.97	7 - 35

HDL= *High Density Lipoproteins*; LDL= L*ow Density Lipoproteins*; AST= aspartate transaminase, ALT= alanine aminotransferase; n=15 subjets; Results expressed in mean ± standard desviation.

APPENDIX 3

Supplementary Material for Part 3

Table 1 – Food composition of meals diary intake by subjects.

Breakfast	**Food Composition**	**Content**
	Carbohydrates	21.43
	Proteins	13.50
	Total fat	16.86
	Dietary Fiber	6.53
	Sodium	480.27
	Calories and energy value (Kcal)	292.32
Lunch	**Food Composition**	**Content**
	Carbohydrates	42.46
	Proteins	36.25
	Total fat	6.59
	Dietary Fiber	2.19
	Sodium	2.48
	Calories and energy value (Kcal)	384.63
Dinner	**Food Composition**	**Content**
	Carbohydrates	75.36
	Proteins	42.47
	Total fat	5.77
	Dietary Fiber	1.68
	Sodium	0.536
	Calories and energy value (Kcal)	523.03
Snacks	**Food Composition**	**Content**
	Carbohydrates	68.00
	Proteins	13.80
	Total fat	17.80
	Dietary Fiber	1.20
	Sodium	536.00
	Calories and energy value (Kcal)	488.00
	Total Calories (Kcal)	1687.98

APPENDIX 3 - Continuation

Table 2 – Metabolites identified comparing to commercial standards by GCMS.

Metabolite Identified	RT	RI	CAS
Oxalic acid	5.753	59	144-62-7
Malonic acid	6.618	101	141-82-2
Fumaric acid	8.323	113	110-17-8
Succinic acid	8.505	115	110-15-6
Benzoic acid	9.63	105	65-85-0
Glyoxylic acid	10.259	75	298-12-4
NADP/NADPH	10.882	138	2646-71-1
beta-Alanine	11.643	88	107-95-9
Pyroglutamic acid	14.16	84	98-79-3
cis-Aconitic acid	14.981	153	585-84-2
Citric acid	14.989	143	77-92-9
Glutamic acid	17.006	174	56-86-0
4-Hydroxyphenylacetic acid	18.125	121	156-38-7
Tartaric acid	18.494	59	87-69-4
Hippuric acid	18.765	105	495-69-2
Phenylalanine	19.056	162	63-91-2
Isocitric acid	19.133	129	320-77-4
Ornithine	23.072	128	70-26-8
Histidine	25.199	139	71-00-1
Tyrosine	27.375	236	60-18-4

RT: retention time; RI: reference íon after MFC derivatization; CAS:

APPENDIX 3 – Continuation

Table 3 – T test results for matrix data of comparison urine at time 0 and urine at 1-2 h and for the comparison between urine at time zero and urine at 2-4 h after GJ intake.

Compounds	Zero x 1-2 h	Zero x 2-4 h
	p.values	p.values
Glyoxylic acid	3.38E-17	1.65E-11
Unknown 070 - (100) 42(28.5) 69(8.0)	2.42E-16	2.97E-07
Unknown 249- (100) 57(31.8) 264(22.9)	2.97E-16	1.56E-08
Heptadecane	6.69E-16	1.64E-08
Unknown 201- (100) 117(89.6) 119(86.2)	8.53E-16	2.87E-11
Unknown 105- (100) 77(48.0) 51(14.2)	2.37E-15	1.06E-08
Pentadecane	3.68E-15	5.81E-08
Dodecane	3.72E-15	1.05E-06
Nicotinamide	3.92E-15	9.39E-07
Unknown 091- (100) 92(23.6) 120(21.2)	8.04E-15	2.90E-06
Unknown 117- (100) 43(68.6) 85(55.8)	9.04E-15	6.61E-06
Unknown 095- (100) 126(31.0) 39(11.2)	9.69E-14	7.54E-07
Unknown 074- (100) 46(21.3) 59(0.97)	2.46E-13	1.97E-08
Unknown 059 - (100) 87(25.7) 91(22.5)	4.29E-13	NV
Unknown 043 - (100) 59(77.1) 103(31.4)	5.77E-13	1.43E-06
Unknown 071(100) 57(94.4) 85(72.9)	6.79E-13	1.78E-06
Tridecane	1.49E-12	3.66E-06
Hexanoic acid	1.69E-12	2.27E-07
Unknown 101(100) 119(80.5) 74(35.3)	1.27E-11	1.50E-07
Unknown 149(100) 167(32.8) 57(17.4)	2.19E-11	8.49E-06
Nonacosane	7.11E-11	8.34E-06
Unknown 091(100) 106(54.7) 105(21.8)	3.68E-10	0.00076179
Oxalic acid	1.70E-09	NV
Unknown 069(100) 41(63.5) 128(36.1)	4.60E-09	5.92E-06
Azelaic acid	8.50E-09	2.37E-08
Unknown 128(100) 129(0.69) 42(0.52)	1.10E-08	3.70E-06
Ornithine	3.07E-08	6.09E-05

Octanoic acid (C8_0)	3.23E-08	7.24E-09
Lysine	5.68E-08	4.96E-05
Unknown 071(100) 57(92.3) 43(75.3)	5.91E-08	0.0024904
Histidine	1.01E-07	9.74E-05
Suberic acid	2.51E-07	0.0050929
Caprylic acid	2.61E-07	1.36E-07
Lactic acid	3.42E-07	0.038063
Nicotinic acid	3.72E-07	0.0014903
2-Hydroxyglutaramic acid	4.37E-07	0.00050236
Tyrosine	5.57E-07	0.00025149
Unknown 088(100) 44(30.3) 59(19.6)	6.64E-07	0.00071282
trans-Vaccenic acid	8.69E-07	0.0011638
Unknown 091(100) 121(90.6) 133(51.3)	1.03E-06	NV
Unknown 135(100) 180(28.0) 77(19.9)	2.85E-05	1.76E-05
Vanillic acid	3.99E-05	0.00048286
Pyroglutamic acid	5.51E-05	NV
Unknown 088(100) 44(23.8) 76(17.7)	7.89E-05	0.0010188
Anthranilic acid	0.000118	2.83E-06
Unknown 161(100) 236(82.4) 121(79.4)	0.000163	0.012924
2-Hydroxybutyric acid	0.00017	8.93E-06
Unknown 128(100) 42(11.1) 129(7.7)	0.000179	0.010766
Malonic acid	0.000179	0.00028042
2-Isopropylmalic acid	0.000187	NV
beta-Alanine	0.000266	0.0077407
Unknown 126(100) 127(46.7) 59(33.2)	0.000694	0.0037031
Tartaric acid	0.000705	0.049993
Malic acid	0.000786	NV
2-Hydroxyisobutyric acid	0.000849	1.98E-05
NADP/NADPH	0.000938	0.00029307
Citraconic acid	0.001048	0.0055427
Itaconic acid	0.001048	0.0051139
Threonine	0.001262	0.0014338
Unknown 143(100) 101(42.3) 201(15.1)	0.001852	NV

Unknown 115(100) 59(65.5) 189(50.1)	0.001871	0.01074
Glutaric acid	0.001884	0.0085103
Asparagine	0.002085	0.046482
Phenylalanine	0.003874	NV
Unknown 130(100) 189(19.9) 77(10.4)	0.005572	NV
Unknown 114(100) 59(15.3) 82(14.4)	0.007913	NV
Adipic acid	0.011242	NV
4-Hydroxyphenylacetic acid	0.012471	NV
Benzoic acid	0.012666	0.0022535
Unknown 151(100) 107(23.6) 254(19.9)	0.015412	NV
Unknown 078(100) 134(95.2) 106(49.3)	0.015658	NV
Citric acid	0.022053	0.012906
2-Aminoadipic acid	0.023872	NV
Unknown 059(100) 57(69.6) 71(61.8)	0.028114	NV
Unknown 045(100) 82(94.9) 55(65.2)	0.030526	0.0065354
Fumaric acid	0.035469	NV
2-Aminobutyric acid	0.03767	NV
Leucine	0.043291	NV
Hippuric acid	NV	0.03916
Pimelic acid	NV	0.031393
Suberic acid	NV	0.005093
Unknown 067(100) 85(95.8) 59(87.6)	NV	0.0014187
Unknown 088(100) 44(23.8) 76(17.7)	NV	0.001019

1: Unknown compounds do not have CAS number; Numbers that followed unknown number is referent to MS profile derivatized ions; NV: Not vary. T test for peak intensity normalized and it was performed by MetaboAnalyst.

APPENDIX 3 - Continuation

Figure 1 - Juice composition

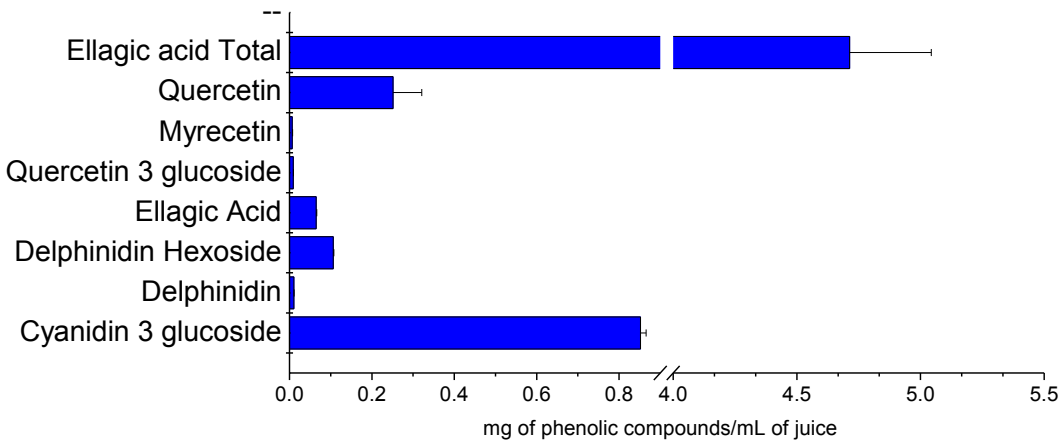


Figure 1 - Phenolic compounds quantified in the grumixama juice (GJ).

APPENDIX 3 - Continuation

Figure 2 - PLS-DA graphics

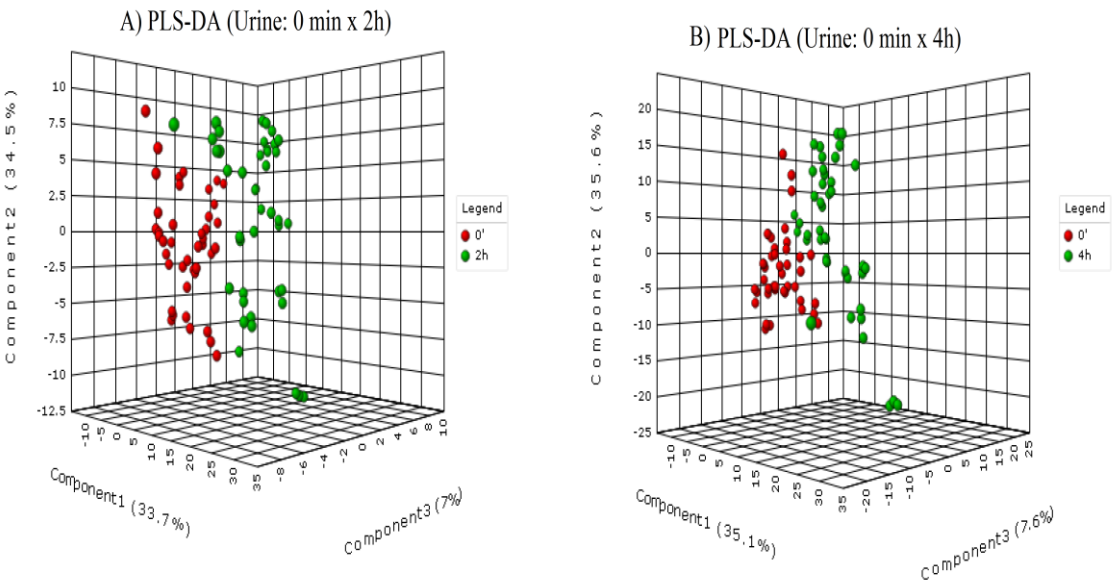


Figure 2 – PLS-DA graphics of metabolites (amino acids and organic acids) found following the acute intake of rich polyphenol juice: A) PLS-DA model of urine samples at time 0 and urine at 1-2 h after juice intake, B) PLS-DA model of urine samples at time 0 and urine at 2-4 h after juice intake.

APPENDIX 3 - Continuation

Figure 3- Permutation tests

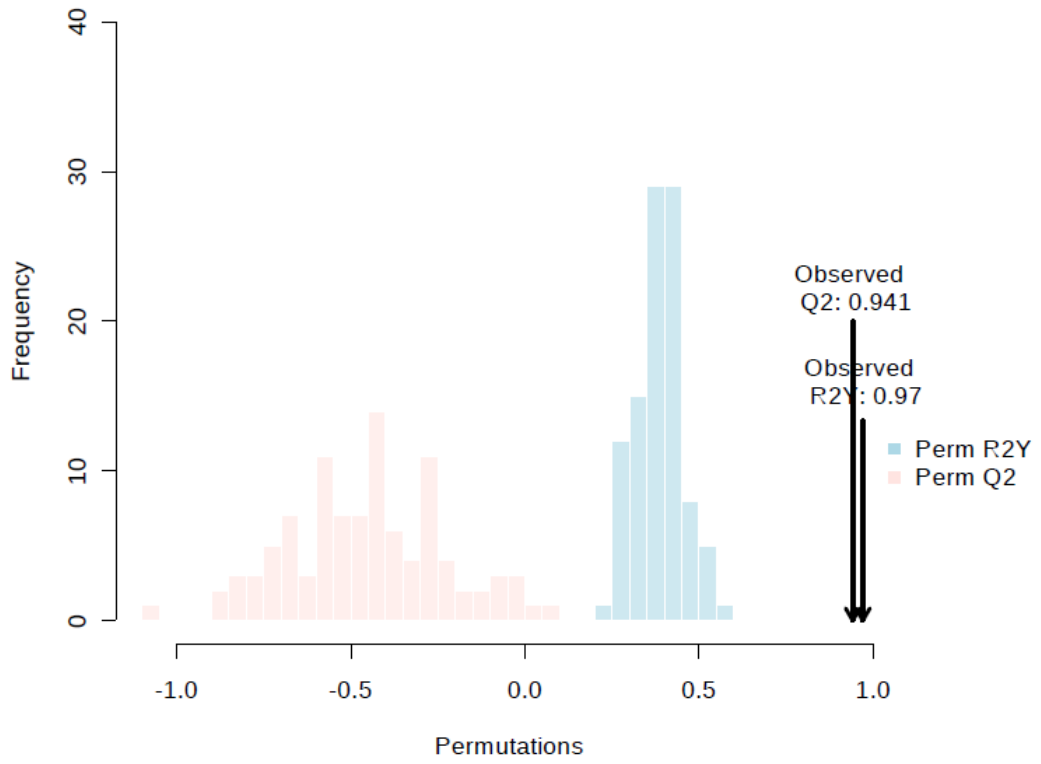


Figure 3 - Result permutation tests (n= 100) of OPLS-DA model for urine at time 0 and urine at time 1-2 h after GJ intake.

APPENDIX 3 - Continuation

Figure 4 - Permutation tests

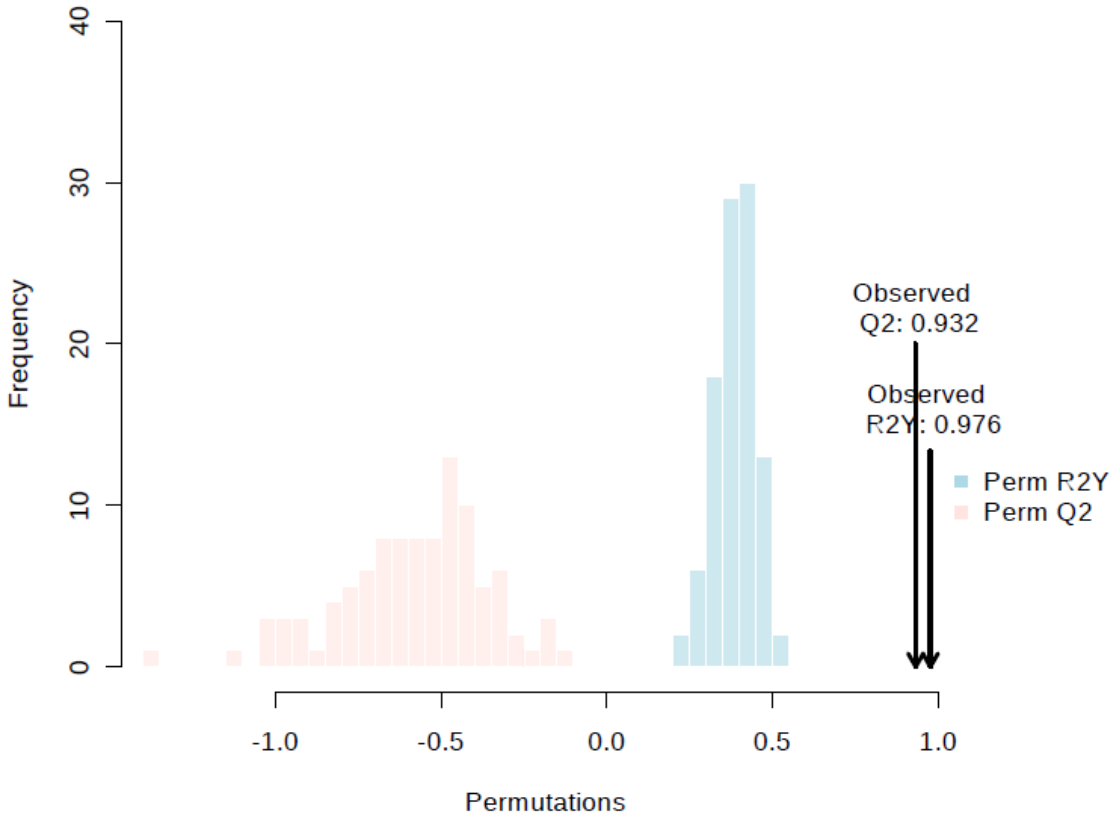


Figure 4 - Result permutation tests (n= 100) of OPLS-DA model for urine at time 0 and urine at time 2-4 h after GJ intake.

APPENDIX 3 - Continuation

Figure 7 – Gluthatione metabolism

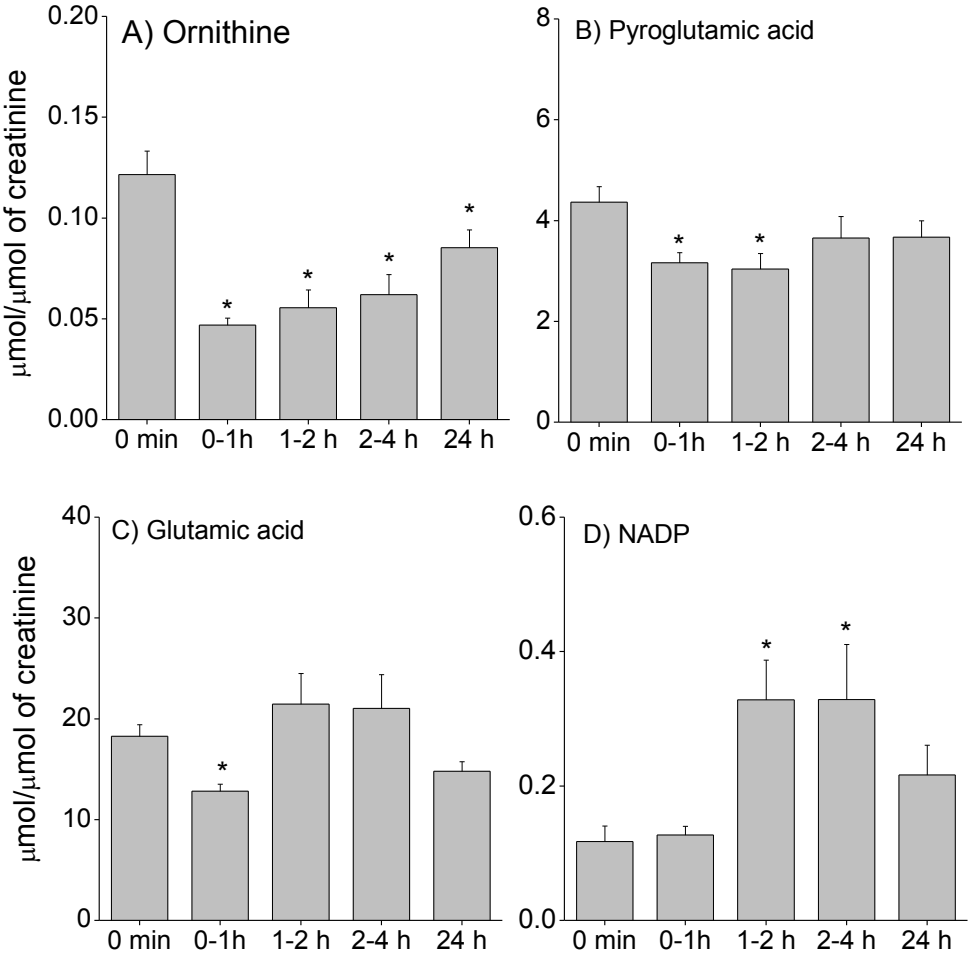


Figure 7 – Metabolites located in glutathione metabolism and quantified by standards curves: A) Ornithine, B) Pyroglutamic acid and C) Glutamic acid. Results expressed as mean ± standard errors, *p-values<0.05, Anova one way following t test comparing to time 0.

APPENDIX 4

Supplementary Material for Part 4

Panel gene list

RT² Profiler™ PCR Array Mouse Fatty Liver

Position	RefSeq Number	Symbol	Description
A01	NM_013454	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1
A02	NM_009593	Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1
A03	NM_133360	Acaca	Acetyl-Coenzyme A carboxylase alpha
A04	NM_007381	Acadl	Acyl-Coenzyme A dehydrogenase, long-chain
A05	NM_134037	Acly	ATP citrate lyase
A06	NM_015729	Acox1	Acyl-Coenzyme A oxidase 1, palmitoyl
A07	NM_027976	Acsl5	Acyl-CoA synthetase long-chain family member 5
A08	NM_016870	Acsm3	Acyl-CoA synthetase medium-chain family member 3
A09	NM_028320	Adipor1	Adiponectin receptor 1
A10	NM_197985	Adipor2	Adiponectin receptor 2
A11	NM_009652	Akt1	Thymoma viral proto-oncogene 1
A12	NM_009692	Apoa1	Apolipoprotein A-I
B01	NM_009693	Apob	Apolipoprotein B
B02	NM_023114	Apoc3	Apolipoprotein C-III
B03	NM_009696	Apoe	Apolipoprotein E
B04	NM_020615	Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1
B05	NM_009810	Casp3	Caspase 3
B06	NM_007643	Cd36	CD36 antigen
B07	NM_009883	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta
B08	NM_013493	Cnbp	Cellular nucleic acid binding protein
B09	NM_013495	Cpt1a	Carnitine palmitoyltransferase 1a, liver
B10	NM_009949	Cpt2	Carnitine palmitoyltransferase 2
B11	NM_021282	Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1
B12	NM_007824	Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1
C01	NM_026384	Dgat2	Diacylglycerol O-acyltransferase 2
C02	NM_017399	Fabp1	Fatty acid binding protein 1, liver
C03	NM_010174	Fabp3	Fatty acid binding protein 3, muscle and heart
C04	NM_010634	Fabp5	Fatty acid binding protein 5, epidermal
C05	NM_007987	Fas	Fas (TNF receptor superfamily member 6)
C06	NM_007988	Fasn	Fatty acid synthase
C07	NM_010446	Foxa2	Forkhead box A2
C08	NM_008061	G6pc	Glucose-6-phosphatase, catalytic
C09	NM_008062	G6pdx	Glucose-6-phosphate dehydrogenase X-linked
C10	NM_010292	Gck	Glucokinase
C11	NM_019827	Gsk3b	Glycogen synthase kinase 3 beta
C12	NM_008194	Gk	Glycerol kinase
D01	NM_008255	Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
D02	NM_008261	Hnf4a	Hepatic nuclear factor 4, alpha
D03	NM_008337	Ifng	Interferon gamma
D04	NM_010512	Igf1	Insulin-like growth factor 1
D05	NM_008341	Igfbp1	Insulin-like growth factor binding protein 1
D06	NM_010548	Il10	Interleukin 10
D07	NM_008361	Il1b	Interleukin 1 beta
D08	NM_031168	Il6	Interleukin 6
D09	NM_010568	Insr	Insulin receptor
D10	NM_010570	Irs1	Insulin receptor substrate 1

APPENDIX 4 – Continuation

Panel gene list

Position	RefSeq Number	Symbol	Description
D11	NM_010700	Ldlr	Low density lipoprotein receptor
D12	NM_010704	Lepr	Leptin receptor
E01	NM_008509	Lpl	Lipoprotein lipase
E02	NM_011949	Mapk1	Mitogen-activated protein kinase 1
E03	NM_016700	Mapk8	Mitogen-activated protein kinase 8
E04	NM_021455	Mlxipl	MLX interacting protein-like
E05	NM_020009	Mtor	Mechanistic target of rapamycin (serine/threonine kinase)
E06	NM_001033305	Ndufb6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6
E07	NM_008689	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105
E08	NM_009473	Nr1h2	Nuclear receptor subfamily 1, group H, member 2
E09	NM_013839	Nr1h3	Nuclear receptor subfamily 1, group H, member 3
E10	NM_009108	Nr1h4	Nuclear receptor subfamily 1, group H, member 4
E11	NM_028994	Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
E12	NM_013743	Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4
F01	NM_008839	Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
F02	NM_001024955	Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
F03	NM_013631	Pklr	Pyruvate kinase liver and red blood cell
F04	NM_026438	Ppa1	Pyrophosphatase (inorganic) 1
F05	NM_011144	Ppara	Peroxisome proliferator activated receptor alpha
F06	NM_011145	Ppard	Peroxisome proliferator activator receptor delta
F07	NM_011146	Pparg	Peroxisome proliferator activated receptor gamma
F08	NM_008904	Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
F09	NM_001013367	Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit
F10	NM_011201	Ptpn1	Protein tyrosine phosphatase, non-receptor type 1
F11	NM_011255	Rbp4	Retinol binding protein 4, plasma
F12	NM_011305	Rxra	Retinoid X receptor alpha
G01	NM_009127	Scd1	Stearoyl-Coenzyme A desaturase 1
G02	NM_008871	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, member 1
G03	NM_009512	Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5
G04	NM_011400	Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1
G05	NM_031197	Slc2a2	Solute carrier family 2 (facilitated glucose transporter), member 2
G06	NM_009204	Slc2a4	Solute carrier family 2 (facilitated glucose transporter), member 4
G07	NM_007707	Socs3	Suppressor of cytokine signaling 3
G08	NM_011480	Srebf1	Sterol regulatory element binding transcription factor 1
G09	NM_033218	Srebf2	Sterol regulatory element binding factor 2
G10	NM_011486	Stat3	Signal transducer and activator of transcription 3
G11	NM_013693	Tnf	Tumor necrosis factor
G12	NM_013842	Xbp1	X-box binding protein 1
H01	NM_007393	Actb	Actin, beta
H02	NM_009735	B2m	Beta-2 microglobulin
H03	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H04	NM_010368	Gusb	Glucuronidase, beta
H05	NM_008302	Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class B member 1
H06	SA_00106	MGDC	Mouse Genomic DNA Contamination
H07	SA_00104	RTC	Reverse Transcription Control
H08	SA_00104	RTC	Reverse Transcription Control
H09	SA_00104	RTC	Reverse Transcription Control
H10	SA_00103	PPC	Positive PCR Control
H11	SA_00103	PPC	Positive PCR Control
H12	SA_00103	PPC	Positive PCR Control

APPENDIX 4 – Continuation

Figure 1 - Chow diet composition.

2018

ENVIGO

Teklad Global 18% Protein Rodent Diet

Product Description- 2018 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. 2018 does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2018C) and irradiated (2918). For autoclavable diet, refer to 2018S (Sterilizable) or 2018SX (Extruded & Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B_{12} supplement, folic acid, biotin, vitamin D_3 supplement, cobalt carbonate.

Standard Product Form: Pellet

Macronutrients		
Crude Protein	%	18.6
Fat (ether extract) [a]	%	6.2
Carbohydrate (available) [b]	%	44.2
Crude Fiber	%	3.5
Neutral Detergent Fiber [c]	%	14.7
Ash	%	5.3
Energy Density [d]	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.4
Glutamic Acid	%	3.4
Alanine	%	1.1
Glycine	%	0.8
Threonine	%	0.7
Proline	%	1.6
Serine	%	1.1
Leucine	%	1.8
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.0
Tyrosine	%	0.6
Methionine	%	0.4
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	1.0
Tryptophan	%	0.2

Vitamins		
Vitamin A [e, f]	IU/g	15.0
Vitamin D_3 [e, g]	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K_3 (menadione)	mg/kg	50
Vitamin B_1 (thiamin)	mg/kg	17
Vitamin B_2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B_6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.7
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.2
C18:2ω6 Linoleic	%	3.1
C18:3ω3 Linolenic	%	0.3
Total Saturated	%	0.9
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	3.4
Other		
Cholesterol	mg/kg	--

[a] Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

[b] Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

[c] Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

[d] Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

[e] Indicates added amount but does not account for contribution from other ingredients.

[f] 1 IU vitamin A = 0.3 µg retinol

[g] 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

0915

Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

APPENDIX 4 – Continuation

Figure 2 – HFHS diet composition.

Description of the high-fat high-sucrose HFHS (casein) diet

Ingredients	g/100g	% of diet	Total amount (g)	g/10kg	Energy provided (kcal/100g)	Energy (%)
Casein high nitrogen*	20.00	0.2247	33 258.4	2 247.2	80.0	14.7
L-cystine	0.18	0.0018	266.4	18.0	1.6	0.3
Sucrose FCC	26.89	0.2689	36 178.8	2 444.5	107.6	19.7
Alphacel non nuritive bulk Peser sous la hotte (très volatil)	5.00	0.0500	7 400.0	500.0		
Mineral mix	6.70	0.0670	9 916.0	670.0	ils sont	
Vitamin mix	1.40	0.0140	2 072.0	140.0	gardés au frigo	
Lard Laisser dans le bécher	19.80	0.1980	29 304.0	1 980.0	178.2	32.7
Corn oil	19.80	0.1980	29 304.0	1 980.0	178.2	32.7
Choline bitartrate	0.20	0.0020	296.0	20.0		
Tert-butylhydrytoluene (BHT)	0.03	0.0003	4.4	~~0.3~~ 3.0		
Total	100.00	1	148 000	10 000	545.6	100

APPENDIX 5

Information for Members of Referee Conmission Doctorate

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Secretaria de Pós-Graduação

Informações para os Membros de Bancas Julgadoras de Mestrado/Doutorado

1. O candidato fará uma apresentação oral do seu trabalho, com duração máxima de trinta minutos.

2. Os membros da banca farão a argüição oral. Cada examinador disporá, no máximo, de trinta minutos para argüir o candidato, exclusivamente sobre o tema do trabalho apresentado, e o candidato disporá de trinta minutos para sua resposta.

2.1 Com a devida anuência das partes (examinador e candidato), é facultada a argüição na forma de diálogo em até sessenta minutos por examinador.

3. A sessão de defesa será aberta ao público.

4. Terminada a argüição por todos os membros da banca, a mesma se reunirá reservadamente e expressará na ata (relatório de defesa) a aprovação ou reprovação do candidato, baseando-se no trabalho escrito e na argüição.

4.1 Caso algum membro da banca reprove o candidato, a Comissão Julgadora deverá emitir um parecer a ser escrito em campo exclusivamente indicado na ata.

4.2 Será considerado aprovado o aluno que obtiver aprovação por unanimidade ou pela maioria da banca.

5. Dúvidas poderão ser esclarecidas junto à Secretaria de Pós-Graduação: pgfarma@usp.br, (11) 3091 3621.

São Paulo, 23 de maio de 2014.

Prof. Dr. Adalberto Pessoa Junior
Presidente da CPG/FCF/USP

Av. Prof. Lineu Prestes, 580, Bloco 13 A - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (11) 3091 3621 - Fax (11) 3091 3141 – e-mail: pgfarma@usp.br

APPENDIX 6

Ethical Committee of the School of Pharmacy at the University of São Paulo

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Investigação metabolômica da ingestão de suco de grumixama (Eugenia brasilienses Lam.)

Pesquisador: Neuza Mariko Aymoto Hassimotto

Área Temática:

Versão: 2

CAAE: 17449613.8.0000.0067

Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo

Patrocinador Principal: CONS NAC DE DESENVOLVIMENTO CIENTIFICO E TECNOLOGICO

DADOS DO PARECER

Número do Parecer: 441.894

Data da Relatoria: 30/09/2013

Apresentação do Projeto:

O presente projeto pretende desenvolver um estudo da ingestão de alimentos fontes de flavonoides e, para tanto, se utilizará de uma investigação metabolômica para obter resultados e inferir acerca da hipótese de que esses alimentos possam vir de fato a trazer benefícios à saúde. Para tanto, selecionou-se, como alimento fonte de flavonoides e em especial de antocianinas, o suco do fruto da grumixama (Eugenia brasilienses Lam.), planta pertencente a família das Myrtaceaes.

Objetivo da Pesquisa:

Como principal objetivo visa-se identificar possíveis alterações no metaboloma, em fluidos biológicos decorrentes da ingestão a curto e a longo prazos do suco de grumixama (Eugenia brasilienses Lam.), alimento fonte de antocianinas, em modelo humano. Identificar potenciais biomarcadores da ingestão do suco de grumixama; Identificar alterações no metaboloma e correlacioná-los com possíveis pontos de regulação.

Avaliação dos Riscos e Benefícios:

Devido a coleta de sangue, os participantes poderão sentir algum desconforto provocado pela picada da agulha no local da punção e pessoas mais sensíveis poderão ficar com a pele arroxeada (pequeno hematoma) por até 48 h. Sendo assim, os riscos são considerados como mínimos.

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

APPENDIX 6 – Continuation

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Continuação do Parecer: 441.894

Comentários e Considerações sobre a Pesquisa:

A pesquisa é inovadora e permitirá avançar o conhecimento sobre substâncias bioativas naturais em alimentos, assim como evidenciar seu potencial para redução do risco de doenças.

Considerações sobre os Termos de apresentação obrigatória:

Adequado.

Recomendações:

Tendo em vista as considerações acima, este CEP entende que o projeto pode ser aprovado.

Conclusões ou Pendências e Lista de Inadequações:

Tendo em vista as considerações acima, este CEP entende que o projeto pode ser aprovado.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Tendo em vista as considerações acima, este CEP recomenda a aprovação do projeto.

SAO PAULO, 31 de Outubro de 2013

Assinador por:
Mauricio Yonamine
(Coordenador)

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

APPENDIX 7

Laval University Animal Ethics Committee

UNIVERSITÉ LAVAL

Demande de modification mineure à un protocole autorisé (VRR-10M)
Comité de protection des animaux
Philippe St-Pierre

2015-06-12

Ce formulaire standardisé doit être utilisé pour soumettre une demande de modification mineure que vous souhaitez apporter à un protocole autorisé et actif. Aucune modification ne peut être effectuée sans l'autorisation du CPA. Notez que pour apporter toute modification majeure (changement d'orientation du projet, changement pouvant entraîner un niveau de douleur accru, changement important dans les manipulations tel que l'ajout d'une chirurgie à un protocole non chirurgical, l'ajout d'une chirurgie majeure, etc.), vous devez déposer une nouvelle demande VRR-10 - Demande d'autorisation d'utiliser des animaux vivants au CPA.

Voir la MOS 094 sur le site https://oraweb.ulaval.ca/pls/vrr/sweb_dsv.html?p_ordre=cat

Nom du chercheur : Philippe St-Pierre

Numéro de demande autorisée : 2012129-3

Date de transmission : 2014-12-02

Modification mineure demandée :

- ☒ Nouveau personnel associé
- ☐ Prolongation de protocole
- ☐ Ajout d'une nouvelle lignée
- ☐ Ajout d'animaux

Modification des manipulations ou des procédures :

- ☐ Substances administrées
- ☐ Prélèvements sanguins
- ☒ Autres modification

Nouveau personnel associé

Nom	Statut	Téléphone	Manipulation	Suivi Santé	Module 1
Luciane De Lira	stagiaire	418-656-8711, 3285	☒	☒	☒

Autre modification

Décrire en détail toute autre modification des manipulations ou des procédures (ex : méthode d'euthanasie modifiée, ajout d'une séance d'imagerie, etc.) :

Changement d'animalerie afin que les expériences ailles lieu au centre de recherche de l'IUCPQ.

Animaux impliqués

Nombre	Espèce	Lignée
48	Souris	C57

Suivi et points limites

Veuillez décrire en détail les changements apportés au suivi de santé des animaux suite à l'application de cette modification.
Décrire s'il y a lieu les nouveaux points limites :

Le même suivi et les mêmes points limites que prévu au protocole autorisé seront observés.

Justification

Veuillez expliquer les raisons pour lesquelles cette modification est requise dans le cadre de ce protocole :

Cette partie du protocole ne requiert pas de mesures en cages métaboliques. De plus, comme il y a des travaux de prévus aux pavillons des services, nous préférons effectuer cette partie du protocole au centre de recherche de l'IUCPQ.

Luciane sera l'étudiante responsable de ce protocole.

APPENDIX 7 – Continuation

UNIVERSITÉ LAVAL

Demande de renouvellement d'autorisation d'utiliser des animaux vivants (VRR-10R)
Comité de protection des animaux
Philippe St-Pierre

2015-06-23

CERTIFICAT DE BONS SOINS AUX ANIMAUX

IDENTIFICATION

Titulaire du projet :
André Marette

Unité de recherche :
Médecine, Faculté de Médecine

Adresse :
CRIUCPQ, Pav M. D'Youville local Y4308
2725 ch. Ste-Foy
Québec, Qc G1V 4G5

Titre du projet :
Étude d'extraits de fruits aux effets anti-obésité et anti-résistance à l'insuline dans un modèle de souris obèse sur diète HFHS

Organisme subventionnaire :
Agence Universitaire de la Francophonie (AUF)

Le Comité de protection des animaux de l'Université Laval (CPAUL) ou du Centre hospitalier universitaire de Québec (CPA-CHUQ) certifie, conjointement avec le titulaire du permis, que les animaux utilisés pour ce projet seront traités conformément aux principes énoncés par le Conseil canadien de protection des animaux dans son document intitulé "Manuel sur le soin et l'utilisation des animaux d'expérimentation, volume I et II", ainsi que selon les directives additionnelles émanant du CPA.

USAGE RÉSERVÉ AU CPA

☒ CPAUL ☐ CPA-CHUQ

Numéro d'autorisation :	2012129-3
Catégorie d'inconfort :	D
But de l'utilisation des animaux :	1
Période de validité :	2014/10/06 au 2015/10/06

André Marette	Rachel Gerard
Titulaire du permis	Président du CPAUL ou CPA-CHUQ
2016-04-07	2016/04/02
Date	Date

APPENDIX 7 – Continuation

UNIVERSITÉ LAVAL

Demande de modification mineure à un protocole autorisé (VRR-10M)
Comité de protection des animaux
Philippe St-Pierre

2015-06-23

Signatures

Identification

Nom du chercheur : Philippe St-Pierre
Numéro de demande autorisée : 2012129-3
Date de transmission :2014-12-02

Titulaire du permis

Signature du chercheur : Julie Morette Date : 2016-04-02

Autorisation du CPA

Signature du président : Rachel Gervais Date : 2016/04/02

APPENDIX 8

Term of Ethical Committee of Research with Animals - USP

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF **35**.2016

CIÊNCIA

A Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, em 19/02/2016, tomou ciência da pesquisa **Procura de terapias alternativas contra o desenvolvimento de disfunções associadas à síndrome metabólica: obesidade e resistência insulínica**, conforme aprovada pela **Université Laval – CPAUL (Canadá)** (CPAUL 2012-129-3), conforme certificado assinado em 02/04/2014.

Orientador(a): **Profa. Dra. Neuza Mariko Aymoto Hassimotto**
Pesquisador(a): **Luciane de Lira Teixeira**

São Paulo, 30 de maio de 2016.

Prof. Dr. Joilson de Oliveira Martins
Coordenador da CEUA/FCF/USP

Av. Prof. Lineu Prestes, 580, Bloco 13 A, Cidade Universitária, CEP 05508-900, São Paulo, SP
Telefone: (11) 3091-3622 - e-mail: ceuafcf@usp.br

APPENDIX 9

Curriculum Lattes

Currículo Lattes

Imprimir currículo

Luciane de Lira Teixeira

Endereço para acessar este CV:http://lattes.cnpq.br/0076797197805817

Última atualização do currículo em 01/02/2016

Resumo informado pelo autor

Possui graduação em Farmácia pela Universidade Federal do Rio Grande do Norte (2010) e mestrado em Química pela Universidade Federal do Rio Grande do Norte (2012). Atualmente é doutoranda em Ciência dos Alimentos na Universidade de São Paulo. Atua nas áreas de análise de alimentos, compostos bioativos, metabolômica aplicada a nutrição experimental e no estudo da obesidade e resistência insulínica em modelo animal. Possui experiência em análises utilizando técnicas de LCMS e CGMS, bem qPCR, e qPCR MicroArray.

(Texto informado pelo autor)

Dados pessoais

Nome Luciane de Lira Teixeira

Nascimento 29/10/1985 - Natal/RN - Brasil

CPF 058.604.124-95

Formação acadêmica/titulação

2012 Doutorado em Ciências dos Alimentos.
Universidade de São Paulo, USP, Sao Paulo, Brasil
com período sanduíche em Université Laval (Orientador : André Marette)
Título: Investigação Metabolômica da Ingestão de Alimento Fonte de Antocianinas
Orientador: Neuza Mariko Aymoto Hassimotto
Co-orientador: Silas G. Villas-Bôas
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2011 - 2012 Mestrado em Química.
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brasil
Título: Análises Bromatológicas e Fitoquímicas em Frutos de Licania Tomentosa (Benth) Fritsch, Ano de obtenção: 2012
Orientador: Maria de Fátima Vitória de Moura
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2006 - 2010 Graduação em Farmacia.
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brasil
Título: ANÁLISES BROMATOLÓGICAS E CONTROLE MICROBIOLÓGICO DE AMOSTRAS DE CHOURIÇO DOS MUNICÍPIOS DE CAICÓ, JARDIM DO SERIDÓ E SÃO JOSÉ DO SERIDÓ
Orientador: Maria de Fátima Vitória Moura

2004 - 2005 Ensino Profissional de nível técnico .
Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Norte, IFRN, Natal, Brasil

Formação complementar

2011 - 2011 Formação Continuada do P. Capacitação Pedagógica. . (Carga horária: 45h).
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brasil

2011 - 2011 Formação Inicial do P. Capacitação Pedagógica. . (Carga horária: 30h).
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brasil

2009 - 2010 Curso de curta duração em VII E VIII CURSO DE VIVÊNCIA NO SUS:VER-SUS. (Carga horária: 80h).
Hospital Universitário Ana Bezerra, HUAB, Brasil

2008 - 2009 Extensão universitária em CONSCIENTIZAÇÃO NA COMUNIDADE DE TANQUES/RN. (Carga horária: 320h).
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brasil

2008 - 2008 Extensão universitária em DIALOGICIDADE NA EXTENSÃO: AS INTERFACES DA PRÁXIS. (Carga horária: 9h).
Universidade Federal do Rio Grande do Norte, UFRN, Natal, Brasil

2007 - 2007 Curso de curta duração em VII E VIII CURSO DE VIVÊNCIA NO SUS:VER-SUS. (Carga horária: 80h).
Hospital Universitário Ana Bezerra, HUAB, Brasil

2006 - 2006 Curso de curta duração em Extração Líquido-Líquido de Proteínas. (Carga horária: 4h).
Sociedade Brasileira de Bioquímica e Biologia Molecular, SBBQ, Sao Paulo, Brasil

2006 - 2006 Curso de curta duração em Francês nível 5. (Carga horária: 72h).
Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Norte, IFRN, Natal, Brasil

Atuação profissional

1. Universidade de São Paulo - USP

Vínculo institucional

2012 - Atual Vínculo: Bolsista , Enquadramento funcional: Doutoranda, Regime: Parcial

2. Université Laval - ULAVAL

APPENDIX 9 - Continuation

Vínculo institucional

2014 - 2015 Vínculo: Bolsista , Enquadramento funcional: Bolsista doutorado sandüiche (CAPES), Regime: Parcial

3. The University of Auckland - U.AUCKLAND

Vínculo institucional

2014 - 2014 Vínculo: Estudante visitante , Enquadramento funcional: Estudante visitante, Regime: Parcial

Producão

Produção bibliográfica

Artigos completos publicados em periódicos

1. doi **TEIXEIRA, LUCIANE LIRA**; BERTOLDI, FABIANO CLEBER; LAJOLO, FRANCO MARIA; HASSIMOTTO, NEUZA MARIKO AYMOTO
Identification of Ellagitannins and Flavonoids from Eugenia brasiliensis Lam. (Grumixama) by HPLC-ESI-MS/MS. Journal of Agricultural and Food Chemistry. JCR , v.63, p.150520055053003 - 5417, 2015.
Citações a partir de 1996 Citações a partir de 1996 Citações a partir de 1996

2. doi SESTARI, IVAN; ZSÓGÓN, AGUSTÍN; REHIDER, GABRIEL GARCIA; **TEIXEIRA, LUCIANE DE LIRA**; HASSIMOTTO, NEUZA MARIKO AYMOTO; PURGATTO, EDUARDO; BENEDITO, VAGNER AUGUSTO; PERES, LÁZARO EUSTÁQUIO PEREIRA
Near-isogenic lines enhancing ascorbic acid, anthocyanin and carotenoid content in tomato (Solanum lycopersicum L. cv Micro-Tom) as a tool to produce nutrient-rich fruits. Scientia Horticulturae. JCR , v.175, p.111 - 120, 2014.
Citações a partir de 1996 Citações a partir de 1996 Citações a partir de 1996

3. TEIXEIRA, L. L.; CARNEIRO, S.E.R.; EMERENCIANO, D.P.; SILVA, H.F.O.; CARVALHO, G.C.; DANTAS, A.S.; DANTAS, M.I.; MOURA, M.F.V.
ANÁLISES BROMATOLÓGICAS EM CHOURIÇO DO RIO GRANDE DO NORTE. Química no Brasil. , v.3, p.109 - 112, 2009.
Citações a partir de 1996 Citações a partir de 1996 Citações a partir de 1996

Trabalhos publicados em anais de eventos (resumo)

1. **TEIXEIRA, L. L.**; LAJOLO, FRANCO MARIA; HASSIMOTTO, NEUZA MARIKO AYMOTO
Bioavailability of anthocyanins and ellagitannins metabolites following acute intake of a polyphenol-rich juice of grumixama (Eugenia brasiliensis Lam) In: 7 th International Congress of Polyphenols and Health, 2015, Tours-France.
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1. Fornari, I. S.; TORRES, K; TRIGUEIRO, A. S.; REGO, A. A. F.; OLIVEIRA, D.N.; Melo, F. M.; MOURA, J.S.; **TEIXEIRA, L. L.**
Diagnóstico Turístico do Município de Guamaré. Cadernos Temáticos. Brasília - Brasil, p.68 - 69, 2005.

Página gerada pelo sistema Currículo Lattes em 29/08/2016 às 08:33:18.

APPENDIX 10 - Continuation

Student Record

29/08/2016

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9131 - 8215564/1 - Luciane de Lira Teixeira

Email:	llirat@usp.br
Data de Nascimento:	29/10/1985
Cédula de Identidade:	RG - 002.340.211 - RN
Local de Nascimento:	
Nacionalidade:	
Graduação:	Farmacêutico - Universidade Federal do Rio Grande do Norte - Rio Grande do Norte - Brasil - 2010
Mestrado:	Mestre em Química (1) - Universidade Federal do Rio Grande do Norte - Rio Grande do Norte - Brasil - 2012

Curso:	Doutorado
Programa:	Ciência dos Alimentos
Área:	Bromatologia
Data de Matrícula:	04/09/2012
Início da Contagem de Prazo:	04/09/2012
Data Limite para o Depósito:	05/09/2016
Orientador:	Prof(a). Dr(a). Beatriz Rosana Cordenunsi Lysenko - 04/09/2012 até 11/02/2014. Email: hojak@usp.br
Co-orientador:	Prof(a). Dr(a). Silas Granato Villas Boas - 12/02/2014 até o presente. Email:
Orientador:	Prof(a). Dr(a). Neuza Mariko Aymoto Hassimotto - 12/02/2014 até o presente. Email: aymoto@yahoo.com
Proficiência em Línguas:	Inglês, Aprovado em 04/09/2012
Data de Aprovação no Exame de Qualificação:	Aprovado em 19/08/2014
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 04/09/2012

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 18/07/2016
Impresso em: 29/08/2016 02:55:25

Janus - Sistema Administrativo da Pós-Graduação

29/08/2016

Faculdade de Ciências Farmacêuticas

Documento sem validade oficial

FICHA DO ALUNO

9131 - 8215564/1 - Luciane de Lira Teixeira

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBA5842-3/1	Fisiologia, Bioquímica e Bioquímica Molecular Pós-Colheita I	13/09/2012	17/10/2012	45	3	100	A	N	Concluída
FBA5871-7/3	Análise de Vitaminas em Alimentos: Aspectos Atuais	24/10/2012	04/12/2012	90	6	100	A	N	Concluída
FBA5896-6/2	Tópicos em Ciência dos Alimentos e Nutrição II	25/02/2013	05/05/2013	30	2	100	A	N	Concluída
FBC5722-2/2	Controle Hormonal da Resposta Inflamatória	06/03/2013	26/03/2013	60	4	100	A	N	Concluída
QFL5944-2/1	Métodos Avançados em Quimiometria (Instituto de Química - Universidade de São Paulo)	19/03/2013	10/06/2013	120	0	-	-	N	Turma cancelada
FBC5780-2/1	Análise de Dados Aplicados às Pesquisas Biológicas	20/05/2013	30/06/2013	90	0	-	-	N	Pré-matrícula indeferida
FBA5900-2/1	Biomodelos Experimentais Aplicados na Avaliação de Alimentos	11/06/2013	01/07/2013	30	2	100	A	N	Concluída
QBQ5749-7/8	Redação Científica (Instituto de Química - Universidade de São Paulo)	15/08/2013	25/09/2013	30	0	-	-	N	Pré-matrícula indeferida
FBC5771-7/2	Bioinformática Aplicada às Análises Clínicas	02/06/2014	15/06/2014	60	0	-	-	N	Matrícula cancelada
FBA5728-3/10	Aprimoramento Didático	19/08/2014	15/09/2014	60	4	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	21
Estágios:			
Total:	0	20	21

Créditos Atribuídos à Tese: 167

Observações:

1) Curso com validade nacional, de acordo com o disposto na Portaria nº 524, de 29.04.2008..

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 18/07/2016

Impresso em: 29/08/2016 02:55:25