UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

GUSTAVO ARAUJO PEREIRA

MUTAMBA (*Guazuma ulmifolia* Lam.) FRUIT AS A POTENCIAL SOURCE OF NATURAL BIOACTIVE AND HIGH ADDED-VALUE COMPOUNDS

FRUTO MUTAMBA (*Guazuma ulmifolia* Lam.) COMO UMA POTENCIAL FONTE DE COMPOSTOS BIOATIVOS NATURAIS E DE ALTO VALOR AGREGADO

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Thesis presented to the School of Food Engineering of the University of Campinas in partial fulfilment of the requirements for the degree of Doctor in Food Science.

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciência de Alimentos.

Orientadora: Profª Drª Gláucia Maria Pastore

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RESUMO

A preocupação dos consumidores com a saúde humana e questões ambientais tem levado a indústria alimentícia a substituir ingredientes sintéticos ou de origem animal por compostos naturais e sustentáveis de plantas, o que representa um grande desafio no desenvolvimento de novos ingredientes alimentares. O fruto mutamba (*Guazuma ulmifolia* Lam.) tem sido usada na América Latina como alimento e medicamento tradicional para o tratamento de doenças, como distúrbios gastrointestinais e cardiovasculares. Os levantamentos etnofarmacológicos identificaram o potencial dessa fruta como alimento e material terapêutico à base de plantas. No entanto, o fruto mutamba não tem sido amplamente explorado pela indústria de alimentos devido à falta de estudos sobre a sua composição fitoquímica e propriedades biológicas e tecnológicas. Os usos populares da mutamba indicam que essa fruta pode ser uma fonte promissora de compostos bioativos e de alto valor agregado, que podem ser explorados pelas indústrias alimentícia e farmacêutica. Nesta abordagem, a presente tese tem como objetivo estudar a recuperação de compostos funcionais com alto valor agregado a partir do fruto mutamba (polpa e sementes), nomeadamente os constituintes mucilaginosos das sementes e os compostos fenólicos da fruta (polpa e sementes) empregando tecnologia ultrassônica. A mucilagem da semente de mutamba foi extraída e suas propriedades químicas e tecnológicas (atividades emulsificante e espessante) foram estudadas. Além disso, o perfil de compostos fenólicos (fenólicos solúveis e insolúveis) dos frutos mutamba foi avaliado por cromatografia líquida acoplada à espectrometria de massas (LC-MS). O fruto mutamba apresentou elevado conteúdo de compostos fenólicos, principalmente proantocianidinas e flavonóides agliconas e glicosilados. O LC-MS identificou 26 compostos fenólicos e a procianidina C1 seguida de procianidina B2, rutina, epicatequina e hiperosídeo como os principais fenólicos. Com relação à mucilagem das sementes de mutamba, a mucilagem foi efetivamente extraída das sementes com auxílio de um ultrassom de alta intensidade (HIUS) sem alterar sua qualidade e estrutura primária e, consequentemente, sua funcionalidade. A mucilagem das sementes de mutamba (MSM) foi avaliada como agente emulsionante em emulsão óleo-em-água. Esse hidrocolóide foi capaz de estabilizar uma emulsão óleo-em-água e reteve efetivamente os compostos voláteis do óleo da casca de laranja, tal como o limoneno. A MSM foi capaz de aumentar a viscosidade da fase contínua e, consequentemente, diminuiu o movimento das gotas de óleo, o que melhorou a estabilidade da emulsão e retardou a liberação dos compostos voláteis. Adicionalmente, a capacidade da MSM de adsorver rapidamente na interface óleo-água ajudou a estabilizar a emulsão. A emulsão estabilizada por MSM apresentou melhor estabilidade e controle de liberação dos compostos voláteis do que as emulsões estabilizadas por goma arábica e amidos modificados. O fruto mutamba pode ser uma fonte natural de compostos bioativos e de alto valor agregado, especialmente compostos fenólicos e polissacarídeos. Os fenólicos do fruto mutamba podem ser usados como potenciais antioxidantes pelas indústrias alimentícia e farmacêutica e o hidrocolóide das sementes de mutamba pode ser usado como um agente emulsificante e espessante natural para preparar emulsões estáveis com alta eficiência de encapsulação de compostos voláteis.

Palavras-chave: compostos fenólicos; emulsão de óleo em água; hidrocolóide; LC-MS/MS; extração assistida por ultrassom.

ABSTRACT

The consumers' concern about human health and environmental issues has driven the food industry to replace synthetic or animal-based ingredients by natural and sustainable compounds from plants, which represent a great challenge in the development of new food ingredients. Mutamba (*Guazuma ulmifolia* Lam.) fruit has been used in Latin America as food and traditional medicine to treat diseases, such as gastrointestinal and cardiovascular disorders. The ethnopharmacological studies have evidenced the potential of this fruit as food and therapeutic plant-based material. Nevertheless, the mutamba fruit has not been extensively exploited by food industry due to the lack of studies about its phytochemical composition and biological and technological properties. The popular uses of mutamba fruit indicate that this fruit could be a promising source of bioactive and high added-value compounds, which it may be explored by food and pharmaceutical industries. In this approach, the current thesis aims to study the recovery of functional compounds with high added-value from mutamba fruits (pulp and seeds), namely the mucilaginous constituents from seeds and the phenolic compounds from fruit (pulp and seeds) by employing ultrasound technology. The mucilage from mutamba seed was extracted and its chemical and technological properties (emulsifying and thickening activities) were studied. Moreover, the phenolic compounds profile (soluble and insoluble-bound phenolics) of mutamba fruit was evaluated by using liquid chromatography coupled to tandem mass spectrometry (LC-MS). Mutamba fruit showed a high content of phenolic compounds, mainly proanthocyanidins, and aglycones and glycosylated flavonoids. LC-MS identified 26 phenolic compounds in which procyanidin C1 followed by procyanidin dimer B2, rutin, epicatechin, and hyperoside were the main phenolic compounds. With regard to mutamba seed mucilage, the mucilage was effectively extracted from seed by using high-intensity ultrasound (HIUS) without altering its quality and the primary structure and, as consequence, its functionality. The mutamba seed mucilage (MSM) was evaluated as emulsifying agent in an oil-in-water emulsion. This hydrocolloid was able to stabilize an oil-in-water emulsion and retained effectively the volatile compounds from orange peel oil, such as limonene. MSM was able to increase the continuous phase viscosity, which improved the emulsion stability and delayed the volatile compounds release by decreasing the oil droplet motion. Additionally, the capability of MSM to adsorb rapidly into the oil-water interface aided to stabilize the emulsion. The emulsion stabilized by MSM showed better stability and control release than emulsions stabilized by gum acacia and OSA modified starches. As a general conclusion, the mutamba fruit could be a natural source of bioactive and high added-value compounds, especially phenolics and polysaccharides. The phenolic compounds from mutamba fruit could be used as potential antioxidant by food and pharmaceutical industries, and the hydrocolloid from mutamba seeds can be used as a natural emulsifying and thickening agent to prepare stable emulsions with high encapsulation efficiency of volatile compounds.

Keywords: emulsion; hydrocolloids; LC-MS/MS; phenolic compounds; ultrasound.

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GENERAL INTRODUCTION

The consumers' knowledge about the impact of diet and lifestyle on human health has been encouraging the food industry to use more natural and sustainable plant-derived ingredients (McClements, Bai, & Chung, 2017). The scientific studies have proved that the daily intake of plant-derived foods, such as fruits, vegetables and teas, improves the human health and well-being. Therefore, the public has been more aware and interested in what they eat. The positive effect of plant-derived foods intake on human body has been attributed to the nutritional and phytochemical (functional compounds) composition of these foods (Santeramo et al., 2018). The food industry following this consumer trends has changed the way of processing foods and invested significant efforts to use natural and plant-based ingredients. Currently, it is easy to find a food product on the market with the functional claim. However, the development of functional-label products containing more label-friendly ingredients is a challenge (Bigliardi & Galati, 2013). The functional compounds can be extracted from different plant parts, such as seed, peel, leaf, and stem bark. These newly ingredients may require government approval (properties and safeness) before they can be used within foods, which may be expensive and time consuming. Furthermore, the food industry uses synthetic ingredients with a specific purpose, as consequence, the food technologists must find several natural compounds to replace them (McClements et al., 2017).

In this regard, an increasing number of studies have been carried out around the world aiming to evaluate the extraction, safeness, and functional and technological properties of natural compounds, especially those from plants. The by-products from food processing, such as seed and peel, and parts of non-conventional foods, including the leaves, stem bark and fruits have been evaluated (Herrero, Sánchez-Camargo, Cifuentes, & Ibáñez, 2015; Silva et al., 2018). These plant materials show high content of essential nutrients and phytochemicals, for example, phenolic compounds and dietary fibre, which can be used as food and non-food ingredients. The food industry has been increasing the demand for novel and natural ingredients from plants. In this scenario, Brazil's Biodiversity could be explored in order to attempt to find these compounds (Carocho, Morales, & Ferreira, 2015).

Brazil has the richest flora in the world counting 56,000 plant species, which represent approximately 19% of the world's flora. Interest in Brazil's flora dates back to the sixteenth century, and numerous scientists from different countries have visited the country since then to study the flora and their ethnobotanical uses (Giulietti, Harley, De Queiroz,

Wanderley, & Van Den Berg, 2005). The pharmaceutical industry has been using Brazil's flora to produce healthcare products, but the food industry has not extensively explored it yet. Brazil's biomes, namely Amazon, Caatinga, Cerrado, Atlantic Forest, Pantanal, and Pampa is home to thousands of plant species, including medicinal and otherwise useful varieties used by the native people (Oliveira et al., 2017). These species could be used as food; moreover, they may be a source of high added-value compounds with potential therapeutic, functional and technological properties.

The *Guazuma ulmifolia* Lam. (Malvaceae), popularly known as mutamba and guácimo, is a tree found in Latina America, especially in Brazil (from Amazon to Cerrado biomes) and Mexico. The native people has used mainly the bark and leaves of this specie as a traditional medicine to treat several pathological conditions, such as diarrhea, hemorrhages, fever, coughs, bronchitis, asthma, gastrointestinal pain and hypertension (Bourdy, Chāvez de Michel, & Roca-Coulthard, 2004; Bourdy, Oporto, Gimenez, & Deharo, 2004; Domínguez & Alcorn, 1985; M Heinrich, Rimpler, & Barrera, 1992; Michael Heinrich et al., 1992). Scientists have been trying to prove the ethnobotanical uses of *G. ulmifolia* Lam. by *in vitro* and *in vivo* studies. For example, the proanthocyanidins isolated from mutamba stem bark showed blood pressure regulation and antidiarrheal properties (Caballero-George et al., 2002; Hör, Heinrich, & Rimpler, 1996; Hor, Rimpler, & Heinrich, 1995). The aqueous extract of mutamba stem bark exerted anti-diabetic effects by stimulating glucose uptake (Alonso-Castro & Salazar-Olivo, 2008). Furthermore, the mutamba leaves showed gastroprotective effect in a model of acute gastric ulcer (Berenguer et al., 2007). Most of the studies carried out on this plant have focused on the stem bark and leaves due to their high concentration of phytochemical compounds, as previously reported (Caballero-George et al., 2002; Hör et al., 1996; Hor et al., 1995). Although the mutamba fruit is less studied, the traditional healers have indicated these fruits as a food and medicine. The mutamba fruit tea has been used to treat diarrhoea, haemorrhage and influenza (Michael Heinrich et al., 1992; Tene et al., 2007).

Mutamba fruit is small (average weight of 3.64 g) and black-coloured at mature stage, and the harvest occurs from August to September in Brazil (Neto & de Aguiar, 1999). Each fruit contains approximately 87 small seeds with the average weight of 0.01 g. Upon imbibition with water, the mutamba seeds liberate a viscous mucilage that envelops the seeds to form a gel-like capsule (Sobrinho & Siqueira, 2008). The fruits show low moisture content (approximately 10%), a high content of dietary fibre (approximately 30%) and a sweet pulp with a unique flavour. The mutamba fruit can be powdered to obtain a flour due to its low

moisture content, and this flour can be used as ingredient in preparation of whole wheat bread aiming to reduce the caloric value of the product (Assis et al., 2019). Moreover, the Latin Americans appreciate the tea prepared from this fruit (Michael Heinrich et al., 1992). The mucilaginous substance isolated from fruit is popularity used as clarifying agent to prepare rapadura and as ingredient in homemade preparations, such as popsicles (Velásquez, Espitia, Mendieta, Escobar, & Rodríguez, 2019).

The traditional uses of mutamba as starting material to prepare tea and mucilaginous solutions indicate that this fruit could be a novel source of bioactive and high added-value compounds. These materials may be used by food and non-food industries as ingredients. In this context, this thesis studied the recovery of functional compounds with high added-value from mutamba fruits (pulp and seeds), namely the mucilaginous constituents from seeds and the phenolic compounds from whole fruit. The phenolic compounds profile from mutamba fruit was carried out by using liquid chromatography coupled to tandem mass spectrometry (LC-MS). Moreover, the mutamba seed mucilage was extracted by using ultrasound technology and its chemical and technological properties (emulsifying and thickening activities) were evaluated. The current thesis intends to supply novel and natural compounds from *G. umilfolia* Lam. to food industry as well as stimulating the use of mutamba fruits by food and non-food industries to develop products. Furthermore, the study of this specie can promote Brazil's biodiversity conservation, which is extremely necessary due to mounting threat of deforestation.

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OBJECTIVES

General objectives

The aim of this thesis was to obtain natural bioactive and high added-value compounds from mutamba (*Guazuma ulmifolia* Lam.) fruit, namely phenolic compounds and functional polysaccharides. The following specific objectives were defined in order to achieve the overall goal of this thesis:

Specific objectives

- To assess and summarize the progress about the phytochemical and healthy properties of *Guazuma ulmifolia*.
- To present the current knowledge and perspectives about *G. ulmifolia* and its potential use as food and therapeutic plant, as well as to uncover the scientific gaps that hinder the use of mutamba as a natural source of bioactive compounds.
- To evaluate the physicochemical and proximate composition of mutamba fruit.
- To extract the soluble and insoluble-bound phenolic compounds from mutamba fruit.
- To characterize the phenolic compounds from mutamba fruit by using liquid chromatography coupled to tandem mass spectrometry (LC-MS).
- To develop a mucilage extraction protocol by using ultrasound technology.
- To evaluate the impact of High-intensity Ultrasound (HIUS) processing conditions (power and process time) on the recovery, quality, and structure of a novel mucilage from mutamba seeds.
- To evaluate the preliminary structure of the mucilage from mutamba seeds.
- To evaluate the use of mutamba seed mucilage as emulsifying agent in the oil-in-water emulsion, as well as to compare its effectiveness with other three biopolymers widely used by food industry, namely gum acacia, and Hi-cap 100 and Snow-Flake E6131, chemically modified starches (OSA-starches).

CHAPTER I

REVIEW ARTICLE

PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF MUTAMBA (*Guazuma ulmifolia* Lam.): A REVIEW

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Phytochemicals and biological activities of mutamba (*Guazuma ulmifolia* Lam.): A review

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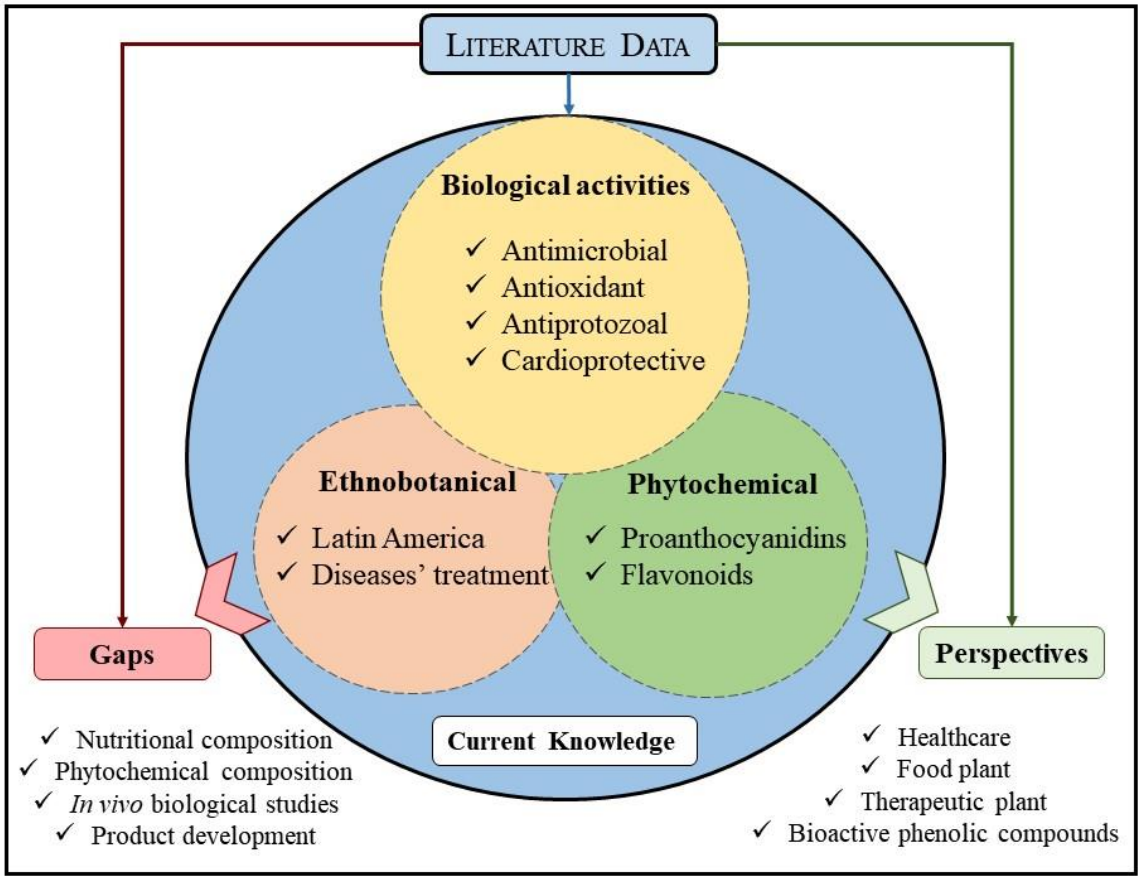
Highlights

- *Guazuma ulmifolia* Lam. contains bioactive compounds, especially proanthocyanidins and flavonoids;
- Traditional uses of *G. ulmifolia* Lam. have been proved by *in vitro* and *in vivo studies*;
- *G. ulmifolia* Lam. could be used as therapeutic plant to treat a wide broad of diseases;
- Proanthocyanidins from stem bark show dual-activity, such as antioxidant and antimicrobial activities.
- This species has potential to be exploited by industrial sector as food and therapeutic plant;

Graphical abstract:



Abstract: *Guazuma ulmifolia* Lam. (Malvaceae), popularly known as mutamba and guácimo, is a tree found in the Latina America, especially in Brazil and Mexico. Mutamba tree has been used as traditional medicine to treat several pathological conditions, such as diarrhoea, coughs, and gastrointestinal and cardiovascular disorders. Phytochemical studies on this plant have resulted in the discovery of secondary metabolites that have shown bioactivities, including antimicrobial, antiprotozoal, antioxidant, and antidiarrheal activities, and cardioprotective effect. The traditional uses of *G. ulmifolia* have been experimentally proved by *in vitro* and *in vivo* studies, in which its bioactivities were associated to its phytochemical composition, mainly proanthocyanidins dimer B and trimer C (condensed tannins). The current review assesses and summarizes the progress about the phytochemical and healthy properties of *G. ulmifolia* published until 2019. This review intends to present the current knowledge and perspectives about *G. ulmifolia* and its potential use as food and therapeutic plant.

Keywords: Malvaceae, proanthocyanidins, phenolic compounds, ethnopharmacology, bioactive compounds, medicinal plant, condensed tannins, dual-activity.

1 Introduction

The humanity has been using the natural resources, especially plants, as food and traditional medicines to treat a broad spectrum of ailments and diseases. The ethnopharmacological studies carried out worldwide have identified the use of edible and non-edible plant parts, such as fruits, leaves, flowers, stem bark, and roots as medicines to treat diseases. It has motivated the scientists to invest efforts in the study of these plants regarding their phytochemical profile and biological properties claimed by folk medicine. Interestingly, a number of compounds with bioactivities widely used by medicine have been discovered from plants, such as the digoxin from *Digitalis spp.*, atropine from *Atropa belladonna*, and cannabinoids from *Cannabis sativa*. Furthermore, the food industry has long been used functional compounds from plants as ingredient, for example, antioxidant from *Rosmarinus officinalis*, hydrocolloid from *Acacia senegal*, and curcuminoids from *Curcuma longa*. It indicates the importance of investigating the chemical composition and biological properties of plants traditionally used by people, as well as the efficacy of natural compounds as bioactive molecules (Pisoschi et al., 2018; Rates, 2001).

Guazuma ulmifolia Lam. (Malvaceae), popularly known as mutamba and guácimo, is a tree found in the Latin America, especially in Brazil and Mexico. This species has been used as traditional medicine in several countries and tribes, such as Mexico, Brazil, Bolivia,

Cuba, Honduras, Ecuador and Guatemala. The parts including leaf, flower, fruit, stem bark, and root of mutamba were reported by ethnopharmacology studies to have a broad range of medicinal properties (Al Muqarrabun & Ahmat, 2015; Navarro, Villarreal, Rojas, & Lozoya, 1996). The Latin Americans have been using the decoction of the stem bark, fruit and leaves as medicines to treat gastrointestinal and cardiovascular disorders. The traditional uses of mutamba have been proved by *in vitro* and *in vivo* studies, which demonstrated its antimicrobial, antioxidant, antiprotozoal, antidiarrheal activities, and cardioprotective effect. Furthermore, the phytochemical studies identified phenolic compounds, especially proanthocyanidins and aglycones and glycosylated flavonoids, as the main secondary metabolites of this species (Assis et al., 2019; dos Santos et al., 2018; Hör, Heinrich, & Rimpler, 1996; Hör, Rimpler, & Heinrich, 1995)

In view of the ethnobotanical uses of *Guazuma ulmifolia* Lam. and its potential as a food and therapeutic plant, a review about the traditional uses, phytochemical composition and pharmacological properties of mutamba is extremely necessary to uncover the scientific gaps that hinder the use of mutamba as a natural source of bioactive compounds. Therefore, the purpose of this review was to assess and summarize the literature about the chemical composition and health properties of *Guazuma ulmifolia* Lam. published until 2019. This review presents the current knowledge and perspectives about *Guazuma ulmifolia* Lam. and its potential use as food and therapeutic plant according to scientific evidences.

2 Literature search and eligibility criteria

A search was performed in Medline (PubMed), Scopus, Web of Science, google scholar and Orbit Intelligence for studies and patents published until 2019. We searched for articles and patents by using the following search terms (Guazuma ulmifolia OR Guazuma tomentosa OR mutamba OR guacimo) on title, abstract and keywords. No restrictions to language were made. An initial screening was performed to exclude unrelated articles due to database search mistakes, such as matching the search terms with authors names (mutamba, for example). The authors studied the related full-text articles and they manually examined the reference lists of the selected studies to complete the literature search. The authors assessed the relevance of studies by using a hierarchical approach based on title, abstract, and the full manuscript. Studies were eligible for this review if they met the following criteria: (i) studies covering the development of products, chemical composition and biological properties of *Guazuma ulmifolia* Lam.; (ii) studies published in high-quality journals that apply a selective

editorial policy and a rigorous peer review. After literature search and study selection, we included 101 studies to write this review.

3 Mutamba (*Guazuma ulmifolia* Lam.): taxonomy and botanical information

3.1 Taxonomy

The species *Guazuma ulmifolia* Lam., previously classified in Sterculiaceae family, is currently classified in Malvaceae family. This repositioning was due to phylogenetic studies (Duarte, Dranka, & Yano, 2011). The scientific classification of this species was taken from the National Inventory of Natural Heritage Database (INPN-FR, 2019).

Domain: Biota.
Kingdom: Plantae.
Subkingdom: Viridaeplantae.
Infrakingdom: Streptophyta.
Class: Equisetopsida.
Clade: Spermatophyta.
Subclass: Magnoliidae.
Superorder: Rosanae.
Order: Malvales.
Family: Malvaceae.
Subfamily: Byttnerioideae.
Tribe: Theobromeae.
Genus: *Guazuma* Mill.
Species: *Guazuma ulmifolia* Lam.

3.2 Botanical information

Guazuma ulmifolia is a semi-deciduous tree (its leaves fall after prolonged dry season) that can reach 8 to 30 m in height and 60 *cm* DBH (Diameter at breast height) (Carvalho, 2007; Neto & Aguiar, 2000; Viana et al., 2011). Its crown is considered dense and wide, presenting an umbeliform shape, with horizontal branches and leaves grouped alternately along the branches (Figure 1A). The trunk is straight to slightly tortuous and presents bifurcation (dichotomous branching) at low height. The bark presents a gray to brown, striated, rough, longitudinally cracked outer surface with easy detachment in the form of rectangular plates (Figure 1B) (Carvalho, 2007).

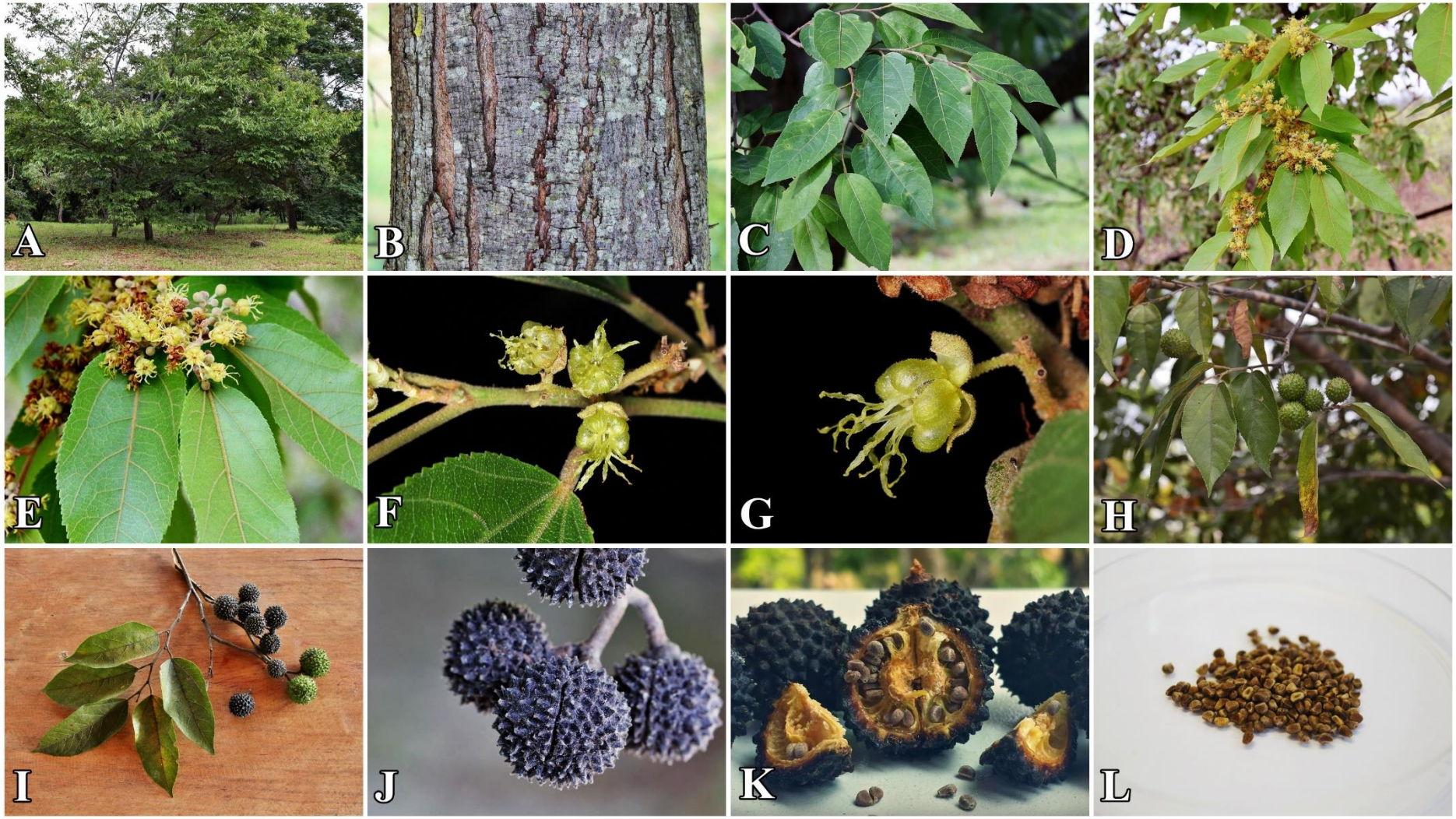


Figure 1. *Guazuma ulmifolia* Lam.. A, Tree; B, Trunk; C, Leaves; D and E, Leaves and flowers; F and G, Flower; H, Unripe fruits; I and J, Ripe fruits (black-coloured); K, Fruit cross-section; L, seeds. Author: Maurício Mercadante (pictures from A to J), Hans Rippel (picture K) and Gustavo Araujo Pereira (picture L).

The leaves show an alternate phyllotaxis, simple, oblong, oval or lanceolate phyllotaxis, 5 to 18 cm long and 2 to 6.5 cm wide, membranous, with acute or long acuminate apex, slightly serrated margin and glaba, upper surface (adaxial surface) shiny with three to five veins emerging from the base, connected to the stem by the petiole which is 0.8 to 2.5 cm long (Figure 1C) (Carvalho, 2007; Mendoza, Terán, & Monzón, 2015).

The flowers are small, hermaphrodites, diclamids, actinomorphic, pedicellate and are grouped forming dense and perfumed inflorescences (Figures D-E) (Galina, Sakuragui, Borguezam Rocha, Lorenzetti, & Palazzo de Mello, 2005; Greuter, 2000; Mendoza et al., 2015; Viana et al., 2011). The calyx (set of sepals) is trilobate, dialysepalous, cream colored, hairy, with sepals of 3 to 4 cm wide. The corolla (set of petals) is pentamera, dialypetals, with petals 4 mm long and 2 mm wide, yellow to yellowish brown when *in natura* (Figures 1 F-G) (Galina et al., 2005; Greuter, 2000; Mendoza et al., 2015). The androecium (male organ) presents 6 stamens grouped in bundle, fillets with 2 mm in length and anthers with 0.5 mm in length. The gynoecium (female organ) is about 1 mm long, presents a superior ovary, pentalocular, with several ovules per locule, and its stigma is pentalobate with the presence of nectaries. The inflorescences are racemous or paniculata, axillary, with about 10 flowers per inflorescence. The peduncle (structure that supports the inflorescence) is brown, covered by starry trichomes (pubescent) and presents from 0.6 to 1.5 cm in length (Galina et al., 2005).

The fruit of *G. ulmifolia* is considered a globular to ovoid capsule, dry, verrucous, green to black when *in natura*, weighing on average 3.64 g, with 1.5 to 4 cm in length and 2.48 cm in diameter, protected with hard tubers and opening (dehiscence) in five narrow slits connected to each other at the apex or irregularly by pores (Figures 1 H-J) (Carvalho, 2007; Flávio, 2010; Mendoza et al., 2015; Neto & Aguiar, 2000; Sobrinho & Siqueira, 2008). In the interior, the fruit is hard and dry, presenting leathery and woody structures, containing on average 87 seeds, which are surrounded by the sweet and mucilaginous mesocarp (Figure 1 K) (Carvalho, 2007; Sobrinho & Siqueira, 2008).

The seeds are ovoid, of greyish color, about 2 mm long, weighing on average 0.72 g and have two distinct layers, characterizing the outer tegument (testa) and the tegma (Figure 1 L). The embryo (part of the seed that develops during germination and gives rise to a new plant) is axial and occupies a good portion of the seed axis, without demarcation between the hypocotyl/root axis and the cotyledons. From the chemical point of view, the seeds are classified as amylaceous (Flávio, 2010; Neto & Aguiar, 2000; Sobrinho & Siqueira, 2008).

3.3 Phenology and agronomic aspects

Guazuma ulmifolia is a heliophite species indicative of mesotrophic soils widely distributed from Mexico to Northern Argentina (Barbosa & Macedo, 1993; Carvalho, 2007; Westerkamp, Soares, & Neto, 2006). In Brazil, where it is popularly known as mutamba, it can be found from the North to the South of the country (Carvalho, 2007). Due to its rapid growth and resistance, the species is highlighted in reforestation programs for the recovery of degraded areas throughout Latin America (Neto & Aguiar, 2000; Westerkamp et al., 2006).

Regarding the edaphoclimatic aspects, *G. ulmifolia* grows with average annual temperatures above 24 ºC and altitudes below 400 m, although in Brazil it can be found in altitudes varying from 15 to 1740 m. In its natural habitat, mutamba is found in areas with annual rainfall of 600 to 1500 mm, but can develop well in areas with annual rainfall of 2500 mm (Costa Filho, Nunes, Costa, Nogueira, & Costa, 2011). The species develops well in fertile, sandy textured soils with a pH greater than 5.5, but can also be found in low fertility soils, tolerates poorly compacted soils (although its development is compromised) and inhabits both dry and flooded environments (Pires, Maria, Piedade, Bart, & Ferreira, 2018).

In the nature, the dispersal of fruits and seeds occurs mainly through animals and their propagation for the production of seedlings is usually carried out sexually (through seeds). The seeds of *G. ulmifolia* have a very resistant integument which limits the absorption of water and oxygen, and for this reason, they must be treated before sowing to increase germination (Carvalho, 2007; Neto & Aguiar, 2000). Among the treatments used to increase the germinative power, it is possible to use thermal scarification (immersion of seeds in hot water at 60 ºC for 16 min) (Sobrinho, Siqueira, Morais, & Silva, 2012) or chemical scarification (immersion of the seeds in sulfuric acid 95 -98% for 50 min) (Costa Filho et al., 2011). Seeds show germination of the epigeal type and emergence of seedlings usually occurs 6 to 14 days after sowing. During the 30 days after emergence, the plant has two pairs of leaves and an average height of 5 cm. At 60 days, the plant presents three pairs of leaves and about 8 cm in height (Sobrinho & Siqueira, 2008).

In Brazil, the reproductive process of *G. ulmifolia* occurs after 5 years of age and the phenological events of flowering and fruiting variable and depend on the region where the species is found (Carvalho, 2007). In the state of Mato Grosso do Sul (Central-West region) the flowering takes place between the months of February and October. In São Paulo and Minas Gerais (Southeastern region) the flowering takes place between the months of September and December while in the North and the Northeast of the country, the flowering takes place

between the months of January and August and November and April, respectively. In the Southeastern region of the country, fruits mature between June and November while in the Northeast, fruiting occurs between July and November of the following year (Carvalho, 2007; Neto & Aguiar, 2000; Sobrinho et al., 2012; Sobrinho & Siqueira, 2008; Viana et al., 2011). Nunes et al. (2005) reported that abiotic factors such as solar radiation intensity, photoperiod (time that the plants are exposed to light during the day) and rainfall influence the phenological phases of flower buds, anthesis (floral opening), leaf fall, foliage, flowering and ripening of mutamba fruits.

G. ulmifolia is a species that has exploration potential for various purposes. For example, leaves and fruits are used as a protein source for animal feed in dry periods (Carvalho, 2007; Mendoza et al., 2015). The wood can be used for the manufacture of boxes, firewood (caloric power of 18,400 kJ kg-1 and ash content of 0.98%), charcoal, besides to cellulose and paper (the stem produces cellulose pulp with up to 44% cellulose) (Costa Filho et al., 2011; Westerkamp et al., 2006). The fruits are edible and can be used for tea, liquor and wine preparation, oil extraction for cosmetic purposes, flour production, ice cream and popsicles. In addition, the fruits provide mucilage which can be used in the making of beverages and sauces (Carvalho, 2007; Greuter, 2000; Viana et al., 2011). In State Ceará (Brazil), the mucilaginous extract obtained from the mutamba stem bark is used as a clarification agent during the artisanal manufacture of rapadura (a traditional sweet from the region produced with sugarcane juice). In Bolivia, the seeds are consumed *in natura* or cooked. The flowers are melliferous and of beekeeping importance (Carvalho, 2007; Pereira et al., 2019b).

4 Nutritional value and phytochemical compounds of *Guazuma ulmifolia* Lam.

4.1 Nutritional value of mutamba fruit

The mutamba ripe fruits showed dry matter of 83.96 g/100 g dw (dry weight), which includes protein (8.01 g/100 g dw), lipid (2.57 g/100 g dw), ashes (15.88 g/100 g dw), total fibre (49.91 g/100 g dw) and carbohydrates (23.62 g/100 g dw) (Ortega, Carranco, Mendoza, & Castro, 1998). The dry matter, protein and lipid content reported by Ortega et al. (1998) was similar to the data published by Mendoza et al. (2015), values of 81.38 g/100 g dw, 8.41 g/ 100 g dw and 2.4 g/100 g dw, respectively. However, the content of ashes (5.62 g/100 g dw) and total fibre (30.41 g/100 g dw) were lower. Rojas-Hernadez et al. (2015) reported that the mutamba fruit contains a high content of protein (8.2 g/100 g dw) and fibre (44.1 g/100 g dw). Recently, a report has described the proximate composition of mutamba fruit containing protein

(3.18 g/100 g dw), lipid (1.27 g/100 g dw), ashes (2.74 g/100 g dw), total fibre (26.90 g/100g dw), and carbohydrates (55.75 g/100g dw) (Assis et al., 2019).

The proximate composition of mutamba fruit varied, probably, due to the environmental, ecology and harvest conditions of the fruits, as well as the method used to perform the analyses. Nevertheless, the data indicate the mutamba fruit is a source of energy (247.15 Kcal/100 g dw) containing significant concentrations of proteins, lipids and carbohydrates (Assis et al., 2019). The data clearly show that the mutamba fruit contains a high content of fibre (26.90 to 49.91 g/ 100 g dw), and it may be a novel source of dietary fibre. Indeed, the ripe fruit is hard to be open due to its hardness and, consequently, it is not appropriate to consume it as fresh fruit. However, the native people appreciate the tea prepared by mutamba fruits, which have a sweet pulp and a unique flavour (Heinrich et al., 1992). Moreover, the mutamba fruit flour has recently been used as ingredient in the preparation of a whole wheat bread aiming to reduce the caloric value of the product (Assis et al., 2019). The bread added of 5% mutamba fruit flour showed a good global acceptance. The fruit (pulp and seed) has been used to prepare popsicles and ice-cream due to a viscous mucilage that is liberated from seeds upon imbibition with water (Pereira et al., 2019b; Sobrinho & Siqueira, 2008).

Mutamba is an edible fruit that has been used to prepare homemade foods due to its sensory properties. However, there are no reports on nutritional profile of this fruit addressing the composition of mineral elements, fat acid, amino acid, mono-, di- and poly-saccharides (glucose, fructose, sucrose, and starch), soluble and insoluble fibre content, and physicochemical properties. The use of mutamba fruit by Food Science and food industry in the development of products depends mainly of its nutritional and phytochemical composition. Therefore, the nutritional composition of mutamba fruit should be evaluated to provide insights on the development of new products. Moreover, the traditional uses of mutamba fruit indicate that this fruit could be valuable to the food industry, in which it could be used to prepare foods that have an impact on the health status of consumers, such as (but not limited to) tea and whole bread.

4.2 Phytochemical compounds of mutamba

Preliminary phytochemical screening revealed the presence of secondary metabolite in different *Guazuma ulmifolia* Lam. parts, such as stem bark, leaves and fruits. Phenolic compounds, namely tannins and flavonoids, and saponins, mucilages, alkaloids,

triterpenes, and steroids were detected in the stem bark (Galina et al., 2005), leaves (Iswantini, Silitonga, Martatilofa, & Darusman, 2011) and fruits (Assis et al., 2019) of *G. ulmifolia*. The total phenolic compounds (TPC), total flavonoid content (TFC), and condensed tannins content (CT) of these plant parts were spectrophotometrically determined. The mutamba fruit showed a high content of TPC (23.0 mg/g dw) and CT (12.0 mg/g dw) (Rojas-Hernandez et al., 2015). Phenolic extracts from leaves showed a high TPC and TFC content, values ranging from 78.0 to 240.0 mg/g extract and 0.81 to 32.5 mg/g extract, respectively (dos Santos et al., 2018; Morais et al., 2017). The stem bark is the plant part that showed the highest TPC (ranging from 160.0 to 469.3 mg/g extract) (dos Santos et al., 2018; Feltrin, Boligon, Janovik, & Athayde, 2012; Sereia et al., 2019), whereas the TFC (from 12.9 to 29.4 mg/g extract) was similar to the leave extracts (dos Santos et al., 2018; Feltrin et al., 2012). The preliminary phytochemical screening and spectrophotometry analysis showed that mutamba tree parts contain secondary metabolites, especially phenolic compounds.

The preliminary phytochemical data were confirmed by reports that used modern analytical techniques, such as HPLC (High-Performance Liquid Chromatograph), MS (Mass Spectrometry) and NMR (Nuclear Magnetic Resonance) to identify and structurally characterize the phytochemical compounds from *G. ulmifolia*. **Table 1** shows the phytochemical compounds present in *G. ulmifolia*.

Table 1. Phytochemical composition of mutamba (*Guazuma ulmifolia* Lam.).

Class	Compound	Plant part	Country	Sample form	Major findings	Reference
Phenolic compound	Proanthocyanidins	Stem bark	Oaxaca, Mexico	Phenolic compounds were extracted with a mixture of ethanol-water (7:3, v/v) and acetone-water (7:3, v/v). The dried-crude extracts were dissolved in water and extracted by using dichloromethane and ethyl acetate. The aqueous and ethyl acetate phases were chromatographed on a Sephadex LH-20 column to obtain different fractions containing proanthocyanidins.	- Thiolysis followed by HPLC analysis revealed proanthocyanidins, namely procyanidin dimer B2 (epicatechin-(4β→ 8)-epicatechin) and B5 (epicatechin-(4β→6)-epicatechin), procyanidin trimer C1 ([Epicatechin-(4β→8)]$_2$-epicatechin), epicatechin-[4β→8]-epicatechin-[4β→6]-epicatechin, epicatechin-[4β→6]-epicatechin-[4β→8]-epicatechin, and a procyanidin tetramer [Epicatechin-(4β→8)]$_3$-epicatechin (Cinnamtannin A2); - Polymeric compounds consisted mainly of epicatechin units; - NMR showed that the flavan-3-ol units were connected by [4β→8] bonds and, less frequently, by [4β→6] bonds.	(Hör et al., 1996)
Terpene	Volatile compounds	Leaves	Ceará, Brazil	The oil from leaves was obtained by steam distillation.	- GC-MS analysis identified 12 volatile compounds, namely precocene I (56.0%), *β*-caryophyllene (13.7%), (2*Z*,6*E*)-farnesol (6.6%), *β*-sesquiphellandrene (3.3%), (*E*)-nerolidol (2.7%), *α*-humulene (2.1%), 10-epi-*γ*-eudesmol (1.8%), bicyclogermacrene (1.6%), germacrene D (1.4%), (*Z*)-*β*-farnesene (1.3%), cis-muurola-4(14),5-diene (1.3%), and *δ*-cadinene (1.1%).	(Arriaga, Machado, Craveiro, Pouliquen, & Mesquita, 1997)
Phenolic compound	Proanthocyanidins	Stem bark	Panama City, Panama	Phenolic compounds were extracted with a mixture of acetone-water (7:3, v/v). The organic solvent was removed and the remaining aqueous phase was extracted successively with chloroform, ethyl acetate and *n*-butanol. The *n*-butanol extract was subjected to chromatography on Sephadex LH-20 column to obtain procyanidin fractions.	- MS and NMR analyses showed proanthocyanidins (procyanidin dimer B2, procyanidin trimer C1, and a procyanidin tetramer, namely Cinnamtannin A2; - Polymeric compounds consisted mainly of epicatechin units.	(Catherina Caballero-George et al., 2002)

Phenolic compound	Proanthocyanidins	Stem bark	Morelos, Mexico	Phenolic compounds were extracted with acetone. The solvent was removed and the dried-crude extract was partitioned with ethyl acetate. The insoluble part (precipitate) was dissolved in methanol-acetone (1:1, v/v). The methanol-acetone soluble part was used as procyanidin extract after drying.	- HPLC-MS analysis identified procyanidins with different degree of polymerization raging from mono- to tetrameric compounds; - Mass spectrometry showed the following compounds: epicatechin (*m/z* 291[M]$^+$), epigallocatechin (*m/z* 307[M]$^+$), procyanidin dimer B (*m/z* 579[M]$^+$), procyanidin trimmer C (*m/z* 867[M]$^+$), and procyanidin tetramer (*m/z* 1155[M]$^+$);	(Magos et al., 2008)
Phenolic compound	Proanthocyanidins	Stem bark	Paraná, Brazil	- Phenolic compounds were extracted with a mixture of acetone:water (7:3, v/v). The soluble part was collected and the solvents were removed to obtain a phenolic compound extract. - The crude extract was dissolved in water and this solution was partitioned with ethyl acetate. The ethyl acetate phase was used to perform the analysis.	- HPLC-UV/Vis analysis identified epicatechin and procyanidin dimer B2; - An HPLC-UV/Vis method was developed and validated to determinate procyanidins in *G. ulmifolia*; - The dried-crude extract was stable at the following storage conditions: 45°C, relative humidity of 75% and 21 days; - Procyanidin B2 can be used as chemical marker to monitor the quality of the *G. ulmifolia* stem bark extract.	(Lopes, Bruschi, & De Mello, 2012; Lopes, Longuini, Klein, & Mello, 2009)
Phenolic compound	Proanthocyanidins	Stem bark	Paraná, Brazil	Phenolic compounds were extracted with a mixture of acetone:water (7:3, v/v), and the solvents were removed to obtain a crude-extract. The crude-extract was dissolved in water and extracted with ethyl acetate. The ethyl acetate phase was chromatographed on a Sephadex LH-20 column to obtain different fractions containing proanthocyanidins. Fractions containing proanthocyanidins were further purified by MLCCC to obtain pure compounds.	- HPLC-UV/Vis, ESI-MS and NMR analyses identified 9 compounds, namely catechin, epicatechin, gallocatechin, epigallocatechin, procyanidin dimer B1(epicatechin-(4β→ 8)-catechin), procyanidin dimer B2, epicatechin-(4β→8)-epigallocatechin, epiafzelechin-(4β→8)-epicatechin, and 4'-O-methyl-epiafzelechin.	(Lopes, Rocha, de Almeida, & de Mello, 2009)

Coumarin and terpene	Bis-coumarins and triterpenes	Root	Rajasthan, India	Bis-coumarins and triterpenes were extracted with ethanol 95% (v/v). The solvent was removed and the dried-extract was successively extracted with petroleum ether, benzene, chloroform, and ethyl acetate. These fractions were chromatographed on a silica gel column to obtain pure compounds.	- FTIR, NMR and MS analyses identified 3,3'-methylenebis(4,6-dihydroxycoumarin), β-sitosteryl stearate, *n*-tetracosanoic acid, friedelin, friedel-1-en-3one, β-sitosterol, 29-norcycloartanol, oleanolic acid, 3-O-acetyloleanolic acid, 6-methoxy-7,8-methylenedioxycoumarin, and methyl-3-acetyloleanolate.	(Agarwal, Saxena, Jain, & Jain, 2010)
Phenolic compound	Flavanocoumarins and proanthocyanidins	Stem bark	Belize	The stem bark was extracted using petroleum ether, chloroform and methanol, sequentially. The methanol extract was chromatographed on a Sephadex LH-20 column to obtain different fractions containing phenolic compounds. These fractions were further purified by HPLC.	- ESI-MS, MALDI-MS, and NMR analysis revelled, epicatechin, flavanocoumarin (epiphyllocoumarin) and two proanthocyanidins, namely epiphyllocoumarin-[4β→8]-epicatechin and epiphyllocoumarin-[4β→8]-epicatechin-[4β→8]-epicatechin.	(Maldini et al., 2013)
Phenolic compound	Phenolic acids and flavonoids	Leaves	Ceará, Brazil	Phenolic compounds were extracted from leaves using ethanol. The solvent was removed to obtain a crude extract.	- HPLC-DAD analysis identified phenolic acids (5-*O*-caffeoylquinic acid (chlorogenic acid) and caffeic acid) and flavonoids (catechin, quercetin 3-*O*-rhamnosyl-glucoside (rutin), quercetin 3-*O*-rhamnosyl (quercitrin), quercetin and luteolin). - Chlorogenic acid (25.3 mg/g extract) and quercetin (24.6 mg/g) were the main compounds followed by rutin (10.0 mg/g), catechin (9.8 mg/g), quercitrin (8.2 mg/g), caffeic acid (7.3 mg/g), and luteolin (4.7 mg/g).	(Calixto Júnior et al., 2016; Morais et al., 2017)
Terpene and others	Volatile compounds	Leaves	Cundinamarca, Colombia	The oil from leaves was obtained by steam distillation.	- GC-MS analysis revelled 38 compounds, namely 26 sesquiterpene (46.7%), 7 monoterpene (19.6%), and 5 aliphatic hydrocarbons (1.0%); - The monoterpene β-citronelol (12.7%) and the sesquiterpene caryophyllene (11.9%) were the main volatile compounds from oil leaves; - *cis*-β-farnesene (6.0%), caryophyllene oxide (5.4%), α-farnesene (4.8%), nerolidol (3.5%), β-humulene (2.9%), citronellal (2.5%), and *cis*-β-terpineol (2.0%) were detected.	(Peláez & Rodríguez, 2016)

Terpene	Volatile compounds	Leaves	Mato Grosso, Brazil	The essential oil was extracted by hydrodistillation using a Clevenger-type apparatus.	- GC-MS identified 31 compounds, in which thymol (20.97%), carvacrol (13.76%), eugenol (10.13%), spathulenol (7.09%), β-caryophyllene (6.74%), sabinene (5.18%), globulol (5.56%), γ-terpinene (3.27) and α-copaene (3.17%) were the main compounds.	(Boligon, Feltrin, Gindri, & Athayde, 2016)
Phenolic compound	Flavonoids	Stem bark and leaves	Mato Grosso do Sul, Brazil	The aqueous extracts of stem bark and leaves of *G. ulmifolia* were prepared by decoction. The liquid was separated from insoluble reside and dried to obtain a crude extract.	- ESI-MS identified flavan-3-ols and condensed tannins in stem bark and glycosylated flavonoids in leaves; - Mass spectrometry showed the following compounds: epigallocatechin, catechin, epicatechin, and procyanidin dimer B in steam bark; and procyanidin dimer B, chlorogenic acid, epicatechin, and glycosylated derivatives of quercetin, kaempferol and luteolin in the leaves.	(dos Santos et al., 2018)
Phenolic compound	Phenolic compounds and flavonoids	Leaves	Pernambuco and Ceará, Brazil	Phenolic compounds were extracted using a mixture of ethanol:water (1:1, v/v).	- TLC analysis revelled caffeic acid, catechin and catechin derivatives; - HPLC-DAD analysis identified quercetin 3-*O*-rhamnosyl-glucoside (rutin).	(de Souza, da Silva, Bezerra, Ferreira, & Soares, 2018)
Phenolic compound	Proanthocyanidins	Stem bark	Paraná, Brazil	Phenolic compounds were extracted as described by Lopes, Rocha et al (2009).	- Capillary Electrophoresis analysis showed epiafzelechin-(4β→8)-epicatechin, epigallocatechin, epicatechin, procyanidin dimer B1, and procyanidin dimer B2.	(Sereia et al., 2019)
Carbohydrate	Mucilage	Seed	Minas Gerais, Brazil	The mucilaginous constituent of mutamba seed were extracted by using high-intensity ultrasound.	- The global yield ranged from 2.1 to 8.4 g/100 g seeds; - HPAEC-PAD and FTIR analyses showed that the mucilage extracted from *G. ulmifolia* seeds is a heteropolysaccharide composed of galactose (33.2%), rhamnose (20.6%), galacturonic acid (19.1%) and glucuronic acid (18.7%). Arabinose (0.4%) and xylose/mannose (0.1%) were found at low amount.	(Pereira et al., 2019b)

ESI-MS: Electrospray Ionization-Mass Spectrometer; **FTIR**: Fourier Transform Infrared Spectroscopy; **GC-MS:** Gas Chromatography-Mass Spectrometry; **HPAEC–PA**D: High Performance Anion Exchange Chromatography-Pulsed Amperometric Detector; **HPLC-DAD:** High-Performance Liquid Chromatography-Diode Array Detector; **HPLC-UV-Vis:** High-Performance Liquid Chromatography-UV/VIS Detector; **MALDI-MS:** Matrix-Assisted Laser Desorption Ionization- Mass Spectrometry; **MLCCC:** Multi-Layer-Coil Counter-Chromatography; **MS**: Mass Spectrometry; **NMR:** Nuclear Magnetic Resonance; **TLC:** Thin-layer Chromatography.

The studies conducted from 1996 to 2019 showed the phenolic compounds, especially proanthocyanidins, flavonoids and phenolic acids as the main class of secondary metabolites in the stem bark and leaves of mutamba. Proanthocyanidins, namely procyanidin dimer B2 and B5, epiafzelechin-(4β→8)-epicatechin, procyanidin trimer C1, epicatechin-[4β→8]-epicatechin-[4β→6]-epicatechin, epicatechin-[4β→6]-epicatechin-[4β→8]-epicatechin, and Cinnamtannin A2, and flavonoids aglycones (catechin, epicatechin, gallocatechin, epigallocatechin, and 4'-O-methyl-epiafzelechin) were identified in stem bark (Hör et al., 1996; Lopes, Rocha, et al., 2009; Magos et al., 2008). Furthermore, Maldini et al. (2013) identified a flavanocoumarin (epiphyllocoumarin) and two proanthocyanidins, namely epiphyllocoumarin-[4β→8]-epicatechin and epiphyllocoumarin-[4β→8]-epicatechin-[4β→8]-epicatechin in these plant part.

Unlike stem bark, the phytochemical studies on mutamba leaves identified phenolic acids (chlorogenic acid and caffeic acid) and flavonoids (catechin, quercetin 3-*O*-rhamnosyl-glucoside, quercetin 3-*O*-rhamnosyl, quercetin and luteolin) as main compounds, mainly chlorogenic acid (25.3 mg/g extract) and quercetin (24.6 mg/g) (Calixto Júnior et al., 2016; Morais et al., 2017). Procyanidin dimer B and glycosylated derivatives of catechin, quercetin, kaempferol and luteolin were detected in these leaves (de Souza et al., 2018; dos Santos et al., 2018). The oil from mutamba leaves showed terpenes as the main class of volatile organic compounds. As expected, the volatile compound profile of leaves varied according to the collection sites, but caryophyllene, nerolidol, humulene, germacrene D, farnesene, etc. were compounds identified in all samples, which indicates a similarity between them (Arriaga et al., 1997; Boligon et al., 2016; Peláez & Rodríguez, 2016). Cyanogenic compounds (tyrosine-derived dhurrin and taxiphyllin) were isolated from leaves of *G. ulmifolia* collected in Costa Rica, 1986. However, the cyanide test was negative when retested in 1988. The presence of these compounds was transitory because the mutamba trees had lost cyanogenic activity and, consequently, the data were inconclusive. Moreover, cyanide tests on more than 200 *G. ulmifolia* trees from Costa Rica and Mexico were uniformly negative (Seigler et al., 2005).

The phytochemical composition of mutamba root was described to contain bis-coumarins and triterpenes, namely 3,3'-methylenebis(4,6-dihydroxycoumarin), β-sitosteryl stearate, *n*-tetracosanoic acid, friedelin, friedel-1-en-3one, β-sitosterol, 29-norcycloartanol, oleanolic acid, 3-O-acetyloleanolic acid, 6-methoxy-7,8-methylenedioxycoumarin, and methyl-3-acetyloleanolate (Agarwal et al., 2010). In another study, the mucilage from mutamba seeds was extracted and its structure was evaluated. The authors showed the mutamba seed

mucilage is a heteropolysaccharide composed of galactose (33.2%), rhamnose (20.6%), galacturonic acid (19.1%) and glucuronic acid (18.7%), and its spectral signature (FTIR) was similar to rhamnogalacturonans and mucilage from *Arabidopsis thaliana* seeds (Pereira et al., 2019b).

The stem bark of *Guazuma ulmifolia* has been used as a traditional medicine to treat several diseases, which may justify the largest number of phytochemical studies on this plant part (**Table 1**). The phytochemical reports have focused on the isolation, identification and structure elucidation of phenolic compounds from stem bark, whereas the secondary metabolites of root, leaves, flower and fruit have not been extensively investigated by using modern analytical techniques. Furthermore, the literature data reveal the qualitative phytochemical profile of mutamba, while studies covering the content of individual bioactive compounds (quantitative analysis) in mutamba plant parts are currently limited. Nevertheless, the literature data clearly showed the phenolic compounds, especially proanthocyanidins, as the main secondary metabolites in *G. ulmifolia*. **Figure 2** shows the chemical structure of these secondary metabolites.

Proanthocyanidins, also known as condensed tannins, are oligomers or polymers of flavan-3-ol units (*e.g.*: catechin and epicatechin), in which they are connected by [4β→8] bonds and less frequently by [4β→6] bonds, such as the procyanidins from mutamba stem bark (Hör et al., 1996). Condensed tannins are widely distributed in plant kingdom in which they accumulate in different plants' organs and tissues to provide protection against biotic and abiotic stresses, while attracting pollinators and dispersing seeds-animals. These class of compounds from plant secondary metabolism impart astringency and flavour to foods, such as wines, teas, and fruit juices due to their capability of forming complexes, both with metal ions and with macromolecules (proteins and polysaccharides) (Shahat & Marzouk, 2013; Xie & Dixon, 2005). This capability has been explored by medicine to treat, for example, diarrhoea. The antidiarrheal activity of proanthocyanidins from mutamba stem bark was attributed because these compounds can specifically interact with the A subunit of Cholera toxin (a protein complex produced by *Vibrio cholerae*) (Hör et al., 1996, 1995). Furthermore, the proanthocyanidins have been showing a broad spectrum of bioactivities directly derived from their structure feature, such as (but not limited to) antioxidant, antiviral, antibacterial, anti-inflammatory, and enzyme-inhibitory activities, and vascular and cardiac activities (Shahat & Marzouk, 2013).

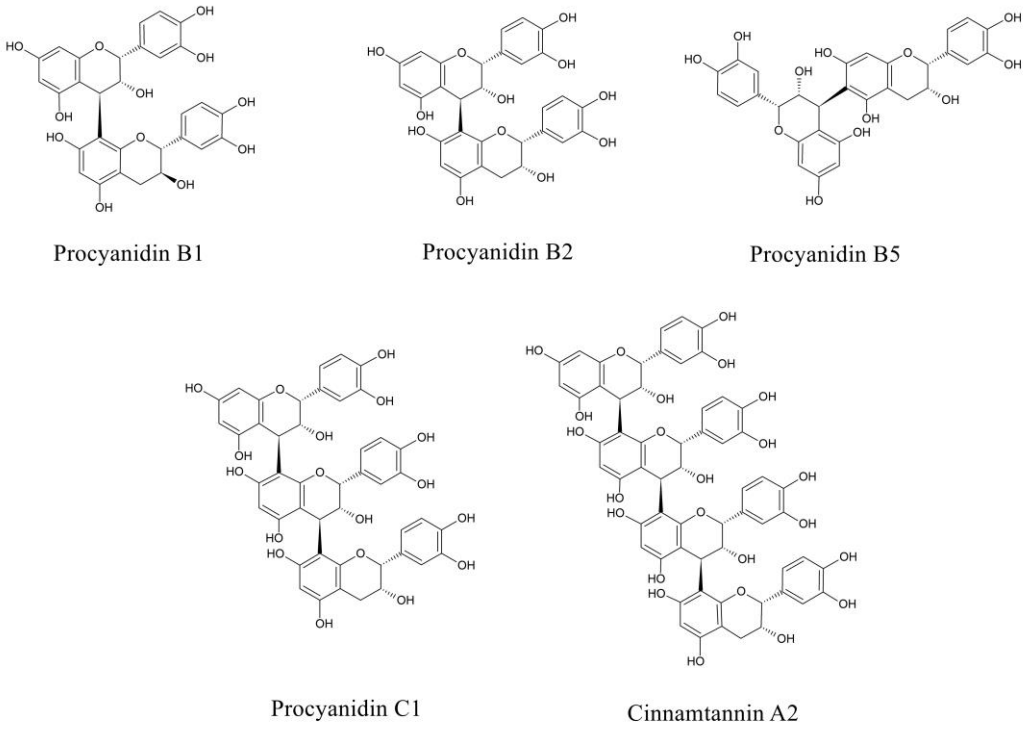


Figure 2. Structure of the main phenolic compounds identified in *Guazuma ulmifolia* Lam.. Procyanidin B1 (epicatechin-(4β→ 8)-catechin), Procyanidin B2 (epicatechin-(4β→ 8)-epicatechin), Procyanidin B5 (epicatechin-(4β→6)-epicatechin), Procyanidin C1 ([Epicatechin-(4β→8)]$_2$-epicatechin), and Cinnamtannin A2 ([Epicatechin-(4β→8)]$_3$-epicatechin). B-type procyanidins represent the dominant class of proanthocyanidins in plant kingdom. These compounds are built by a single interflavan linkage usually between C_4 of the first flavan-3-ol and C_8 ([4β→8]) or C_6 ([4β→6]) of the second unit. Procyanidins B1 and B2 differ only in the arrangement of the catechin and epicatechin, while the procyanidin B5 show a [4β→6] interflavan linkage. The procyanidin trimer C1 and tetramer A2 are both oligomers of epicatechin connected by [4β→8] bonds (Shahat & Marzouk, 2013; Xie & Dixon, 2005).

5 Biological activities of *Guazuma ulmifolia* Lam.

5.1 Traditional use of mutamba

Guazuma ulmifolia Lam. has been used as traditional medicine in several countries and tribes, such as Mexico, Brazil, Bolivia, Cuba, Honduras, Ecuador and Guatemala. The parts including leaf, flower, fruit, stem bark, and root of this species were reported by ethnopharmacology studies to have a broad range of medicinal properties (Al Muqarrabun & Ahmat, 2015; Navarro et al., 1996). Huastec Mayans of Northeastern Mexico used the stem bark to treat gastrointestinal disorders, diarrhoea, dysentery, and fever (Domínguez & Alcorn,

1985), while the Izoceño-guaraní people, a Bolivian lowland ethnic group, has used the mutamba leaf and bark decoction by oral administration to treat cough and lungs ailment with fever (Bourdy, Chāvez de Michel, & Roca-Coulthard, 2004). Moreover, the stem bark is used by the Brazilian people to treat seborrheic dermatitis, bursitis, tendonitis, and hemorrhoids, and a shampoo against scalp's parasitic diseases is produced by using this plant part (Campelo, 1988; Magalhães et al., 2019; Ricardo, Paula-Souza, Andrade, & Brandão, 2017). Stem bark decoction is used in Cuba for renal afflictions, such as renal calculus (Cano & Volpato, 2004), and in Mexico the tea prepared from mutamba stem bark and fruit is used to treat diarrhoea and hemorrhage (Heinrich, Rimpler, & Barrera, 1992; Heinrich et al., 1992). An ethnobotanical study on Guatemala plant's traditional medicine showed that the mutamba stem bark and fruit are used for diarrhoea and gastrointestinal disorders (Kufer, Förther, Pöll, & Heinrich, 2005). The leaf and stem bark are used in Honduras to treat physical discomforts, such as dental, digestive, and urinary tract aches (Lentz et al., 1998). In Mexico, the tea from the leaves is used in diabetes mellitus control (Alarcon-Aguilara et al., 1998) and an aqueous extract from stem without bark is used for the treatment of stomachache and diarrhoea (Moreno-Salazar, Robles-Zepeda, & Johnson, 2008). The aqueous infusion of mutamba fruit is used by Ecuadoreans to treat influenza (Tene et al., 2007).

Ethnopharmacological studies have shown that traditional healers have used parts of mutamba tree to treat diseases. The stem bark from *Guazuma ulmifolia* Lam. is the most used part by folk medicine, mainly to treat gastrointestinal disorders and diarrhoea. Latin Americans have been used the decoction of the stem bark, fruit and leaves as medicines. The study of plants as therapeutic agents to maintain health and to treat physical and mental illnesses is useful in the discovery and development of new drugs. The societies have resorted to nature, mainly plants, as a medical and health source. Therefore, the world population, especially in developing countries, has been using plants for facing primary needs of medical assistance. The ethnopharmacological studies indicate that the species *Guazuma ulmifolia* Lam. could be a source of compounds with pharmacological properties. As presented previously, proanthocyanidins had been detected in different parts of *G. ulmifolia*, especially in the stem bark and leaves (*see Section 4.2*). Therefore, the condensed tannins could be the precursor of the therapeutic effects of *G. ulmifolia* reported by folk medicine. In the next section, we summarized the biological activities of *G. ulmifolia* supported by scientific evidences.

5.2 Biological activities of mutamba

The traditional use and phytochemical studies on *G. ulmifolia* have been leading the scientists to investigate its pharmacological properties in order to validate its use as therapeutic plant. The extracts and pure compounds from different mutamba parts have shown several biological activities, such as antimicrobial, antioxidant, antiprotozoal and cardioprotective activities. **Table 2** shows the biological activities of *G. ulmifolia* with additional information on studies reviewed. We found out 47 studies reporting the biological activity of mutamba. The antimicrobial (40.4% of studies) and antioxidant (25.5%) activities were the bioactivities most evaluated, followed by antiprotozial (12.8%), cardioprotective (8.5%), anti-obesity (6.4%), antidiabetic (6.38%), anti-inflammatory (4.26%), anticancer (4.26%), anticholinesterase (4.26% of studies), anticholera (4.26%), insecticide (4.26%), apoptotic inductor (2.13%), neuroprotective (2.13%), gastroprotective (2.13%), antiviral (2.13%), and molluscicide (2.13%) activities. These activities were mainly tested by using *in vitro* assays (93.6% of studies), while just five studies (10.6 %) used animal model. We did not find studies regarding the therapeutic effect of mutamba on human body. The data in **Table 2** on plant part shows that the biological properties of stem bark (46.8% of studies) and leaves (46.8%) were extensively studied, whereas the fruits (6.4%), roots (2.13%) and flowers (2.13%) were less investigated. Interestingly, the phenolic compounds, mainly proanthocyanidins and flavonoids, were reported as the biological active compounds (44.7 % of studies), while 51.1% of studies did not report the related compounds to bioactivities. As a general observation, the antimicrobial, antioxidant and antiprotozoal were the bioactivities most investigated. Furthermore, the *in vivo* studies could reflect the therapeutic potential of this species on human body, since the *in vitro* studies did not take into account the biochemical, metabolic and physiological parameters, such as bioavailability and drug metabolism. We discuss below the promising bioactivities of *G. ulmifolia* according to the aforementioned literature analysis.

Table 2. Biological activities of mutamba (*Guazuma ulmifolia* Lam.).

Bioactivity	Plant part	Sample form	Method/model	Major findings	Related compounds	Reference
Anti-obesity	Leaves	Ethanolic extract	-3T3-L1 mouse preadipocytes cells	-↓Relative expression of adipogenesis-related genes (PPARγ, C/EBPα and SCD1) in 3T3-L1 cells (50 µg/mL)	-	(Hidayat et al., 2016)
Anti-obesity	Leaves	Aqueous, hydroethanolic (ethanol 70%) and saponin extracts	-*In vitro* pancreatic lipase inhibition assay and toxicity assay on shrimp larvae of *Artemia salina*	-Hydroethanolic extract showed the highest pancreatic lipase inhibitory activity (25.31% at 60 µg/mL) and the lowest toxicity (LC_{50} 1070.93 µg/mL), followed by aqueous and saponin extracts. -Inhibitory effect of hydroethanolic extract (25.31% at 60 µg/mL) was higher than positive control, Xenical®/orlistat (17.53% at 100 µg/mL).	Flavonoids and tannins	(Iswantini et al., 2011)
Antidiabetic and anti-obesity	-	Hydroethanolic extract (ethanol 60%)	-*In vitro* α-glucosidase and pancreatic lipase inhibition assays	-Inhibition of α-glucosidase (23% at 1 mg/mL) and pancreatic lipase (13.1% at 0.25 mg/mL) activities.	-	(Ramírez, Zavala, Pérez, & Zamilpa, 2012)
Antidiabetic	Stem bark	Aqueous extract	-3T3-F442A preadipocytes cells	-No anti-adipogenic or pro-adipogenic effects on 3T3-F442A adipocytes (1-70 µg/mL). -Extract stimulated glucose uptake by insulin-sensitive and insulin-resistant 3T3-F442A adipocytes.	-	(Alonso-Castro & Salazar-Olivo, 2008)
Antidiabetic	Leaves	Aqueous decoctions	-New Zealand adult male rabbits	-↓Hyperglycaemic peak (22.2%) and ↑glucose tolerance (21.2%) when	-	(Alarcon-Aguilara et al., 1998)

				administrated at 46 mg/kg body weight.		
Anti-inflammatory	Stem bark	Flavanocoumarins isolated from methanolic extract	-STAT1 protein based *in vitro* assays	-Epiphyllocoumarin and epiphyllocoumarin-[4β→8]-(–)-epicatechin showed strong affinity for STAT1 and inhibited IFN-γ induced STAT1–DNA binding.	Flavanocoumarins	(Maldini et al., 2013)
Anticancer	Leaves	Tiliroside isolated from mutamba	-*In vitro* antitumor activity against human tumour cell lines T47D and MCF7	-Tiliroside has higher antitumor activity on T47D compared to MCF7 cancer cell lines (IC_{50} 67.79 and 112.77 µg/mL, respectively). -Tiliroside induced apoptosis through extrinsic pathways by Caspases 8 and 9 activation.	Tiliroside (flavonoid)	(Dai et al., 2016)
Anticancer and antimicrobial	Stem bark	Ag, Au and Ag/Au alloy nanoparticles containing aqueous extract	-Human cervical cancer cell line (HeLa) -*In vitro* antimicrobial activity against 14 species of microbial pathogens (*Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Proteus vulgaris, Shigella dysenteriae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Trichoderma viridae, Fusarium solani, Aspergillus niger, Nigrospora oryzae, Aspergillus fumicatus* and *Candida albicans*) using the disc diffusion method	-Ag/Au alloy nanoparticles reduced the proliferation of human cervical cancer cell line (IC_{50} 24.83 µM). -Ag and Ag/Au alloy nanoparticles exhibited good antimicrobial activity on 14 species of microbial pathogens.	-	(Karthika et al., 2017)
Antimicrobial (antigonorrhoeic)	Stem bark	Hydroethanolic extract (ethanol 50%)	-*In vitro* activity against penicillin-resistant strain of *Neisseria gonorrhoeae* isolated from symptomatic patients using the disc diffusion method	-Inhibition of *Neisseria gonorrhoeae* growth (IZ = 6.0 mm at 50 mg/disk).	-	(Cáceres et al., 1995)

Antimicrobial (antituberculotic)	Stem bark and stem	Dichloromethane extract	-*In vitro* activity against *Mycobacterium tuberculosis*	-Weak antimicrobial activity (less than 50% inhibition of *M. tuberculosis* at 50 µg/mL).	-	(Graham et al., 2003)
Antimicrobial	Stem bark	Aqueous extract	-Antimicrobial activity against 11 human multi-drug resistant pathogens (*Enterococcus faecalis, Staphylococcus aureus, Staphylococcus haemolyticus, Acinetobacter baumannii, Acinetobacter lwoffii, Burkholderia cepacia, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Candida tropicalis* and *Trichosporon belgeii*) using the disc diffusion method	-Potent antimicrobial effects against *A. lwoffii, E. faecalis* and *C. albicans* (IZ > 15 mm). -Antimicrobial effect (IZ = 26 mm at 75 µg/disk) significantly higher than the positive control neomycin (IZ = 23 mm at 25 µg/disk) against *C. albicans*.	-	(Jacobo-Salcedo et al., 2011)
Antimicrobial	Stem bark	Ethanolic extract and its hexane, dichloromethane, ethyl acetate and hydromethanolic (methanol 50%) fractions	-Antimicrobial activity against 11 microorganisms (*Candida albicans, C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, Cryptococcus neoformans, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis* and *Staphylococcus aureus*) using microbroth dilution assay	-Ethanolic extract and ethyl acetate fraction were the most active against most microorganisms. -*C. krusei, C. neoformans, E. faecalis* and *S. aureus* were the strains most sensitive to tested extract and fractions (MIC ranging 62.5 to > 1000 µg/mL).	-	(Violante et al., 2012)
Antimicrobial	Leaves and stem bark	Hydroethanolic extract (ethanol 80%)	-Antimicrobial activity against 11 microorganisms (*Cryptococcus neoformans, Candida albicans, Saccharomyces cerevisiae, Aspergillus flavus, Aspergillus fumigatus, Trichophyton mentagrophytes, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Mycobacterium intracellulare*) and 6 oral	-Active against *B. subtilis, M. intracellulare, C. neoformans, C. albicans* and all oral pathogenic bacteria at 20 mg/mL.	-	(Lentz et al., 1998)

			pathogenic bacteria (*Streptococcus mutans, Streptococcus sanguis, Actinomyces viscosus, Fusobacterium nucleatum, Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*) using the disc diffusion method			
Antimicrobial	Leaves	Peptides were extracted with buffers: B1 (pH 7.0) KCl, EDTA; B2 (pH 5.2) KCl, CH_3COONa, β-mercaptoethanol; and B3 (pH 7.0) KCl, EDTA, phenylmethylsulfonyl fluoride, polyvinylpyrrolidone, thiourea, while metabolites were extracted with hexane, methanol and dichloromethane	-Antimicrobial activity against 4 phytopathogens (*Sclerotium cepivorum, Fusarium oxysporum, Xanthomonas campestris* and *Pseudomonas aeruginosa*)	-All extracts were active against at least one phytopathogen. -Dichloromethane extract showed the greatest inhibition against *S. cepivorum* (88.4% at 10000 µg/mL), B2 against *F. oxysporum* and *X. campestris* (60.0% at 10000 µg/mL and 86.5% at 78 µg/mL, respectively), and hexane extract against *P. aeruginosa* (70.0% at 625 µg/mL).	Bioactive peptides in buffer extracts and secondary metabolites in organic extracts	(Ramírez Salcedo, Virgen-Calleros, Vargas-Radillo, Salcedo-Pérez, & Barrientos-Ramírez, 2015)
Antimicrobial	Fruits	Methanolic extract	-Antimicrobial activity against 4 pathogenic strains (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*)	-Significant antimicrobial activity against all pathogenic strains (MIC ranging 10-40 mg/mL). -Extract was more active against *S. aureus* (MIC 10 mg/mL).	-	(Navarro et al., 1996)
Antimicrobial	Leaves	Hydroethanolic extract (ethanol 50%)	-Antibacterial activity against 5 enteropathogenic bacteria (*Escherichia coli, Salmonella enteritidis, Salmonella typhi, Shigella dysenteriae* and *Shigella flexneri*) using the disc diffusion method	-Extract was active only against *S. typhi* and *S. dysenteriae* (IZ ≥ 6 mm at 200 mg/mL).	-	(Caceres, Cano, Samayoa, & Aguilar, 1990)

Antimicrobial	Leaves	Hydroethanolic extract (ethanol 88%)	-Antibacterial activity against 3 Gram-positive bacteria causing respiratory infections (*Staphylococcus aureus, Streptococcus pneumoniae* and *Streptococcus pyogenes*) using the disc diffusion method	-Extract was active only against *S. pneumoniae* (IZ ≥ 6 mm at 500 mg/mL).	-	(Caceres, Alvarez, Ovando, & Samayoa, 1991)
Antimicrobial	Leaves	Methanol, acetone and hexane extracts	-Antibacterial activity against 3 enteropathogenic bacteria (*Escherichia coli, Salmonella typhi* and *Shigella flexneri*) using the disc diffusion method	-All extracts equally inhibited the 3 enteropathogenic bacteria (IZ = 6 mm at 50 mg/disk). -Methanolic extract exhibited MICD > 10 mg/disk for *S. typhi*.	-	(Cáceres et al., 1993)
Antimicrobial	Stem bark	Hexane, chloroform and methanolic extracts	-Antibacterial activity against 4 aerobic reference bacterial strains (*Escherichia coli, Pseudomonas aeruginosa Staphylococcus aureus* and *Enterococcus faecalis*) using broth microdilution method	-Antibacterial activity against *E. coli* for hexane extract (MIC 2.5 mg/mL) and *P. aeruginosa* for methanolic extract (MIC 2.5 mg/mL).	-	(Camporese et al., 2003)
Antimicrobial	Stem bark	Ethanolic extract	-Antibacterial activities against *Escherichia coli*, a susceptible strain of *Staphylococcus aureus* and two resistant strains of *S. aureus*	-Active against fluoroquinolone- and macrolide-resistant strains of *S.* aureus (↓growth by at least 10-fold at 100 µg/mL compared to negative control).	-	(de Lima et al., 2006)
Antimicrobial and antiprotozoal (amebicidal and giardicidal)	Leaves	Decoction of 2 leaves each of *Teucrium cubense, Psidium guajava, Guazuma ulmifolia* and *Senna atomaria* and a pinch of baking soda	-Antimicrobial activity against 5 bacterial strains (*Escherichia coli, Klebsiella pneumoniae, Shigella flexneri* and *Salmonella typhi*) based *in vitro* assay -Amebicidal activity against *Entamoeba histolytica* trophozoites based *in vitro* assay -Giardicidal activity against *Giardia lamblia* trophozoites based *in vitro* assay	-Formulation was active only against *S. typhi* (MIC 0.5 mg/mL). -High amebicidal (IC_{90} 139.67 µg/mL) and giardicidal (IC_{90} 769.66 µg/mL) activities.	-	(Vera-Ku et al., 2010)

Antimicrobial and antiprotozoal (amebicidal)	-	Ethanolic extract	-Antibacterial activity against 5 microoganisms (*Bacillus subtilis, Escherichia coli, Micrococcus luteus, Cladosporium cucumerinum* and *Penicillium oxalicum*) based *in vitro* assay -Amebicidal activity against *Entamoeba histolytica* based *in vitro* assay	-Active against all microorganisms (10-25 µg/spot), except *E. coli*. -*B. subtilis* and *C. cucumerinum* were more sensitive to extract (extract was active at 10 µg/spot) compared to other microorganisms. -No amebicidal activity in concentrations below 250 µg/mL.	-	(Heinrich et al., 1992)
Antioxidant, antimicrobial and antiprotozoal (antileishmanial and trypanocidal)	Leaves	Methanolic and aqueous extracts	-Scavenger activity against DPPH, $OH^{\bullet}$ and O_2^- based *in vitro* assays -*In vitro* lipid peroxidation of rat liver membranes -Antibacterial activity with 3 bacterial strains (*Pseudomonas aeruginosa, Salmonella typhi* and *Staphyloccocus aureus*) -Epimastigotes and trypomastigotes of *Trypanosoma cruzi*, and promastigotes of *Leishmania brasilensis* and *L. mexicana*	-No toxicity against *A. salina* nauplii at doses of < 1000 µg/mL. -Methanolic extract showed the highest antioxidant capacity against all radicals (IC_{50} 50.21, 250 and 72.8 µg/mL for DPPH, $OH^{\bullet}$ and O_2^- radicals), as well as for lipid peroxidation (7.6% inhibition at 60 µg/mL). -Both extracts were active against all bacterial strains (> 2 mg/mL). -No antiprotozoal activity.	Flavonoids and condensed tannins	(Navarro et al., 2003)
Antioxidant and antimicrobial	Leaves	Petroleum ether, ethyl acetate, methanolic and aqueous extracts	-DPPH based *in vitro* assay -Antibacterial activity against 5 bacterial strains (*Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium* and *Enterobacter aerogenes*) using the disc diffusion method	-Methanolic extract showed better antioxidant activity (IC_{50} 655 µg/mL). -Petroleum ether extract was active against *B. subtilis* (IZ = 11 mm) and *S. aureus* (IZ = 13 mm), while ethyl acetate and aqueous extracts were active against only *B. subtilis* (IZ = 14 mm) and *P. aeruginosa* (IZ	Mainly cardiac glycosides, tannins, triterpenes, steroids and saponins	(Kaneria, Baravalia, Vaghasiya, & Chanda, 2009)

				= 14 mm), respectively (all extracts at 500 µg/well).		
Antioxidant and antimicrobial	Leaves	Essential oil extracted by hydrodistillation	-DPPH based *in vitro* assay -Antimicrobial activity against 12 microorganisms (*Candida albicans, Cryptococcus neoformans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Malassezia* sp., *Aspergillus* sp., *Aeromonas* sp. *and Escherichia coli*) by the microdilution broth method.	-Antioxidant capacity (IC_{50} 7.61 µg/mL) superior to vitamin C (IC_{50} 15.98 µg/mL). -High activity against *P. aeruginosa* and *S. aureus* (MIC 62.50 and 125.00 µg/mL, respectively).	Phenolic compounds, mainly carvacrol, thymol, spathulenol and eugenol	(Boligon et al., 2016)
Antioxidant, anticholinesterase and antimicrobial	Leaves	Ethanolic extract	-DPPH based *in vitro* assay -*In vitro* acetylcholinesterase inhibition assay -Antifungal activity (3 standard strains: *Candida albicans* (CA 77), *C. krusei* (CK 01) and *C. tropicalis* (CT 23); and 3 multiresistant clinical strains: CA 40006, CK 40095 and CT 40042) by the microdilution broth method	-High antioxidant capacity (IC_{50} 119.85 µg/mL). -Anticholinesterase activity (IZ = 1 cm at 4 mg/mL) similar to standard physostigmine (IZ = 0.9 cm at 4 mg/mL). -Low antifungal activity against all strains of *Candida* (MIC = 1024 µg/mL). -Potentiated the antifungal effect of the fluconazole against multiresistant strains of *C. tropicalis*.	Phenolic compounds	(Morais et al., 2017)
Antioxidant and anticholinesterase	Stem bark	Hydro-acetone extract (acetone 70%) and its ethyl acetate fraction	-DPPH, FRAP, TEAC and xanthine oxidase based *in vitro* assays -*In vitro* acetylcholinesterase inhibition assay	-Both crude extract and ethyl acetate fraction showed high antioxidant and anticholinesterase activities. -In general, ethyl acetate fraction had better antioxidant (IC_{50} 10.10 and 0.75 µg/mL for DPPH and	Epiafzelechin-(4β→8)-epicatechin, epigallocatechin, epicatechin, procyanidin dimers B1 and B2	(Sereia et al., 2019)

				xanthine oxidase assays, and 6.41 and 3.12 mmol TE/g for FRAP and TEAC assays) and anticholinesterase (IC_{50} 1.14 mg/mL) activities.		
Antioxidant	-	Prolipid capsule (20% w/w mutamba extract, 10% w/w murraya extract and 10% w/w souchi extract)	-DPPH, TEAC and FRAP based *in vitro* assays	-High antioxidant capacity (275.81, 74.98 and 62.44 µmol TE/g dw for TEAC, FRAP and DPPH assays).	Phenolic compounds	(Jastrzebski, Medina, Moreno, & Gorinstein, 2007)
Antioxidant	Stem bark	Acetone extract (acetone 70%)	-DPPH based *in vitro* assay	-Antioxidant capacity (IC_{50} 8.94 µg/mL) similar to vitamin C (IC_{50} 7.52 µg/mL).	Phenolic compounds	(Munhoz, Lonni, de Mello, & Lopes, 2012)
Antioxidant	Stem bark	Hydroethanolic extract (ethanol 70%) and its dichloromethane, ethyl acetate and *n*-butanol fractions	-DPPH based *in vitro* assay	-Hydroethanolic extract and its fractions had high antioxidant capacity (IC_{50} 8.09-67.69 µg/mL). -Ethyl acetate was the most active fraction (IC_{50} 8.09 µg/mL).	Phenolic compounds	(Feltrin et al., 2012)
Antioxidant	Fruit	Hydroethanolic and chloroform extracts	-DPPH and β-carotene system by bioautography	-Both assays revealed the presence of antioxidant compounds bands.	Flavonoids, tannins, phenolic acids, steroids, saponins and resins	(Assis et al., 2019)
Antioxidant and anti-inflammatory	Leaves	Hydromethanolic extract (methanol 80%)	-DPPH and reducing power based *in vitro* assays -Lipopolysaccharide-stimulated RAW264.7 macrophages	-High antioxidant activity (19.7 and 307.6% for electron donating ability and reducing power assays, respectively, at 0.5 µg/mL). -Medium activity inhibitory on nitric oxide release (↓51.4%) and low cytotoxicity (maintained 93.7% viable cells) at 50 µg/mL.	-	(Choi & Hwang, 2005)
Antioxidant and apoptotic inductor	Leaves	Flavonoid extract	-Inhibition of malonaldehyde formation based *in vitro* assay	-High antioxidant capacity (88.52% inhibition of	Flavonoids	(Syaefudin, Wahyuni,

			-Apoptosis induction using *Saccharomyces cerevisiae*	malonaldehyde formation at 100 µg/mL compared to α-tocopherol that inhibited 71.57% at 200 µg/mL). -Apoptotic inductor (induction of petite colony formation and membrane blebbing, and ↓cell proliferation).		Artika, & Sulistiyani, 2014)
Antioxidant and cardioprotective	Stem bark and leaves	Aqueous extract	-DPPH based *in vitro* assay -Cellular antioxidant activity using human erythrocytes and chronic myeloid leukemia (K562) cell line -DOX-induced cardiotoxicity in C57Bl/6 mice	-High antioxidant capacity (IC_{50} 25.2 and 39.3 µg/mL for stem bark and leaves extracts, respectively). -Both extracts ↓AAPH-induced haemolysis, ↓lipid peroxidation, ↓DOX-induced haemolysis and ↓DOX-induced lipid peroxidation in human erythrocytes. -Stem bark extract showed a higher overall antioxidant potential. -Stem bark extract ↓H_2O_2-induced ROS production in K562 cells and ↓DOX-induced leukocyte death. -Mice treated with stem bark extract (2000 and 5000 mg/kg body mass) showed no signs of toxicity, mortality, or physical and behavioural changes. -Stem bark extract ↓lipid peroxidation in the cardiac tissue of DOX-treated mice.	Flavonoids	(dos Santos et al., 2018)
Cardioprotective	Stem bark	Procyanidin fraction from acetone extract	-Sugar-fed hypertensive rats	-Antihypertensive and vasorelaxant properties.	Procyanidins	(Magos et al., 2008)

				-↓Both the systolic arterial pressure and the heart rate in sugar-fed hypertensive rats (10 mg/kg orally administered). -↓Contraction induced by norepinephrine in isolated aortic rings of normotensive and sugar-fed hypertensive (IC_{50} 35.3 and 101.3 ng/mL, respectively) rats.		
Cardioprotective and neuroprotective	Stem bark	Hexane and methanol-dichloromethane extracts, chloroform, ethyl acetate and aqueous fractions from methanol-dichloromethane extracts, tannin-free, tannin-free chloroform and tannin-free aqueous	-Chinese hamster ovary cells (CHO-K1) stably transfected with the cDNA for the human angiotensin II AT1 receptor.	-Inhibition of [^3H]-angiotensin II binding to the AT1 receptor and of [^3H]-neuropeptide Y binding to the Y1 receptor. -Methanol-dichloromethane extract and its ethyl acetate and aqueous fractions were the most active.	Tannins	(C Caballero-George et al., 2001)
Cardioprotective	Stem bark	*n*-Butanol fraction from crude extract (acetone 70%) and its proanthocyanidins fractions	-Wild-type Chinese hamster ovary cells (CHO-K1) stably transfected with the cDNA for the human angiotensin II AT1 receptor.	-High inhibition of the [^3H]-angiotensin II binding to the human AT1 receptor. -Fractions containing highly polymerized proanthocyanidins showed the highest activities.	Proanthocyanidins, mainly highly polymerized proanthocyanidins	(Catherina Caballero-George et al., 2002)
Gastroprotective	Leaves and flowers	Ethanolic extracts	-Male and female Wistar rats/model of acute gastric ulcer induced by diclofenac	-↓Ulcerated area in a dose-dependent way (125-500 mg/kg body weight). -↓Oxidative stress parameters in gastric mucosa (myeloperoxidase activity and lipid peroxidation). -↑SOD and GSH-Px levels activities. -↑PGE_2 and non-protein SH levels.	Phenolic compounds, especially proanthocyanidins and flavonoids	(Berenguer et al., 2007)

Antiprotozoal (trypanocidal)	Fruits	Methanolic extract	-Epimastigotes of *Trypanosoma cruzi*	-Trypanocidal activity (MC_{100} > 500 µg/mL).	-	(Abe et al., 2005)
Antiprotozoal (antileishmanial and trypanocidal)	Leaves	Ethanolic extract	-Epimastigotes of *Trypanosoma cruzi*, and promastigotes of *Leishmania brasiliensis* and *Leishmania infantum*	−↓*Leishmania* spp. and *T. cruzi* viability. −Extract was more active against *Leishmania* spp. (inhibition > 92% at 500 µg/mL) than *T. cruzi* (63.86% inhibition at 1000 µg/ mL).	Phenolic compounds, such as catechin, chlorogenic acid, caffeic acid, rutin, quercetrin, quercetin and luteolin	(Calixto Júnior et al., 2016)
Antiprotozoal (antimalarial)	Leaves, roots and stem bark	Hydroethanolic extract (ethanol 70%)	-*Plasmodium falciparum* chloroquine sensitive strain, and on ferriprotoporphyrin IX biocrystallisation inhibition test (FBIT) based *in vitro* assays	-Only leaves extract had antimalarial potential (IC_{50} > 10 µg/mL and 1.00 mg/mL for *P. falciparum* inhibition and FBIT assays, respectively).	-	(Bourdy, Oporto, Gimenez, & Deharo, 2004)
Anticholera	Stem bark	Hydroethanolic extract (ethanol 70%) and its fractions	-Rabbit distal colon	-Extract (40 µg/mL) completely inhibited cholera toxin-induced secretion when added to the mucosal prior to the toxin. -Aqueous fraction dose-dependently inhibited cholera toxin-induced secretion (IC_{50} 15 µg/mL).	Proanthocyanidins	(Hör et al., 1995)
Anticholera	Stem bark	Ethanol-water-acetone (7:7:6) fraction from ethanolic extract	-SDS-PAGE based *in vitro* assay	-High activity against cholera toxin (the lowest dose at which no A-band of the toxin was detectable ranging 7.5-30 µg).	Proanthocyanidins	(Hör et al., 1996)
Antiviral	Stem bark	Hydro-acetone (acetone 70%) extract and its aqueous and ethyl acetate fractions	-Poliovirus 1 (P-1) and bovine herpesvirus 1 (BHV-1) in HEp-2 cultured cells	-No cytotoxicity against HEp-2 cells (5-50 µg/mL). -Hydro-acetone extract and its fractions showed high antiviral activity at 5 µg/mL (inhibited BHV-1 and P-1 replication by 97-100% and 62-99% for, respectively).	Condensed tannins	(Felipe et al., 2006)

Insecticide	Leaves	Ethanolic extract	-*Aedes aegypti* larvae	-Control of fourth instar larvae of *A. aegypti* (35% mortality at 500 µg/L).	-	(de Mendonça, da Silva, dos Santos, Ribeiro Júnior, & Sant'Ana, 2005)
Insecticide	Leaves	Ethanolic extract, aqueous infusion and distillation with acetone, ethanol, or water	-Ants (*Atta mexicana*) and wild turkeys (*Meleagris gallopavo*) naturally infested with lice and mites	-Distilled with acetone and ethanol showed the highest insecticide activity against ants (average mortality of 18.4/20 and 16.3/20 at 100 mg/mL, respectively). -Distilled with acetone and ethanol at 100 mg/mL reduced the exoparasites infestation in wild turkeys.	-	(Garrido et al., 2017)
Molluscicide	Stem bark	Ethanolic extract	-*In vitro* assay against adult *Biomphalaria glabrata*	-Control of adult *B. glabrata* (20% mortality at 100 µg/mL).	-	(A. F. dos Santos & Sant'Ana, 2000)

dw: dry weight; *SDS-PAGE:* sodium dodecyl sulphate polyacrylamide gel electrophoresis; *IZ:* inhibition zones; *MIC:* minimum inhibitory concentration; *MICD:* minimal inhibitory concentration in disk; LC_{50}*:* lethal concentration (extract concentration which causes 50% mortality); IC_{50} *or* IC_{90}*:* extract concentration that resulted in a 50% or 90% reduction in the response; MC_{100}*:* minimum extract concentration at which all the epimastigotes were terminated; *DPPH:* DPPH radical scavenging activity; *TEAC:* Trolox equivalent antioxidant capacity; *FRAP:* ferric reducing antioxidant power; *TE:* Trolox equivalents; $OH^{\bullet}$*:* hydroxyl radical; O_2^-*:* superoxide radical; *ROS:* reactive oxygen species; *SOD:* superoxide dismutase; PGE_2*:* prostaglandin E_2; *GSH-Px:* glutathione peroxidase; *PPARγ:* peroxisome proliferator-activated receptor gamma; *C/EBPα:* CCAAT/enhancer-binding protein alpha; *SCD1:* stearoyl-CoA desaturase 1; *STAT:* signal transducer and activator of transcription proteins; *IFN-γ:* interferon gamma; *T47D:* breast cancer cell line; *MCF7:* human breast cancer cell line; *DOX:* doxorubicin.

The extracts from stem bark, leaves and fruits of mutamba were active against a wide spectrum of pathogenic bacteria, including multi-drug resistant pathogens (Cáceres et al., 1995; Jacobo-Salcedo et al., 2011; Karthika et al., 2017; Morais et al., 2017). Microbial pathogens cause several human diseases and, consequently, the discovery of new antimicrobial compounds can be useful in their treatment, especially in developing countries. In addition to this, the number of human-pathogenic bacteria that are resistant to antibiotics has increased in the past decades, which has encouraged the scientists to develop new antimicrobial agents. In this regard, the natural antimicrobial agents from plants could be an alternative to the common antibiotics. Moreover, they could be an important strategy in the fight against bacterial resistance because plant-derived antimicrobials interact with multiple bacterial cellular targets instead of displaying a particular single mode of action and, as consequence, they can inhibiting the pathogens to acquiring resistance. Furthermore, the plant-derived antimicrobials can be labelled as GRAS (Generally Recognized as Safe), which creates a friendly image that would be more acceptable to consumers (Martelli & Giacomini, 2018; Pisoschi et al., 2018). The *G. ulmifolia* extracts can be explored as antimicrobial agent against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Candida albicans*, *Neisseria gonorrhoeae, Salmonella typhi*, and *S. dysenteriae*. The extracts from stem bark and leaves showed potent antimicrobial activity against these pathogenic microorganisms (**Table 2**). Moreover, the mutamba extracts were active against *Staphylococcus aureus, Pseudomonas aeruginosa, and Neisseria gonorrhoeae.* These pathogens developed resistance to a large number of antibiotics and pose great threat to human health as published by World Health Organization (WHO). Therefore, the mutamba-derived antimicrobials could be explored to treat globally important diseases and foodborne illnesses, such as skin, urinary tract, and sexually transmitted genitourinary infections, pneumonia, gastrointestinal infections, typhoid, and shigellosis.

In addition to the antimicrobial activity, the extracts from stem bark, leaves, roots and fruits of mutamba were efficient antiprotozoal agents, including antileishmanial, trypanocidal, amebicidal, giardicidal and antiplasmodial activities (Abe et al., 2005; Bourdy, Oporto, et al., 2004; Calixto Júnior et al., 2016; Heinrich et al., 1992; Navarro et al., 2003; Vera-Ku et al., 2010). Furthermore, the stem bark extract showed molluscicide activity against *Biomphalaria glabrata* (schistosomiasis vector), whereas the leaf extract was active against *Aedes aegypti* larvae, which is the principal dengue vector (de Mendonça et al., 2005). These bioactivities indicate that the mutamba-derived antiprotozoal may help to prevent neglected

tropical diseases, such as leishmaniasis, Chagas disease, amebiasis, giardiasis, malaria, and schistosomiasis. Neglected tropical diseases is a term used to a diverse group of diseases that prevail in tropical and subtropical conditions, especially in populations living in poverty without adequate sanitation and in close contact with infections vectors and domestic animals and livestock. These diseases cause human suffering and death, and create an obstacle to health and remain a serious impediment to poverty reduction and socioeconomic development. The plant-derived medicines may be the only antiprotozoals viable and available to populations living in poverty, which can therefore enhance the local access to essential medicines to prevent and control neglected tropical diseases.

The extracts from different mutamba parts have shown potential as antimicrobial and antiprotozoal agents against a great number of diseases. Interestingly, these extracts also demonstrated high antioxidant activity (Boligon et al., 2016; Kaneria et al., 2009; Morais et al., 2017; Navarro et al., 2003). Oxidative stress is defined as an imbalance between the production of free radicals and the antioxidant system. It occurs when the concentration of reactive species is not controlled by internal defence mechanisms and, as consequence, oxidative damage occurs to proteins, lipids, and DNA, which could lead to cytotoxicity, genotoxicity, and carcinogenesis when mutated (damaged) cells can proliferate. Oxidative stress is generally considered the starting point of a wide number of chronic degenerative diseases, such as tissues chronic inflammation and cancer (Choi & Hwang, 2005; Feltrin et al., 2012; Jastrzebski et al., 2007; Munhoz et al., 2012; Syaefudin et al., 2014). In this regard, it would be favourable to find agents that have, for example, antioxidant and antimicrobial activities combined, which could lead to better therapeutic solutions against complex multifactorial diseases (Martelli & Giacomini, 2018). For example, oxidative stress could result from the activation of the immune system in response to invading microorganisms (inflammation), which could lead to oxidative damage of surrounding cells and, consequently, increase the risk of degenerative diseases development. Therefore, the use of a drug with multi-active properties, such as antimicrobial, antioxidant, and anti-inflammatory could be more effective in the treatment of chronic diseases (Martelli & Giacomini, 2018). The stem bark and leaves of mutamba showed antimicrobial, antioxidant, anti-inflammatory and anti-cancer activities, which demonstrates the potential of this species as therapeutic plant (Choi & Hwang, 2005; Dai et al., 2016; Karthika et al., 2017; Maldini et al., 2013; Syaefudin et al., 2014).

Dos Santos et al. (2018) evaluated the *in vivo* cardioprotective effect of mutamba stem bark extract in doxorubicin-treated mice. Doxorubicin (DOX) is a chemotherapy agent

widely used to treat cancer, but the DOX treatment can induce the increase of reactive species that damage the cardiac tissue due to oxidative stress mechanism. The DOX-induced cardiotoxicity can be reduced by the co-administration of DOX and antioxidant extracts from medicinal plants, such as *G. ulmifolia*. The heart shows higher susceptibility to oxidative stress than other organs, such as liver, due to its lower activity of antioxidant enzymes. Therefore, the administration of dual-active agent could have a synergic effect in counteracting cardiac oxidative stress (Martelli & Giacomini, 2018). Indeed, the stem bark extract prevented the malondialdehyde generation in the cardiac tissue of DOX-treated mice, which demonstrated its cardioprotective effect (dos Santos et al., 2018). Furthermore, the steam bark showed *in vivo* (rat's cardiovascular system) antihypertensive and vasorelaxing properties mediated at least partially by an endothelial L-arginine/Nitric Oxide pathway (Magos et al., 2008). The procyanidins from mutamba stem bark *in vitro* inhibited the [^{3}H]-angiotensin II binding to the human AT1 receptor, which indicates a potential hypotensive effect (C Caballero-George et al., 2001; Catherina Caballero-George et al., 2002). However, this mechanism has not been proved by using *in vivo* studies. The cardioprotective effect of mutamba stem bark has been attributed to condensed tannins, especially proanthocyanidins B (Caballero-George et al., 2001). The stem bark has great potential to be used as a natural medicine in the reduction of the risk of stroke and myocardial infarction due to its antihypertensive activity (dos Santos et al., 2018).

The proanthocyanidins from stem bark showed antidiarrheal activity due to the capability of these compounds to specifically interact with the A subunit of Cholera toxin, a protein complex produced by *Vibrio cholerae* that leads to rapid fluid loss from the intestine. It may explain the use of *G. ulmifolia* as antidiarrheal drug in developing countries (Hör et al., 1996, 1995). Furthermore, the areal parts of mutamba (leaves and flowers) combated the diclofenac-induced gastric lesions by antioxidant and anti-inflammatory mechanisms. The non-steroidal anti-inflammatory drugs suppressing prostaglandin E_2 biosynthesis, which decreases the gastric mucus production and increases acid secretion in stomach. It eventually can cause a gastric ulcer and additionally oxidative stress due to the fact that the gastric mucus protects the mucosa from reactive species attack. The leaves and flowers of mutamba can act preserving the antioxidant endogenous enzymes, such as superoxide dismuthase (SOD), in exposed mucosa, scavenging free radicals by its antioxidant activity (phenolic compounds), and inhibiting the release of pro-oxidant agents (anti-inflammatory). The protective action of *G. ulmifolia* was evidenced by ulcerated area's reduction as well as by the decrease of oxidative stress markers in gastric mucosa (Berenguer et al., 2007).

G. ulmifolia has shown other promising bioactivities. The aqueous decoction of mutamba leaves decreased the hyperglycemic peak (22.2%) of hyperglycemic rabbits (Alarcon-Aguilara et al., 1998), and the stem bark aqueous extract showed *in vitro* antidiabetic activity by stimulated glucose uptake (Alonso-Castro & Salazar-Olivo, 2008). Furthermore, the leaves extracts showed potential anti-obesity assayed by *in vitro* studies (Hidayat et al., 2016; Iswantini et al., 2011). Obesity currently affects 600 million people worldwide and it is closely associated with comorbidities, such as diabetes and cardiovascular diseases. It is estimated that about 90% of diabetes mellitus is attributed to excess of weight. With regard to obesity and diabetes, the use of medicinal plants contenting dual-active molecules, antioxidant and anti-inflammatory, could enhance the potential of this agent in the control of obesity and disorders that compose the metabolic syndrome (Verma & Hussain, 2017). The stem bark and leaves of *G. ulmifolia* have potential as therapeutic plant in the control of obesity and diabetes. Nevertheless, this bioactivity is still elusive and not well understood, and strong scientific evidences should be presented to validate the anti-diabetic and anti-obesity activities of mutamba.

The studies covering the biological activities of *G. ulmifolia* have demonstrated that this species has great potential as therapeutic plant due to its antioxidant, antimicrobial, antiprotozoal, and antidiarrheal activities, as well as the cardioprotective effect. Furthermore, the mutamba shows promising bioactivities such as antidiabetic and anti-obesity activities, and gastroprotective. The traditional uses of mutamba have been proved by *in vitro* and *in vivo* studies (*see Section 5.1*), which demonstrates the importance of ethnopharmacological surveys as a scientist tool. Furthermore, pharmacological validation of medicinal plants could greatly benefit people living in poverty since they do not have easily access to modern healthcare.

The stem bark and leaves were the plant parts most evaluated, whereas the flowers, roots and fruits were underexploited despite their therapeutic potential indicated by traditional healers. Certainly, the studies on phytochemical compounds and biological properties of mutamba should be evaluated by using clinical trial experiments, but the currently scientific evidences clearly show this species as potential food and therapeutic plant. It is important to note that the proanthocyanidins (stem bark) and aglycones and glycosylated flavonoids (leaves) were reported as the biological active compounds from mutamba. The phenolic compounds indeed have shown a wide broad spectrum of biological activities, and the scientific evidences about it have been daily increasing (Shahat & Marzouk, 2013). Therefore, the literature clearly shows *Guazuma ulmifolia* as a potential source of bioactive phenolic compounds.

The discovery of compounds from plants that can have bioactivities with no toxic effects on health is highly estimated. Unfortunately, the toxic effect of both extracts and isolated compounds from *G. ulmifolia* has not been extensively evaluated (Assis et al., 2019; Calixto Júnior et al., 2016; dos Santos et al., 2018). The toxicity studies on *Guazuma ulmifolia* Lam. seem to be inconclusive, except for the stem bark aqueous extract (GUEsb). GUEsb showed no acute toxicity tested in C57Bl/6 Mice at 2000 and 5000 mg/kg of extract orally administrated. The intake of GUEsb induced no physical and behavioural changes in the animal tested, such as food and water intake, body mass, relative organ mass, and biochemical and haematological parameters (dos Santos et al., 2018). The authors concluded that the aqueous extract from stem bark is safe for consumption. Nevertheless, toxicological, pre-clinical and clinical trials should be carried out to further confirm the bioactivity effects of different botanical parts of mutamba on human health and to assure the safety and well-being of the consumers.

6 Commercial scenario: Potential products development and patent grants

Product development and commercial exploitation of *Guazuma ulmifolia* Lam. (mutamba) may be linked to the traditional popular knowledge, considering that leaves, mucilage, roots, and other plant parts, are used in homemade remedies and treatments. In traditional medicine, for example, it is reported the use of its stem bark and leaves for the treatment of hemorrhages and diarrhea, and against inflammatory diseases, fever, kidney disorders, alopecia, cough, among several others (Maldini et al., 2013; Morais et al., 2017). One of the main sources of its consumption is from the preparation of tea from stem bark and leaves, used against gastrointestinal diseases, treatment of asthma, fever, diarrhea and dysentery, with an anti-inflammatory and radical-scavenging recognized potential (Magos et al., 2008). The mutamba stem bark and leaves show phytochemical compounds with bioactivities (*see section 5.2*) that can be explored by pharmaceutical industry. Therefore, these millenarian uses can be an interesting key to extend the commercial studies with mutamba tree.

Moreover, considering the complex composition of mutamba tree, as well as its proven biological activity, this plant appears as a promising alternative for the application and development of new commercial products, which may affect the valorization and increase knowledge on the properties of mutamba tree.

Among some potential applications, Morais et al. (2017) presented a detailed chemical study of the ethanolic extract from leaves containing flavonoids and phenolic acids, with recognized antioxidant potential. Therefore, the antioxidant activity of mutamba leaves

highlights the potential of new drugs development. Additionally, the antifungal activity of ethanolic extract has also been widely evaluated (Navarro et al., 2003). One of the main example showed the potential of the antifungal action against *Candida tropicalis*, besides a significant fluconazole modulatory effect (Morais et al., 2017). This kind of approaches increases the commercial potential of pharmacological applications.

Besides leaves and stem bark, the extraction and characterization of mucilage can be considered as an essential step in establishing their potential as food and non-food ingredient (Prajapati, Jani, Moradiya, & Randeria, 2013). In this approach, a novel and natural mucilage was extracted from mutamba seeds applying high-intensity ultrasound (Pereira et al., 2019b). The technology employed gradually enhanced the extraction of a novel polysaccharide without any negative influence on its properties, quality or structure. It is worth noting the wide applications of plant-derived gums and mucilages in pharmaceutical, cosmetic and food industries (Prajapati et al., 2013). The mutamba seed mucilage can be used as a natural emulsifying and thickening agent to prepare stable emulsions with high encapsulation efficiency of volatile compounds (Pereira et al., 2019a).

Moreover, Assis et al. (2019) performed the chemical and phytochemical characterization of mutamba fruit flour and used it for the partial substitution in whole wheat bread development. Mutamba flour presented a low lipid content and a high crude fibre content ($26.90 \pm 0.37\%$) while the formulation with 5% addition resulted in a bread with the best global acceptance. Authors observed the potential of mutamba for new products development with a focused approach for the food industry due to the composition of this flour. Still, *Guazuma ulmifolia* Lam. flowers is popularly known for hair growth applications (Albuquerque, Monteiro, Ramos, & de Amorim, 2007), increasing its commercial potential for this kind of products and sector.

In addition to scientific advances, patents development and registration can be considered one important step for technological innovation and for transferring technology (Figueroa & Serrano, 2019). In this approach, some advances have already been presented with the use of mutamba as main or as part of products and techniques, bringing greater approximation to commercial products.

A Spanish patent comprised a cosmetic based on vegetable gum obtained from *Guazuma ulmifolia* Lam with other additives used for topic application (ES2179791A1, 2001). With a wide range application product, *Guazuma ulmifolia* extract was used to obtain an

excellent lipid peroxidase inhibitor involved with lipid oxidation, showing potential for the food, pharmaceutical and cosmetic industries application (JP2000198740, 2000).

The scientific association of mutamba with hair treatments was also the target of technical production, considering that mutamba extract was used in a gray hair-preventing and improving agent by the activation of melanocytes formulated in association with several other plant extracts (JP2002020243, 2002). In a close approach, another Japan granted patent promoted a hair-growing substance formulated with a complex mixture of plant extracts, including *Guazuma ulmifolia* Lam. extract (JP2001220320, 2001). *Guazuma ulmifolia* extract was also applied as the main ingredient for a hair-care shampoo that could affect hair growth, broadening the spectrum of applications for this purpose (WO2018158691).

An Indian invention has provided knowledge about an herbal formulation prepared with *Guazuma tomentosa* extract after decoction for fractures healing. Authors of this patent proposed that the extract could be considered as an effective and low-cost method for fracture healing in animals and without side effects (1112/MUM/2011A, 2014).

A Russian claimed patent showed weight loss agent based on natural components combined with natural instant lyophilized coffee. This substance was composed of several plant dry extracts, including from *Guazuma ulmifolia* Lam., aiming to prevent the fat absorption and stimulating their break down and removal (RU2310342, 2006).

Despite being a promising source of bioactive compounds with potential for application and development of commercial products, the data available in the scientific and technical literature are still very scarce. Therefore, this fact opens up many possibilities for research, development and mainly for new studies aimed at elucidating the potential applications of mutamba tree, and further *in vivo* studies and clinical trials are required in order to attest its safety of use in commercial products.

7 Conclusion and perspectives

The available information about the phytochemical and biological properties of *Guazuma ulmifolia* Lam. indicated that this species has great potential to be used as food and therapeutic plant. The findings show the *G. ulmifolia* as a potential source of phenolic compounds, especially proanthocyanidins, with a broad spectrum of bioactivities, including antimicrobial, antioxidant, antiprotozoal, and antidiarrheal activities, and cardioprotective effect. The interesting medicinal properties possessed by this species and its chemical

composition prospect a significant opportunity to discover novel molecules with promising pharmacological properties. The leaves and stem bark from *Guazuma ulmifolia* Lam. have been used by folk medicine, which led the scientists to investigate their phytochemical composition and pharmacological properties, whereas the fruits, roots and flowers have not been extensively investigated. Although the literature data about the mutamba fruit is scarce, the traditional uses of this fruit indicate that it could be valuable for the food industry to prepare foods that have an impact on the health status of consumers, such as (but not limited to) tea and whole bread. This review uncovered gaps that open up scientific opportunities, namely (i) evaluating the mutamba fruit nutritional value, (ii) phytochemical quantitative and qualitative composition of different mutamba botanical parts, especially the fruits, roots and flowers, (iii) the study of mutamba' biological activities and toxicity by using randomised and controlled clinical trials, and (iv) the development of products. The biological and toxicological properties of *G. ulmifolia* should be proved by clinical trials to confirm its health effects as well as to assure the safety and well-being of the consumers. The species *Guazuma ulmifolia* Lam. could be used by food and pharmaceutical industries as a source of functional compounds due to its phytochemical composition and biological properties. The use of this species can go beyond ethnobotanical reports through the study of uncovered gaps.

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CHAPTER II

RESEARCH ARTICLE

MUTAMBA (*Guazuma ulmifolia* Lam.) FRUIT AS A NOVEL SOURCE OF DIETARY FIBRE AND PHENOLIC COMPOUNDS

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Mutamba (*Guazuma ulmifolia* Lam.) fruit as a novel source of dietary fibre and phenolic compounds

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Highlights

- Mutamba fruit showed a high content of dietary fibre (37%);
- Proanthocyanidins, and aglycones and glycosylated flavonoids composed mutamba fruit;
- Procyanidin trimer C1 was the main soluble phenolic compound;
- Phenolic acids and flavan-3-ols were the main bound phenolic compounds;
- Mutamba fruit may contribute to the intake of dietary fibre and bioactive phenolic compounds.

Abstract: This study covers the proximate and phytochemical composition of mutamba (*Guazuma ulmifolia* Lam.) fruit. This fruit showed high dietary fibre (36.9%) and low moisture (10.0%) contents which justify its hardness and dryness. Nevertheless, this fruit is very appreciated due to its sweet pulp (high sucrose content, 16.3%) and attractive taste. The soluble and insoluble-bound phenolic compounds from mutamba fruit were analysed by using liquid chromatography coupled to tandem mass spectrometry (LC-MS). LC-MS identified 26 compounds. Mutamba fruit was composed mainly by soluble flavonoids (1385.9 µg/g dw), namely proanthocyanidins, and aglycones and glycosylated flavonoids. Procyanidin trimer C1 (972.8 µg/g dw) followed by procyanidin dimer B2, rutin, epicatechin, and hyperoside were the main soluble phenolics, accounting 1435.5 µg/g dw. Conversely, the main cell wall bound phenolics (228.8 µg/g dw) were catechin, followed by protocatechuic acid, epicatechin, and gallic acid. In conclusion, mutamba fruit could be a novel source of dietary fibre and bioactive phenolic compounds.

Keywords: Brazilian fruits, Flavan-3-ol, HPLC-ESI-MS/MS, insoluble-bound polyphenols, Procyanidin C1, Proximate composition.

1 Introduction

Guazuma ulmifolia Lam. (Malvaceae), popularly known as mutamba (Brazil) and guácimo (Mexico), is a tree commonly found in Latin America, such as in Mexico, Cuba Guatemala, Honduras, Ecuador, Bolivia, and Brazil. Ethnopharmacological studies have reported the use of different botanical mutamba parts, for example leaves, stem bark, and fruits, as traditional medicines to treat a broad spectrum of diseases, mainly diarrhoea, cough and fever, and gastrointestinal and cardiovascular disorders (Al Muqarrabun & Ahmat, 2015; Navarro, Villarreal, Rojas, & Lozoya, 1996; Tene et al., 2007). These biological properties

claimed by traditional healers have been proved by scientific studies. For example, the aqueous extract obtained by decoction of stem bark and leaves of mutamba have shown to be efficient antimicrobial, antioxidant, antiprotozoal, and cardioprotective agents (dos Santos et al., 2018). The bioactivities of these botanical plant parts have been correlated with their phytochemical composition in which the phenolic compounds, namely proanthocyanidins (condensed tannins) and aglycones and glycosylated flavonoids, have been reported as the main bioactive molecules (Hör, Heinrich, & Rimpler, 1996; Lopes, Rocha, de Almeida, & de Mello, 2009; Magos et al., 2008). The phytochemical studies revealed procyanidin dimer B1, B2, B5, procyanidin trimer C1 and Cinnamtannin A2 as the main phenolic compounds of stem bark (Hör et al., 1996). Unlike stem bark, the leaves showed phenolic acids (chlorogenic acid and caffeic acid) and flavonoids (catechin, quercetin 3-O-rhamnosyl-glucoside, quercetin 3-O-rhamnosyl, quercetin and luteolin) as main compounds. Procyanidin dimer B and glycosylated derivatives of catechin, quercetin, kaempferol and luteolin were detected in mutamba leaves (Calixto Júnior et al., 2016; Morais et al., 2017).

The tea from mutamba fruit is also used as traditional medicine to treat diarrhoea, haemorrhage, and influenza (Tene et al., 2007). However, scientists have not evaluated the biological properties of this plant part, probably, due to the lack of studies reporting the nutritional and phytochemical composition of mutamba fruit. The fruit of *Guazuma ulmifolia* Lam. is a globular capsule that weighs on average 3.64 g, with 1.5 to 4 cm of length and 2.48 cm of diameter (at ripening stage). Its colour changes from green to black during ripening and its moisture content decreases to approximately 10-15%, which characterizes the fruit as dry. The fruit is hard and dry, presenting leathery and woody structures, and containing on average 87 seeds (Neto & Aguiar, 2000; Sobrinho & Siqueira, 2008). The flowering and fruiting events vary and depend on the region in which the species is found, but in Brazil the fruits can be harvested from June to November (Carvalho, 2007). This fruit is difficult to be open due to its hardness and, consequently, it is not appropriate to consume it as fresh fruit. However, the fruit has been used as a flour to prepare tea and whole bread due to its sweet pulp and unique flavour (Assis et al., 2019; Tene et al., 2007). Furthermore, the seeds upon imbibition with water liberates a mucilaginous substance that may improve the taste of these products (Pereira et al., 2019).

The traditional use of mutamba fruit to treat diseases and as food, as well as the phytochemical composition of other plant parts (stem bark and leaves) has motivated us to investigate the secondary metabolites of this fruit, mainly the phenolic compounds.

Furthermore, the fruit shows low moisture (from 10 to 15%) and high dietary fibre contents indicated by its hardness and the seed mucilage, which demonstrates its potential to be used by food industry (Assis et al., 2019; Ortega, Carranco, Mendoza, & Castro, 1998). Nevertheless, the nutritional and phytochemical composition of mutamba fruit should be evaluated to prove it. According to the aforementioned background, we extracted the soluble and insoluble-bound phenolic compounds from mutamba fruit by using ultrasound technology, and these extracts were quantitatively characterized by liquid chromatography coupled to tandem mass spectrometry (LC-MS) (Arruda, Pereira, de Morais, Eberlin, & Pastore, 2018). In addition to LC-MS analysis, the proximate composition and physicochemical properties of mutamba fruit were assessed to identify the main primary metabolites and to encourage further research about this interesting fruit.

2 Material and methods

2.1 Chemicals and reagents

Analytical standards (*p*-hydroxybenzoic acid, caffeic acid, 5-*O*-caffeoylquinic acid, ellagic acid, ferulic acid, gallic acid, gentisic acid, *p*-coumaric acid, protocatechuic acid, catechin, epicatechin, epigallocatechin, kaempferol, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-rhamnosyl-glucoside, luteolin, luteolin 7-*O*-glucoside, naringenin, procyanidin dimer B1, procyanidin dimer B2, procyanidin trimer C1, quercetin, quercetin 3-*O*-galactoside, quercetin 3-*O*-methyl ether, quercetin 3-*O*-rhamnosyl-glucoside, and vanillin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents (methanol, acetone, and hexane), as well as LC/MS grade formic acid, were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18 $M\Omega\,cm^{-1}$) obtained from a Milli-Q water purification system (Millipore, Bedford, USA) was used. All other chemicals and reagents used in the experiments were of analytical grade.

2.2 Plant material

The mature (black-coloured fruit) and morphologically perfect mutamba (*Guazuma ulmifolia* Lam.) fruits were manually collected in August 2017 in natural areas of the Cerrado Biome located in the municipality of Uberlândia (18°55′07″ South latitude, 48°16′38″ West longitude and 863 m altitude), Minas Gerais, Brazil. The fruits (1 kg) were transported from the collection site to the laboratory up to 48 h after collection. The mutamba fruits were subjected to a sieving step to remove dirt and physical contaminants, and, after that, they were

(whole fruits) immediately ground to a fine powder by using a knife grinder. The mutamba fruits (pulp and seeds) were powdered without drying treatment due to their low moisture content (approximately 10%) (Ortega et al., 1998). The average size of the particles after grounding was as follows: 0.98% >2.4 mm; 1.72% between 2.0 and 2.4 mm; 17.45% between 1.4 and 2.0 mm; 41.19% between 1.0 and 1.4 mm; 18.93% between 0.70 and 1.0 mm; 19.73% <0.70 mm. Finally, the mutamba fruit powder (MF) was placed to vacuum packs, and it was stored at -18 °C until analysis. The Genetic Heritage Management Board (CGen) under number AA56F0A, following the Law n° 13.123/2015 and its regulations, regimented the activity of access to Genetic Heritage (**Annex 3**).

2.3 Physicochemical properties and proximate composition of mutamba fruit

The mutamba fruit was characterized by pH (method n° 970.21), total titratable acidity (TTA; n° 942.15), total soluble solids (TSS; n° 932.12), moisture (n° 925.09) and proteins (n° 920.87; protein factor equal to 6.25), ashes (n° 923.39), lipids (n° 945.16), and total dietary fibre (n° 985.29) contents according to the protocols described by the Association of Official Analytical Chemists, AOAC. The measurement of mono- and disaccharides content (glucose, fructose, and sucrose) was carried out by using a HPAEC-PAD (High Performance Anion Exchange Chromatography Coupled to Pulsed Amperometric Detection) method as exactly as described by Pereira et al. (2018). The total carbohydrates content was determined by using the following formula: 100 – (moisture + lipid + protein + ash + dietary fibre). The total energy value was estimated by using the conversion factors of 4 kcal/g for protein and carbohydrate content and 9 kcal/g for the lipid content. The physicochemical and proximate composition analyses were performed three times.

2.4 Extraction of phenolic compounds from mutamba fruit

The soluble and insoluble-bound phenolic compounds from MF were extracted according to the method described by Arruda et al. (2018). The extraction of soluble and insoluble-bound phenolic compounds was carried out three times.

2.4.1 Obtaining the soluble phenolic compounds

MF (1 g) was homogenized with 15 mL of a solution composed of methanol-acetone-deionized water (7:7:6, v/v/v). This mixture was ultrasonicated for 30 min at room temperature by using an ultrasonic bath (UNIQUE, model UCS-2850, 25 kHz, 120 W, Brazil). The supernatant was collected after centrifugation (4000g, 15 min, 5 °C) and the residue was re-extracted under the same conditions two more times. Therefore, the aforementioned

procedure was carried out three times and the supernatants were combined. The supernatant was evaporated under vacuum at 35 °C and the remaining aqueous phase was acidified to pH 2 using 6 M HCl, and centrifuged (4000g, 5 min, 5 °C) to remove precipitates. Subsequently the interfering lipids from aqueous phase were extracted three times with an equal volume of hexane. The aqueous phase was used as soluble phenolic compounds extract. The solid residue from soluble phenolic compounds extraction was used to extract the insoluble-bound phenolic compounds as described in the next section.

2.4.2 Obtaining the insoluble-bound phenolic compounds

The solid residue from soluble phenolics extraction (after solid-liquid extraction) was mixed with a 4 M NaOH solution containing 10 mM EDTA and 1% ascorbic acid at a solid to solvent ratio of 1:20 (w/v). The alkaline hydrolysis was performed for 4 h in a water bath shaker set up at room temperature and 150 rpm to release insoluble-bound phenolics. After alkaline treatment, the pH of the hydrolysate was adjusted to 2 using 6 M HCl and it was centrifuged (4000*g*, 5 min, 5 °C). The aqueous supernatant was collected and the interfering lipids from it were extracted three times with an equal volume of hexane. The insoluble-bound phenolics released from cell wall were recovered by liquid-liquid extraction by using diethyl ether-ethyl acetate (1:1, v/v) at a solvent to supernatant phase ratio of 1:1 (v/v). The organic phase was dehydrated with anhydrous sodium sulfate, and evaporated to dryness under vacuum at 35 °C. The dry material was dissolved into 5 mL of deionized water (Millipore®), and this solution was centrifuged so that precipitates were removed. This aqueous solution was used as insoluble-bound phenolic compounds extract.

The aqueous soluble and insoluble-bound phenolic extracts were filtered with a 0.22 μm filter and stored at - 80 °C until the LC-ESI-MS analysis.

2.5 Targeted analysis of phenolic compounds by HPLC-ESI-MS/MS

The targeted analysis of phenolic compounds was performed on a high-performance liquid chromatography - electrospray ionization - triple quadrupole mass spectrometer (HPLC-ESI-MS/MS) system (Shimadzu, model LCMS-8040, Kyoto, Japan) according to the method described by Arruda et al. (2018). The phenolic compounds separation was performed on a Shimpack XR-ODS III column (2.2 μm, 2.0 mm i.d., and 150 mm) at 40 °C using a binary mobile phase at flow rate of 0.4 mL/min. The mobile phase components were (A) 0.1% formic acid in water (v/v) and (B) methanol. The gradient elution was as follows: 0–1 min, 5% B; 1–4 min, from 5 to 60% B; 4–7 min, from 60 to 70% B; 7–10 min, from 70 to

100% B; 10–10.50 min, 100% B; 10.50–11 min, from 100 to 5% B; 11–15 min, 5% B. The extracts were diluted in 0.1% formic acid in water and injected (10 µL) into the column using an autosampler (10 °C).

Identification of the phenolic compounds was performed by using a tandem quadrupole mass spectrometer equipped with an ESI source in the negative mode. The mass spectrometer parameters were as follows: capillary voltage, 3.5 kV; heat block temperature, 300 °C; desolvation line temperature, 250 °C; drying gas flow (N_2), 20 L/min; nebulizing gas flow (N_2), 3 L/min; collision induced dissociation gas pressure (Ar), 224 kPa. The phenolic compounds from MF extracts were identified by comparing their retention time (r.t.) and ESI(-)-MS/MS dissociation patterns with those of authentic standards. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode and the dissociation patterns of the phenolic standards were evaluated by direct injection of each standard solution at 0.1 µg/mL. The precursor ions $[M-H]^-$ and their both most abundant product ions were selected to perform the analysis. **Table 1** shows the mass-to-charge ratio (*m/z*) of the precursor and product ions, as well as the best cone voltage, and collision energy.

Table 1. Multiple Reaction Monitoring (MRM) conditions of the phenolic compounds determined by HPLC-ESI-MS/MS.

Compound	Precursor ion (m/z)	Product ion (m/z)	Q1 Pre Bias (V)	Collision energy (V)	Q2 Pre Bias (V)
p-hydroxybenzoic acid	137.10	93.30 a	15	18	14
		65.30 b	14	34	21
Caffeic acid	179.10	135.10	11	16	22
		85.10	12	25	20
5-*O*-caffeoylquinic acid (Chlorogenic acid)	353.10	191.20	16	15	18
		85.10	12	46	29
Ellagic acid	301.00	145.20	14	37	10
		229.00	20	30	24
Ferulic acid	193.10	134.10	21	19	22
		178.00	22	13	30
Gallic acid	169.10	125.10	11	16	20
		79.20	17	24	27
Gentisic acid	153.10	109.30	16	16	16
		108.30	10	24	17
p-Coumaric acid	163.10	119.20	18	15	19
		93.10	18	30	15
Protocatechuic acid	153.10	109.30	16	16	16
		108.30	10	24	17

Catechin	289.10	109.20	14	25	17
		245.10	20	16	23
Epicatechin	289.10	109.20	14	25	17
		245.10	20	16	23
Epigallocatechin	305.10	125.00	14	20	21
		179.10	14	14	20
Kaempferol	285.10	93.10	13	39	30
		117.10	13	45	18
Kaempferol 3-*O*-glucoside (Astragalin)	447.10	284.10	15	28	28
		255.10	15	41	24
Kaempferol 3-*O*-rhamnosyl-glucoside (Nicotiflorin)	593.00	285.00	20	36	25
		284.00	20	40	25
Luteolin	285.10	133.20	13	36	22
		151.10	13	27	16
Luteolin 7-*O*-glucoside (Cynaroside)	446.90	285.10	15	30	27
		133.20	15	55	22
Naringenin	271.10	151.10	13	19	25
		119.30	13	26	19
Procyanidin dimer B1	577.10	407.20	20	30	19
		289.00	20	30	30
Procyanidin dimer B2	577.10	407.20	20	30	19
		289.00	20	30	30
Procyanidin trimer C1	865.00	289.00	20	50	19
		125.00	20	50	20
Quercetin	301.10	151.10	14	22	26
		179.10	14	18	30
Quercetin 3-*O*-galactoside (Hyperoside)	463.10	300.00	16	28	29
		271.00	16	44	26
Quercetin 3-*O*-methyl ether (Isorhamnetin)	315.10	300.10	15	23	28
		150.90	23	33	24
Quercetin 3-*O*-rhamnosyl-glucoside (Rutin)	609.00	300.20	22	39	29
		301.00	22	35	29
Vanillin	151.10	136.20	10	15	22
		92.20	10	22	16

The signal of the first transition reaction (a) was used as quantitative parameter, and the second transition (b) was used to confirm the compound identity.

The phenolic compounds quantification was performed by constructing calibration curves using the peak areas of the first transitions. The primary standard stock solution of each standard (1 mg/mL) was prepared in methanol. The working solution containing all standards (1.25 µg/mL) and the series of working solutions (concentration ranging from 20 to

1000 ng/mL) were made in 0.1% formic acid in water. Results were expressed as μg per gram of dried mutamba fruit (μg/g dw) (Arruda et al., 2018; Pereira et al., 2018).

2.7 Statistical analysis

The data were submitted to one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$). Statistical analyses of the data were carried out by using the STATISTICA software (Statsoft, version 12.0, Oklahoma, USA). The data are presented as mean values with standard deviation of three measurements.

3 Results and discussion

3.1 Physicochemical properties and proximate composition of mutamba fruit

Table 2 shows the physicochemical properties and proximate composition of mutamba fruit.

Table 2. Proximate composition (g/100 g fw) and physicochemical properties of mutamba fruit.

Component	Mutamba fruit
Physicochemical parameter	
pH	4.64 ± 0.02
Total titratable acidity (TTA)	1.41 ± 0.07
Total Soluble Solids (TSS)	40.71 ± 2.17
TSS/TTA ratio	28.80 ± 0.45
Proximate composition	
Moisture	10.03 ± 0.37
Protein	7.02 ± 0.35
Ash	4.17 ± 0.63
Total lipid	3.52 ± 0.50
Total dietary fibre	36.93 ± 0.89
Carbohydrate, by difference	38.33 ±1.05
Glucose	4.17 ± 0.06
Fructose	5.43 ± 0.11
Sucrose	16.34 ± 0.12
Total mono- and disaccharides	25.94 ± 0.10
Unknown carbohydrates	12.39 ± 1.15
Energy value (kcal/100 g fw)	213.07 ± 2.82

The contents of components are presented as mean values with standard deviation.

fw: Data expressed on a fresh weight basis; TTA, total titratable acidity (g citric acid equivalent/100g fw); TSS, total soluble solids (°Brix).

Fruit taste is closely related to the composition and concentration of sugars and their balance with acids; that means, the sweetness and acidity proportion (TSS/TTA ratio) can indicate the fruit taste in which low values can represent the predominance of sour taste (Colaric, Veberic, Stampar, & Hudina, 2005). Mutamba fruit showed pH of 4.6, TSS of 40.7 °Brix, TTA of 1.4 g citric acid equivalent/100 g fw, and low TSS/TTA ratio (28.8), the last value was similar to guava (from 18.9 to 25.5) (Batista, de Lima, da Trindade, & Alves, 2015) and pineapple (from 25.0 to 35.0) (Saradhuldhat & Paull, 2007). Nevertheless, this fruit showed a high content of mono-, and disaccharides, mainly sucrose (high sweetness factor; 100), which can conferee a sweet taste to mutamba fruit as reported by ethnobotanical studies (Tene et al., 2007). Mutamba fruit has an interesting sweetness and sourness balance like guava and pineapple, which may explain why the consumers appreciate this fruit. Interestingly, mutamba fruit showed a very low moisture content (10.0%) as compared to other fruits, for example (but not limited to) banana, avocado, papaya, and melon (values ranging from 74 to 93%) (Jovanovic-Malinovska, Kuzmanova, & Winkelhausen, 2014; Morais et al., 2016). Its low moisture content could enable a high stability during the post-harvests, transport, and fruit processing. Moreover, the drying process to obtain a flour may be discharged as this fruit is naturally dried (10.0%), which can decrease the fruit processing cost; according to Codex Standard 152-1985, the moisture content of flour cannot be more than 15%.

Mutamba fruit has not been consumed as fresh fruit due to its hardness, but it has been used as in flour form to prepare, for example, tea, whole bread, and popsicles (Assis et al., 2019; Tene et al., 2007). Although the fruit is hard and dry, the ethnobotanical reports as well as the physicochemical data presented on Table 2 demonstrated that this fruit has a good flavour (sweet pulp and unique aroma) (Tene et al., 2007). The fruit indeed showed a high content of total dietary fibre (36.93 ± 0.89 g/100 g fw), which can justify its hardness. Nevertheless, its high fibre content could be explored by food industry, as the daily intake of dietary fibre has been associated with human health and wellness (Stephen et al., 2017). With regard to dietary fibre intake, the World Health Organization (WHO) recommends the daily intake of at least 25 g of dietary fibre. The use of mutamba fruit as food ingredient (*e.g.*: as a flour) can contribute to the increase in the consumption of fibre as well as indirectly help the prevention of diseases, such as obesity and its comorbidities (diabetes and cardiovascular dysfunctions) (Dhingra, Michael, Rajput, & Patil, 2012; Stephen et al., 2017). Moreover, it has been recently reported a mucilaginous substance (soluble fibre) from mutamba seed (Pereira et al., 2019). Certainly, the use of mutamba fruit as a source of dietary fibre and its bioactivities should be evaluated by using *in vivo* model, but its high fibre content and the strong scientific evidences about the

dietary fibre's health effects indicate a promising use of this fruit by food industry. It is important to point out that the mutamba fruit showed fibre content higher than barley (17.3 g/100 g fw), wheat whole grain (12.6 g/100 g fw), white beans (17.7 g/100 g fw), coconut (9.0 g/100 g fw) and flaxseed (22.3 g/100 g fw), which have been reported as fibre source (Dhingra et al., 2012).

Mutamba fruit showed a remarkable content of protein (7.72 g/100 g dw), ash (4.58 g/100 g dw), and lipid (3.87 g/100 g dw) as compared with tropical fruits commonly consumed by Brazilian people, such as avocado, pineapple, banana, passion fruit, watermelon, and melon. The protein content was higher than pineapple (5.2 g/100 g dw), banana (4.4 g/100 g dw), papaya (6.5 g/100 g dw), and lower than avocado (12.5 g/100 g dw), and passion fruit (9.9 g/100 g dw). Mutamba fruit showed ash content lower than melon (6.1 g/100 g dw), but it was higher than avocado, pineapple, banana, papaya, passion fruit, and watermelon (from 1.8 to 4.2 g/100 g dw). The lipid content was higher than pineapple, banana, papaya, passion fruit, and melon (from 1.0 to 1.8 g/100 g dw), and lower than avocado (28.6 g/100 g dw) (de Morais et al., 2016). Furthermore, the mutamba fruit showed high content of total carbohydrates (42.2 g/100 g dw) containing 28.5 g/100 g dw of mono-, and disaccharides, namely glucose, fructose, and sucrose, and 13.6 g/100 g dw of unknown carbohydrates. The content of glucose (4.6 g/100 g dw) and fructose (6.0 g/100 g dw) was lower than apple, melon, strawberry, blueberry, watermelon, and grape, values ranging from 15.7 to 71 g/100 g dw and 15.3 to 39.2 g/100 g dw, respectively. Unlike glucose and fructose, the sucrose content (18.0 g/100 g dw) of mutamba fruit was higher than apple, blueberry, grape, strawberry, and watermelon (from 3.2 to 16.8 g/100 g dw) (Jovanovic-Malinovska et al., 2014). This fruit showed energy value (213.1 kcal/100 g fw) higher than, for example, banana, guava, orange and avocado (NEPA-UNICAMP, 2011). The proximate composition data reported herein corroborated with previous reports (Assis et al., 2019; Ortega et al., 1998).

The data clearly indicated that the mutamba fruit could be a source of energy contributing to the dietary intake of nutrients, namely protein, lipid, ash and carbohydrates. Furthermore, the physicochemical and proximate composition data indicated that the mutamba fruit has great potential to be explored by food industry in the development of functional foods, such as (but not limited to) tea and whole bread. Nevertheless, the nutritional profile of this fruit (pulp and seed) addressing, for example, the composition of mineral elements, fat acids, amino acids, organic acids, and soluble and insoluble fibre content should be evaluated to support the next scientific studies on food development and biological properties of mutamba fruit.

3.2 Individual phenolic compounds from mutamba fruit by HPLC-ESI-MS/MS

The phytochemical profile of stem bark and leaves of mutamba (*Guazuma ulmifolia* Lam.) has been determined, and the literature data show phenolic compounds as the main bioactive compounds. Unlike stem bark and leaves, the phenolic compounds from mutamba fruit have not been evaluated despite its potential evidenced by ethnopharmacological studies. In this study, the soluble and insoluble-bound phenolic compounds from mutamba fruit were extracted, and their individual phenolic compounds determined by HPLC-ESI-MS/MS (Arruda et al., 2018). **Figure 1** shows the chromatograms obtained by HPLC-ESI-MS/MS of soluble (A) and insoluble-bound (B) mutamba fruit extracts as well as the identified compounds, while the content (µg/g dw) of the identified phenolic compounds is shown on **Table 3**.

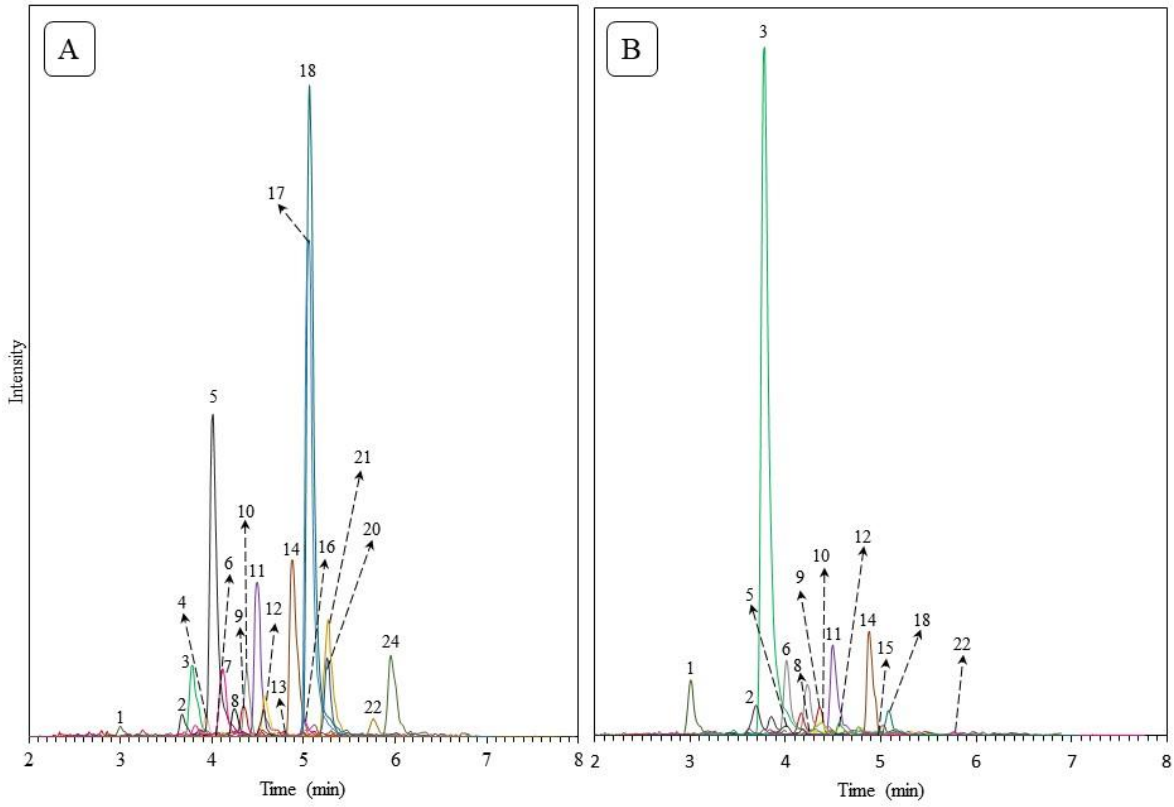

Figure 1. Chromatograms obtained by HPLC-ESI-MS/MS showing the phenolic compounds identified in soluble (A) and insoluble-bound (B) mutamba fruit extracts. The compounds eluted as follows: ***1-*** gallic acid, 2.98 min; ***2-*** procyanidin dimer B1, 3.66 min; ***3-*** protocatechuic acid, 3.77 min; ***4-*** epigallocatechin, 3.93 min; ***5-*** procyanidin dimer B2, 4.00 min; ***6-*** catechin, 4.01 min; ***7-*** procyanidin trimer C1, 4.10 min; ***8-*** chlorogenic acid, 4.23 min; ***9-*** p-hydroxybenzoic acid, 4.33 min; ***10-*** epicatechin, 4.38 min; ***11-*** caffeic acid, 4.50 min; ***12-*** gentisic acid, 4.57 min; ***13-*** vanillin, 4.77 min; ***14-*** p-Coumaric acid, 4.87 min; ***15-*** ferulic acid, 4.95 min; ***16-*** cymaroside, 5.01 min; ***17-*** rutin, 5.04 min; ***18-*** hyperoside, 5.06 min; ***19-*** ellagic acid, 5.24 min; ***20-*** nicotiflorin, 5.27 min; ***21-*** astragalin, 5.29 min; ***22-*** quercetin, 5.76 min; ***23-*** naringenin, 5.86 min; ***24-*** luteolin, 5.96 min; ***25-*** kaempferol, 6.35 min; ***26-*** isorhamnetin, 6.52 min.

Table 3. Content of individual phenolic compounds of mutamba fruit determined by HPLC-ESI-MS/MS method.

Compound	Content expressed as µg/g dw (µg/g fw)		
	Soluble Phenolic	Insoluble-bound Phenolic a	Total
Phenolic acids			
p-hydroxybenzoic acid	1.91 ± 0.31 (1.73 ± 0.28)E	1.71 ± 0.42 (1.55 ± 0.38)E *ns*	3.61 ± 0.63 (3.28 ± 0.57)G
Caffeic acid	5.44 ± 0.68 (4.94 ± 0.62)E	2.46 ± 0.09 (2.23 ± 0.08)E *	7.90 ± 0.72 (7.18 ± 0.66)G
5-*O*-caffeoylquinic acid (Chlorogenic acid)	2.96 ± 0.11 (2.69 ± 0.10)E	1.45 ± 0.28 (1.32 ± 0.26)E *	4.41 ± 0.38 (4.01 ± 0.34)G
Ellagic acid	Traces	Traces	Traces
Ferulic acid	Traces	7.24 ± 0.25 (6.58 ± 0.23)D	7.24 ± 0.25 (6.58 ± 0.23)G
Gallic acid	3.50 ± 0.67 (3.18 ± 0.61)E	14.92 ± 1.23 (13.56 ± 1.12)C *	18.41 ± 0.83 (16.74 ± 0.76)EFG
Gentisic acid	12.64 ± 1.02 (11.49 ± 0.93)DE	2.88 ± 0.17 (2.62 ± 0.16)E *	15.52 ± 1.16 (14.11 ± 1.05)FG
p-Coumaric acid	10.73 ± 0.32 (9.76 ± 0.29)DE	3.20 ± 0.46 (2.91 ± 0.42)E *	13.93 ± 0.24 (12.66 ± 0.21)FG
Protocatechuic acid	5.39 ± 0.50 (4.90 ± 0.46)E	57.82 ± 0.94 (52.56 ± 0.85)B *	63.21 ± 1.02 (57.46 ± 0.93)CDE
Flavonoinds			
Catechin	5.21 ± 3.22 (4.74 ± 2.92)E	98.59 ± 1.95 (89.63 ± 1.77)A *	103.81 ± 5.03 (94.37 ± 4.57)BC
Epicatechin	81.09 ± 11.40 (73.72 ± 10.37)C	17.44 ± 3.66 (15.86 ± 3.33)C *	98.54 ± 14.85 (89.58 ± 13.50)BCD
Epigallocatechin	18.20 ± 1.07 (16.54 ± 0.98)DE	n.d.	18.20 ± 1.07 (16.54 ± 0.98)EFG
Kaempferol	Traces	n.d.	Traces
Kaempferol 3-*O*-glucoside (Astragalin)	8.67 ± 0.72 (7.88 ± 0.66)E	Traces	8.67 ± 0.72 (7.88 ± 0.66)G
Kaempferol 3-*O*-rhamnosyl-glucoside (Nicotiflorin)	3.95 ± 0.38 (3.59 ± 0.34)E	n.d.	3.95 ± 0.38 (3.59 ± 0.34)G
Luteolin	3.90 ± 0.40 (3.54 ± 0.36)E	Traces	3.90 ± 0.40 (3.54 ± 0.36)G
Luteolin 7-*O*-glucoside (Cynaroside)	6.37 ± 1.03 (5.79 ± 0.93)E	n.d.	6.37 ± 1.03 (5.79 ± 0.93)G
Naringenin	Traces	n.d.	Traces
Procyanidin dimer B1	13.38 ± 0.98 (12.17 ± 0.89)DE	14.76 ± 2.33 (13.41 ± 2.12)C *ns*	28.14 ± 3.29 (25.58 ± 2.99)EFG
Procyanidin dimer B2	139.02 ± 4.27 (126.38 ± 3.88)B	4.26 ± 0.14 (3.88 ± 0.13)DE *	143.29 ± 4.38 (130.26 ± 3.99)B
Procyanidin trimer C1	972.83 ± 78.64 (884.39 ± 71.49)A	n.d.	972.83 ± 78.64 (884.39 ± 71.49)A
Quercetin	4.06 ± 0.23 (3.69 ± 0.21)E	1.00 ± 0.09 (0.91 ± 0.08)E *	5.05 ± 0.26 (4.59 ± 0.24)G
Quercetin 3-*O*-galactoside (Hyperoside)	54.65 ± 1.27 (49.68 ± 1.16)CD	1.10 ± 0.10 (1.00 ± 0.09)E *	55.75 ± 1.20 (50.68 ± 1.09)DEF
Quercetin 3-*O*-methyl ether (Isorhamnetin)	Traces	n.d.	Traces
Quercetin 3-*O*-rhamnosyl-glucoside (Rutin)	74.60 ± 1.31 (67.82 ± 1.19)C	Traces	74.60 ± 1.31 (67.82 ± 1.19)CD

Other Phenolics			
Vanillin	7.01 ± 1.32 (6.37 ± 1.20)E	n.d.	7.01 ± 1.32 (6.37 ± 1.20)G
Total Phenolic acids	*42.57 ± 0.82 (38.70 ± 0.75)*	*91.67 ± 2.00 (83.33 ± 1.82) **	*134.23 ± 2.43 (122.03 ± 2.21)*
Total Flavonoids	*1385.94 ± 72.72 (1259.95 ± 66.10)*	*137.15 ± 4.51 (124.68 ± 4.10) **	*1523.09 ± 74.25 (1384.63 ± 67.50)*
Total Other Phenolics	*7.01 ± 1.32 (6.37 ± 1.20)*	*n.d.*	*7.01 ± 1.32 (6.37 ± 1.20)*
Total Phenolic Compounds	**1435.51 ± 73.41 (1305.01 ± 66.74)**	***228.82 ± 6.44 (208.02 ± 5.86) ****	***1664.33 ± 75.30 (1513.03 ± 68.46)***

fw: Data expressed on a fresh weight basis. dw: Data expressed on a dry weight basis. Data were originally expressed on fw but they were transformed to dw according to the moisture content of fresh fruit (10%, *see Table 2*). n.d.: Not detected. Total phenolic compounds content was calculated as the sum of the contents of all detected phenolic compounds.

The compound content is presented as mean value with standard deviation. Mean values within a column followed by same letters are not significantly different by Tukey's HSD test ($p \leq 0.05$).

a: *, mean values within a line (soluble and insoluble-bound) were statistically different by Student's t-test ($p \leq 0.05$); *ns*, non-significant.

HPLC-ESI-MS/MS identified 26 compounds (9 phenolic acids, 16 flavonoids, and 1 other phenolic) and 18 compounds (9 phenolic acids and 9 flavonoids) in soluble and insoluble-bound mutamba fruit extracts, respectively. The soluble fraction showed procyanidin trimer C1 (972.8 µg/g dw) followed by procyanidin dimer B2 (139.0 µg/g dw), rutin (74.6 µg/g dw), epicatechin (81.1 µg/g dw), and hyperoside (54.7 µg/g dw) as main phenolic compounds. Conversely, the insoluble-bound fraction showed catechin (98.6 µg/g dw), protocatechuic acid (57.8 µg/g dw), epicatechin (17.4 µg/g dw), gallic acid (14.9 µg/g dw), and procyanidin dimer B1 (14.8 µg/g dw) as the main phenolic compounds. The total phenolic compounds (expressed as the sum of soluble and insoluble-bound) was composed mainly by flavonoids, namely procyanidin trimer C1 (972.8 µg/g dw), procyanidin dimer B2 (143.4 µg/g dw), catechin (103.8 µg/g dw), epicatechin (98.5 µg/g dw), and rutin (74.6 µg/g dw). Indeed, the mutamba fruit showed flavonoid content (1523.3 µg/g dw) up to 11 times higher than phenolic acid content (134.2 µg/g dw). These flavonoids were found mainly in the soluble form (1385.9 µg/g dw), which indicates that the mutamba fruit was composed by soluble phenolic compounds, especially procyanidins and aglycones and glycosylated flavonoids. Certainly, the insoluble-bound phenolics contribute to the secondary metabolic profile of mutamba fruit, but comparing the number of identified compounds and the content of them between both fractions, the soluble phenolic compounds stand out of insoluble-bound. Therefore, the data clearly showed that the phenolic compounds of mutamba fruit were found mainly as soluble forms. This result can be easily observed in the **Figure 2** in which the soluble data covers an area higher than insoluble-bound data.

The literature data report the phenolic compounds, especially proanthocyanidins, flavonoids and phenolic acids, as the main class of secondary metabolites in the stem bark and leaves of mutamba. Proanthocyanidins, namely procyanidin dimer B2 and B5, epiafzelechin-(4β→8)-epicatechin, procyanidin trimer C1, epicatechin-[4β→8]-epicatechin-[4β→6]-epicatechin, epicatechin-[4β→6]-epicatechin-[4β→8]-epicatechin, and Cinnamtannin A2, and flavonoids aglycones (catechin, epicatechin, gallocatechin, epigallocatechin, and 4'-O-methyl-epiafzelechin) were identified in stem bark (Hör et al., 1996; Lopes et al., 2009; Magos et al., 2008). Unlike stem bark, the phytochemical studies on mutamba leaves identified phenolic acids (chlorogenic acid and caffeic acid) and flavonoids (catechin, quercetin 3-*O*-rhamnosyl-glucoside, quercetin 3-*O*-rhamnosyl, quercetin and luteolin) as main compounds (Calixto Júnior et al., 2016; S. M. Morais et al., 2017). Procyanidin dimer B and glycosylated derivatives of catechin, quercetin, kaempferol and luteolin were detected in these leaves (de Souza, da

Silva, Bezerra, Ferreira, & Soares, 2018; dos Santos et al., 2018). Interestingly, the phenolic profile of mutamba fruit was similar to other plant parts, such as stem bark and leaves. Mutamba fruit showed proanthocyanidins, such as procyanidin dimer B1, B2, and procyanidin trimer C1, aglycones and glycosylated flavonoids, and phenolic acids (**Table 3**). This similarity between different mutamba parts indicates that the biosynthesis of phenolic compounds on *Guazuma ulmifolia* Lam. may follow a common metabolic pathway, regardless of botanical plant part (Caretto, Linsalata, Colella, Mita, & Lattanzio, 2015). Unfortunately, we did not find reports covering the content of phenolic compounds in the stem bark and leaves of mutamba, which hampers a quantitative comparison.

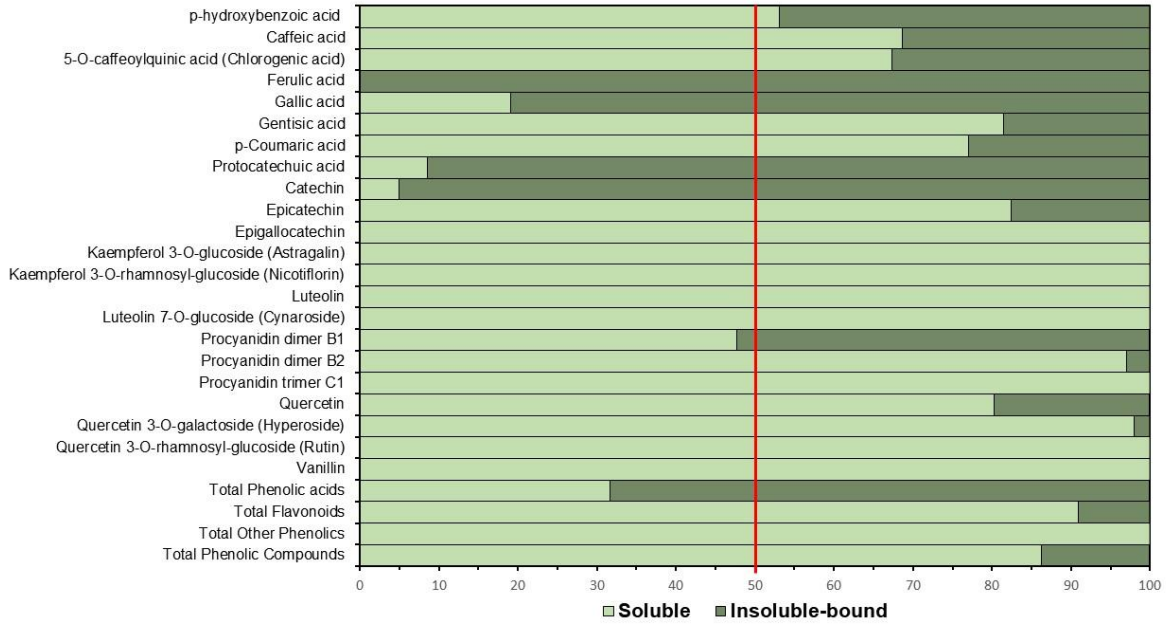

Figure 2. Soluble and insoluble-bound phenolic compounds from mutamba fruit. Content expressed as percentage.

Phenolic compounds play a significant role in plants as secondary metabolites closely responsible for protecting the plants from biotic and abiotic stresses, as well as serving as attractant pollinators, chemical signalling, and regulating plant architecture and growth. This class of compounds is widely found in food, especially plant-derived food (*e.g.*: fruits, vegetables, wine, beer, coffee, cocoa, and fruit juices), therefore, people have daily intaken them. The phenolic compounds have been associated with the astringency of food, especially condensed tannins. Furthermore, phenolic compounds have attracted great interest from different fields of study due to their health-promoting effects, for example, antioxidant, antimicrobial, anti-inflammatory and anti-diabetic agent, as well as to their abundance in the

nature and human diet (Shahidi & Yeo, 2016; Tsao, 2010). It is therefore very important to detail the nature and quantities of phenolics in mutamba fruit aiming to evaluate the relative contribution of this food or its products to the intake of a particular phenolic as compared to other food sources, as well as studying its association with health and disease (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010; Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010).

Mutamba fruit is not commonly consumed as a fresh fruit due to its hardness. As consequence, the phenolic compounds from this fruit would be available by means of intaking of mutamba fruit products, such as whole bread and tea. The ethnopharmacological studies reported that the mutamba fruit has been used to prepare tea. Traditional healers have indicated this tea as medicine to treat cough, influenza, diarrhoea, and haemorrhage. The findings of this study showed that mainly soluble phenolic compounds, especially condensed tannins, and flavonoids aglycones and glycosylated, composed mutamba fruit. Therefore, the effect of mutamba fruit tea reported by traditional medicine could be linked to these bioactive molecules.

Mutamba fruit showed a remarkable total phenolics content, value of 130.5 mg/100 g fw (total soluble phenolics content), comparing it with the Phenol-Explorer data base (an online comprehensive database on polyphenol contents in foods) (Phenol-Explorer, 2019). Total phenolics content (130.5 mg/100 g fw) was lower than the richest source of phenolics, such as cocoa powder (3448.0 mg/100 g fw), dark cocoa (1664.0 mg/100 g fw), flaxseed meal (1582.0 mg/100 g fw), and plum (377 mg/100 g fw). However, the mutamba fruit showed phenolics content similar to apple (136 mg/100 g fw), and higher than peach (59 mg/100 g fw), and broccoli (45 mg/100 g fw). Nevertheless, the mutamba fruit may be among the 100 richest dietary sources of polyphenols (Pérez-Jiménez et al., 2010). This fruit can contribute to the intake of phenolic compounds, especially procyanidin trimer C1, procyanidin dimer B2, rutin, epicatechin, and hyperoside. For example, the procyanidin trimer C1 content (87.4 mg/100 g fw) was higher than dark chocolate (26.0 mg/100 g fw), cocoa powder (25.8 mg/100 g fw), plum (10.0 mg/100 g fw), and dessert apple without peel (7.0 mg/100 g fw). The procyanidin dimer B2, rutin, epicatechin, and hyperoside contents were similar to apple and plum, values ranging from 5.0 to 12.6 mg/100 g fw (Phenol-Explorer, 2019).

Phenolic compounds occur in both soluble and insoluble-bound forms. Insoluble-bound phenolics form covalent bonds with cell wall matrix via ester, ether and C-C bonds, and account a high content in foods, especially cereals. Unlike soluble phenolic compounds, the bound phenolics cannot be absorbed at the small intestine because they are bound to insoluble macromolecules such as cellulose, hemicellulose, structural protein and pectin. Thus, they pass

into the large intestine (colon) in which they undergo fermentation by the colon microbiota by a number of microorganisms, which release the bound phenolics. These released phenolic compounds may promote a number of health benefits, such as lowering the pH, develop fermentative microflora and preventing the growth of pathogenic and carcinogenic bacteria (Mudenuti et al., 2018; Shahidi & Yeo, 2016). Therefore, evaluating the insoluble-bound compounds is very important to find the actual functional composition of mutamba fruit, as well as providing scientific support to studies about its effect on human health (Arruda et al., 2018; Pereira et al., 2018).

The cell wall matrix should be hydrolysed to liberate the insoluble-bound phenolic compounds from mutamba fruit in order to measure their contents. Enzyme treatment is close to what happens in human gastrointestinal tract, in which a variety of enzymes, such as carbohydrases, proteases and other types of enzymes, lead to the disruption of cell wall matrix, followed by phenolic liberation (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2016). As consequence, alkaline hydrolysis (chemical treatment) is considered less specific than enzyme treatment, but it has been used as a first level screening due to its low cost and time consumption, which is the purpose of this study (Mudenuti et al., 2018). Therefore, we performed an alkali hydrolysis of residue from soluble phenolics extraction (*see Section 2.4*). This alkaline hydrolysis released phenolic acids, mainly ferulic acid, gallic acid, and protocatechuic acid, and flavonoids, including catechin and epicatechin (**Table 3**). These bound phenolic acids and flavonoids have been commonly identified after hydrolysis treatment of foods (Shahidi & Yeo, 2016). The bound phenolic compounds from mutamba fruit showed very low content (from 6.6 to 89.6 µg/g fw) as compared with the bound phenolics of raw chocolate, namely protocatechuic acid (4465 µg/g fw), epicatechin (287.0 µg/g fw), and catechin (2193 µg/g fw) (Mudenuti et al., 2018), and different millets variety, including ferulic acid (from 178.2 to 1685 µg/g defatted millet) (Chandrasekara & Shahidi, 2010). Besides that, the bound phenolic compounds content in mutamba fruit were similar to other foods (Shahidi & Yeo, 2016), such as lentils (Alshikh, de Camargo, & Shahidi, 2015).

Mutamba fruit showed a major contribution of soluble than insoluble-bound phenolics, as previously discussed. Nevertheless, the insoluble-bound phenolics cannot be inconsiderate, regardless of its low content, once some phenolic compounds, such as catechin and epicatechin, have shown high antioxidant activity even at low concentration, which indicates the potential health-promoting effect of bound phenolics from mutamba fruit (Pereira et al., 2018). The mutamba fruit can contribute to the intake of soluble and insoluble-bound

phenolic compounds. We suggested the intake of soluble phenolics from mutamba fruit by means of tea. Conversely, the insoluble-bound can be intaken by means of products using mutamba flour, such as whole bread (Assis et al., 2019). Therefore, mutamba fruit (especially the mutamba flour) may contribute to local gut health after liberation of insoluble-bound phenolics by human microbiota, as well as due to its high fibre content (*see Section 3.1*) (Alshikh et al., 2015; Dhingra et al., 2012; Mudenuti et al., 2018; Shahidi & Yeo, 2016; Stephen et al., 2017). Certainly, this expectation should be confirmed by *in vitro* and *in vivo* studies.

As a general remark, the mutamba fruit showed a high content of soluble phenolics, namely proanthocyanidins, and aglycones and glycosylated flavonoids, and insoluble-bound phenolics, including phenolic acids and flavan-3-ols. Mutamba fruit showed a diversity of phenolic compounds from simple molecules, such as gallic acid, to oligomeric compounds (procyanidin C1). The phenolic structure feature has been associated with a broad spectrum of health effects, for example, oxidative stress management (antioxidant), in addition to other properties (Shahat & Marzouk, 2013; Xiao, 2015). With regard to procyanidin trimer C1 (the main phenolic compound of mutamba fruit), this isolated compound has shown bioactivities, such as (but not limited to) anti-inflammatory (Byun et al., 2013), anti-allergic (Nakano et al., 2008), anti-cancer (Kin et al., 2013), cardioprotective (Byun et al., 2014), and neuroprotective effects (Song, Lee, & Kang, 2019), and the capability of modulating immune responses (Sung et al., 2013). Therefore, the consumption of mutamba fruit and its products may contribute to the intake of bioactive phenolics compounds and, consequently, promote beneficial effects on human health and wellbeing. The effect of mutamba fruit on human health should be evaluated by means of *in vivo* studies, in which the complexity of human metabolism is taken in account. Nevertheless, the data reported herein clearly demonstrated that mutamba fruit could be a potential source of bioactive phenolic compounds in both soluble and insoluble-bound forms.

4 Conclusion

The proximate composition and physicochemical data indicated that mutamba fruit is hard and dry due to its high dietary fibre (36.9 %) and low moisture (10.0 %) contents. Although the mutamba fruit is not consumed as fresh fruit, it has been used to prepare tea and whole bread due to its sweet pulp (high sucrose content, 16.3 %) and attractive taste (sweetness and sourness balance). Furthermore, the HLPC-ESI-MS/MS analysis identified 26 phenolic compounds, especially proanthocyanidins, and aglycones and glycosylated flavonoids. These compounds were found mainly as soluble form, whereas phenolic acids and flaval-3-ols were

the main bound phenolics. Mutamba fruit showed procyanidin trimer C1 followed by procyanidin dimer B2, rutin, epicatechin, hyperoside, catechin, protocatechuic acid, epicatechin, gallic acid, and procyanidin dimer B1 as the main phenolic compounds. This fruit showed a diversity of phenolic compounds accounting a high content.

Mutamba (*Guazuma ulmifolia* Lam.) is a hard and dry fruit commonly used to prepare tea due to its sweet pulp and attractive taste. Latin Americans appreciate this tea as well as they have used it as traditional medicines to treat diseases, such as influenza, diarrhoea, and haemorrhage. These ethnopharmacological reports indicate a therapeutic potential of mutamba fruit despite the lack of scientific evidences covering its biological activities and phytochemical composition. The data reported herein bring an insight about the potential of mutamba fruit as food and therapeutic plant since this fruit showed an interesting proximate composition and phytochemical profile. The bioactivities of mutamba fruit reported by ethnopharmacological studies could be related to its high dietary fibre and phenolic compounds contents.

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CHAPTER III

RESEARCH ARTICLE

OBTAINING A NOVEL MUCILAGE FROM MUTAMBA SEEDS EXPLORING DIFFERENT HIGH-INTENSITY ULTRASOUND PROCESS CONDITIONS

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Obtaining a novel mucilage from mutamba seeds exploring different high-intensity ultrasound process conditions

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ABSTRACT

We evaluated the effect of ultrasonic power (200–600 W) and process time (1–7 min) on the recovery of a novel polysaccharide from mutamba (*Guazuma ulmifolia* Lam.) seeds applying high-intensity ultrasound. Ultrasound process conditions intensification gradually was removing the mucilage layer around the hydrated seeds. Then, the scanning electron micrographs showed that the mucilage was removed completely at the highest applied energy density (10,080 J/mL). Although the colour of mutamba seed mucilage (MSM) have been changed due to increase of energy density, it not affects its practical use because the MSM can be purified to remove impurities. The results obtained in this study demonstrated that the ultrasound process conditions intensification did not affect the primary structure of MSM according to ζ-potential, FTIR spectrum, and monosaccharide residues data. In conclusion, ultrasound process conditions intensification allows the full recovery of the MSM at a short process time (7 min) without altering its quality and the primary structure.

1. Introduction

Natural gums and mucilages are water-soluble polymers that can be extracted from plants, animals, and seaweed, and they also can be produced by bioprocess. In addition, gums can be chemically synthetized (synthetic gums), and natural gums can also be semi synthetically modified to produce derivatives with unique physicochemical and rheological properties (modified gums). These hydrocolloids are widely used in food and pharmaceutical industries as an emulsifying, foaming, thickening, gelling, binding, fat replacing, and stabilizing agent. They also can be used as a source of dietary fibre, pharmaceutical excipient, and biodegradable edible film [1]. Over the past few decades, natural polymeric gums and mucilages have been extensively studied due to their many advantages as compared to synthetic polymers, such as biodegradable, nontoxic, economical and easily available in the environment. Furthermore, novel and natural gums/mucilages with unique properties could be widely used in both food and non-food industries for a broad range of applications [2,3].

A novel and natural mucilage can be extracted from mutamba (*Guazuma ulmifolia* Lam.) seeds. The species *Guazuma ulmifolia* Lam. belongs to Sterculiaceae family and occurs naturally throughout Latin America. Mutamba fruit is small (average weight of 3.64 g) and black-coloured at mature stage, and the harvest occurs from August to September [4]. Each fruit contain approximately 87 small seeds with the average weight of 0.01 g. Upon imbibition with water, the mutamba seeds liberate a viscous mucilage that envelops the seeds to form a gel-like capsule [5]. To the best of our knowledge, the extraction and the physicochemical, structural, rheological, and functional properties of mutamba seed mucilage have not been reported.

The extraction and structural characterization of mucilages is an essential step in establishing their potential as food and non-food ingredient [1]. The extraction of mucilages using conventional procedures is tedious due to very time-consuming and experimental conditions, such as the heating of extraction medium, pH alteration, addition of chemical agents, and organic solvent use. Furthermore, the conventional extraction procedures can promote loss of polysaccharide, and chemical structure changes, and therefore loss of its functionalities [6–10]. The extraction of polymeric hydrocolloids from food plant-based materials have therefore recently been carried out by using emergent technologies, among which we can mention the high-intensity ultrasound (HIUS) technology [6–10].

In ultrasound-assisted recovery, the ultrasound's waves cause

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mechanical vibration of the liquid of extraction system and bubbles are formed from gas nuclei existing within the fluid. These bubbles can grow to a critical size and thus violently collapse, phenomenon known as acoustic cavitation. The phenomenon of acoustic cavitation generate several physical effects, such as shock waves, micro-jets, turbulence, shear forces, and increasing of temperature and pressure [11,12]. Ultrasound can be operated in two different levels of frequency: high (100 kHz–1 MHz) and low (16–100 kHz) frequencies applying intensity levels of ≤ 1 W/cm^2 and 10–1000 W/cm^2, respectively. As ultrasound frequency increases, the production and intensity of acoustic cavitation in liquid decreases. Therefore, low-frequency ultrasound (high-intensity ultrasound, HIUS) has become an important, inexpensive, simple, and efficient processing tool in the food sector to improve through its physical effects the process response, such as extraction yield, decrease droplet size in emulsion, and enhance the flux in ultrafiltration or microfiltration [11,13,14]. Furthermore, it is easy to operate, control and clean an HIUS apparatus, and HIUS is associated with low production costs and low time consuming.

The ultrasound treatment has been used to recovery mucilage from seeds [6–8,10,15,16]. However, the effect of HIUS process conditions intensification on quality, and physical and chemical properties of polysaccharides has not been clearly studied. In this approach, the aim of this study was to evaluate the impact of HIUS processing conditions (power and process time) on the recovery, quality, and structure of a novel mucilage from mutamba seeds. In addition, a preliminary structural evaluation of the mucilage was also carried out.

2. Material and methods

2.1. Plant material

The morphologically perfect and mature mutamba (*Guazuma ulmifolia* Lam.) fruits were collected in August 2017 in natural areas of the Cerrado Biome located in the municipality of Uberlândia (18°55′07″ south latitude, 48°16′38″ west longitude and 863 m altitude), Minas Gerais, Brazil. All fruits collected (1 kg) were transported from the collection site to the laboratory up to 48 h after collection. The fruits were manually opened with the aid of a knife and the seeds were easily separated from the pulp. The selected seeds (free of defects) were placed to vacuum packs, and it was stored in a desiccator until mucilage extraction. The Genetic Heritage Management Board (CGen) under number AA56F0A, following the Law n° 13.123/2015 and its regulations, regimented the activity of access to Genetic Heritage.

2.2. HIUS processing

Approximately 400 mg of seed was precisely weighed in 50 mL plastic tubes with conical bottom containing 30 mL of deionized water (Millipore®). The mixture was kept at room temperature for 20 h to ensure complete hydration and swelling. The mucilage layer was extracted using a 13 mm diameter, 19 kHz ultrasonic probe (Unique, Desruptor, 800 W, Indaiatuba, Brazil) at different power levels (200, 400 and 600 W) and process time (1, 3, 5 and 7 min). The operating extraction conditions with the different ultrasonic power and extraction time were determined according to previous studies [6–8,10,15,16]. The probe contact height with the medium was standardized to 30 mm. An ice bath was used to prevent overheating of the extraction medium. The maximum temperature of the sample, as measured at the outlet of the system, was 30.4 °C. After ultrasound extraction, the viscous liquid (mucilage suspension) was naturally separated from seeds with the aid of a stainless-steel sieve until the viscous liquid flow stopped (± 2 min).

2.3. Total sugars

Total sugars were determined with a phenol-sulfuric assay based on the method of Dubois et al. [17]. Briefly, 200 µL of diluted mucilage suspension was incubated with 200 µL freshly made 5% (w/v) aqueous phenol and 1000 µL concentrated sulfuric acid for 15 min at room temperature. Absorbance was detected at 490 nm and each sample was repeated three times. A linear response curve was obtained with glucose standards from 10 to 70 µg/mL and the results were expressed as g/100 g fw (fresh weight) seeds.

2.4. Global yield

The mucilage suspensions were freeze-dried to calculate the global yield of mucilage extracted from mutamba seeds. The global yield was calculated as the ratio of the total mass extracted (M_{ext}) to the mass of the raw seeds used (S), according to the following Eq. (1):

$$Global\ Yield\ (\text{g/100 g fw}) = \left(\frac{M_{ext}}{S}\right) * 100 \quad (1)$$

2.5. Colour analysis

The colour of the mucilage suspensions was measured six times using a colorimeter (Hunter Lab colorimeter, model Colour Quest II, Virginia, USA). The average values of luminosity (L^*) and of the chromaticity coordinates a^* and b^* were used to calculate the total colour-difference (ΔE^*) according to the Eq. (2), where subscript "o" refers to the colour reading of mucilage suspension extracted by gentle shaking (reference). A larger ΔE^* denotes greater colour change from the reference material.

$$\Delta E* = \sqrt{(L^* - L_o^*)^2 + (a^* - a_o^*)^2 + (b^* - b_o^*)^2} \quad (2)$$

2.6. Scanning electron microscopy (SEM)

After mucilage extraction, the seeds were vacuum dried at 60 °C for 24 h. The dry seeds were attached to a double-sided adhesive tape mounted on aluminium stubs, sputter-coated with 92 Å of gold (VG Microtech, Sputter Coater Polaron SC 7620, Uckfield, England) and viewed using a 440i Scanning Electron Microscope with EDS 6070 Energy Dispersive X-ray Detector (LEO Electron Microscopy/Oxford, Cambridge, England). The micrographs were obtained with an accelerating voltage of 15 kV, a beam current of 50 pA, and magnification of 100×. The thickness of the gold coating was calculated according to the Eq. (3), where K = 0.17 Å/mA.V.s; i = 3 mA; V = 1 V, and t = 180 s.

$$Å = K \times i \times V \times t \quad (3)$$

2.7. Visual observation of mucilage layer

The mucilage extraction was performed as previously described (*see* Section 2.2) using a freshly made aqueous fluorescein (25 µg/mL, w/v) instead deionized water at hydration step. After HIUS treatment, the hydrated seeds were placed on a microscope slide and photographs were taken using a digital camera Nikon D3200 (Melville, USA) operating in the automatic mode. The photographs were computationally transformed using the PowerPoint Pictures Tools to increase brightness (+40% brightness and +40% contrast), which improved the visualization of the mucilage around the mutamba seeds and the effect of different process conditions on the mucilage layer.

2.8. ζ-potential measurements

The surface charge density of the mucilage suspensions was determined by measuring the ζ-potential using a chamber microelectrophoresis (Malvern Instruments, ZetaSizer Nano Z, Worcestershire, England). The mucilage suspensions (pH equal to 5.7 ± 0.2) were

diluted in deionized water (1:100 v/v) according to the optimal detection range of the equipment. Six measurements were performed for each sample at 25 ± 2 °C.

2.9. Fourier transform infrared spectroscopy (FTIR)

The mucilage suspensions were freeze-dried. The KBr disk standard technique was used in the preparation of samples for infrared measurements. The mixture of 2 mg sample and 200 mg KBr powders were finely powdered in an agate mortar. The mixture was squeezed with a tablet press machine according to the manufacturer's instructions. FTIR spectroscopy in absorbance mode was performed on a Shimadzu IRPrestige-21 spectrometer. The measurements were performed at room temperature and the spectra ranging from 4000 to 800 cm^{-1} were selected with total of 10 scans at a resolution of 4 cm^{-1}.

2.10. Monosaccharide composition

The monosaccharide composition of mucilage extracted by gentle shaking (untreated) and by HIUS condition (600 W/7 min) was analysed as previously described [18,19]. In brief, 2500 µL of 2 M TFA was added to 20 mg of freeze-dried mucilage in a screw capped test tube, and it was heated at 121 °C and 9.8 × 10^4 N/m^2 by 60 min in a sterilizer autoclave. After that, the hydrolysate was diluted 100-fold with 18 mM NaOH (pH equal to 6.0), followed by filtration with a 0.22 µm filter, and 25 µL of diluted sample was injected into chromatography column at 30 °C (Carbopac PA1 (250 × 4 mm, 10 µm particle size)). Ion chromatography coupled to pulsed amperometric detection system model DIONEX ICS-5000 (Thermo Fisher Scientific, Waltham, USA) was used to perform the identification and quantification of monosaccharides. The mobile phase consisted of 0.2 M NaOH (A), ultrapure water (B), and 1 M sodium acetate containing 0.2 M NaOH (C) at flow rate of 1.0 mL/min. The gradient was performed as follows: 0–22 min, 4% A and 96% B; 22–24 min, 4–50% A and 96–50% B; 24–45 min, 50% A and 50% B; 45–55 min, 16% C and 84% B; 55–60 min, 100% A; and 60–65 min, 4% A and 96% B. All sugar standards from Sigma-Aldrich (St. Louis, USA) with purity ≥ 96%, namely rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl), mannose (Man), galacturonic acid (GalA), and glucuronic acid (GlcA) were diluted in ultrapure water and they were used to constructing calibration curves (0.25–12.5 µg/mL).

2.11. Protein, ash, and moisture content

The total protein (protein factor equal to 6.25) was measured using a Dumas Nitrogen Analyzer (VELP Scientifica, NDA 701, Usmate, Italy) according to the manufacturer's instructions. The total ash (n°940.26) and moisture (n°931.04) were measured according the protocols described by Association of Official Analytical Chemists, AOAC. The mineral concentration (K, Ca, Zn, Mg, Mn, Na, Cu, Fe) was carried out by using Atomic Absorption Spectrometer as exactly described by Silva et al. [20].

2.12. Statistical analysis

The HIUS process parameters of ultrasonic power (200, 400 and 600 W) and process time (1, 3, 5 and 7 min) were evaluated with a randomized full factorial design (3 × 4) with two blocks, totalling 24 experimental runs (Table 1). The effects of ultrasonic power (W), process time (min), and the interactive effects (W*min) on total sugars, global yield, ζ-potential, and total colour-difference were performed by ANOVA ($p < 0.05$) using the Minitab 18® software (Minitab Inc., State College, PA, USA).

Table 1
Influence of the ultrasonic power (W) and process time (min) on the sugar content, yield, total colour-difference (ΔE^*) and ζ-potential (ζ).

Ultrasonic Power (W)	Process Time (min)	Yield (%)	Sugar (g/100 g fw)	ΔE^*	ζ
200	1	2.10	1.75 ± 0.04	0.37 ± 0.03	−30.8 ± 2.2
	3	2.32	2.30 ± 0.1	0.33 ± 0.01	−36.0 ± 4.4
	5	3.55	2.63 ± 0.1	0.22 ± 0.03	−34.4 ± 5.8
	7	3.39	2.34 ± 0.1	1.07 ± 0.02	−36.6 ± 4.5
400	1	4.53	2.67 ± 0.1	0.61 ± 0.2	−35.2 ± 2.8
	3	4.73	3.01 ± 0.1	0.84 ± 0.04	−36.0 ± 3.3
	5	5.61	3.23 ± 0.03	2.90 ± 0.3	−31.9 ± 2
	7	6.45	3.56 ± 0.1	3.50 ± 0.4	−35.8 ± 3.6
600	1	4.78	3.25 ± 0.1	1.26 ± 0.04	−30.4 ± 3.1
	3	6.76	3.66 ± 0.2	3.51 ± 0.1	−39.5 ± 2
	5	7.83	4.37 ± 0.1	5.59 ± 0.1	−35.5 ± 1.4
	7	8.41	5.00 ± 0.1	5.95 ± 0.2	−39.3 ± 0.6

3. Results and discussion

3.1. Effect of the HIUS on mutamba seed mucilage extraction

The extraction of mutamba seed mucilage (MSM) was performed using HIUS applying different ultrasound process conditions. We evaluated the extraction process conditions aiming to obtain a high amount of a high-quality mucilage; it means without structural changes and, therefore, without loss of functionality. In this way, the aim of the present study was not to found the optimal extraction conditions, but to investigate the alternative use of HIUS in the clean and green extraction of MSM. In addition, a preliminary structural investigation was carried out to identify the monosaccharides residues and the proximate composition of the MSM.

3.1.1. Effect of the process parameters on global yield and total sugar content

Table 1 shows the mean values of global yield and sugar content, and the Fig. 1 the effect of HIUS process conditions on these responses. The applied power (W) and process time (min) significantly influenced the global yield and sugar content ($p < 0.0001$), whereas the interaction (W*min) only significantly affected the sugar content ($p < 0.0001$). The global yield and sugar content ranged from 2.10 to 8.41 g/100 g fw and 1.75 to 5.00 g/100 g fw, respectively. The global yield of MSM extraction (8.41 g/100 g) was higher than mucilage extracted from chia (6.52 g/100 g) [6] and flaxseed (6.75 g/100 g) [7] using HIUS technology.

The increase of the ultrasonic power and process time had a positive effect on global yield and sugar content (Fig. 1A and B), it means, an increase in the extraction of mucilage from mutamba seeds. Increasing the applied power at a fixed process time (Fig. 1D and E) it is possible to increase by up to 3-fold the global yield and sugar content, whereas the increase of process time at a fixed applied power can increase by up to 1.8-fold the MSM extraction. Fabre et al. [7] also verified that the intensification of applied power (100 W/5 min to 400 W/5 min) increase the extraction of flaxseed mucilage.

The intensification of HIUS process conditions allows to increase the recovery of MSM, which can be mainly attributed to increase of energy density (Eq. (4)).

$$\mathrm{ED}\left(\frac{\mathrm{J}}{\mathrm{mL}}\right) = \frac{\text{Nominal ultrasonic power (W)} \times \text{Extraction time (s)}}{\text{Sample volume (mL)}} \quad (4)$$

The effectiveness of HIUS in the compounds extraction from plants is due to the phenomenon of acoustic cavitation that can be intensified with the increase of energy density [21]. The intensification of HIUS process conditions consequently promoted the increase of acoustic

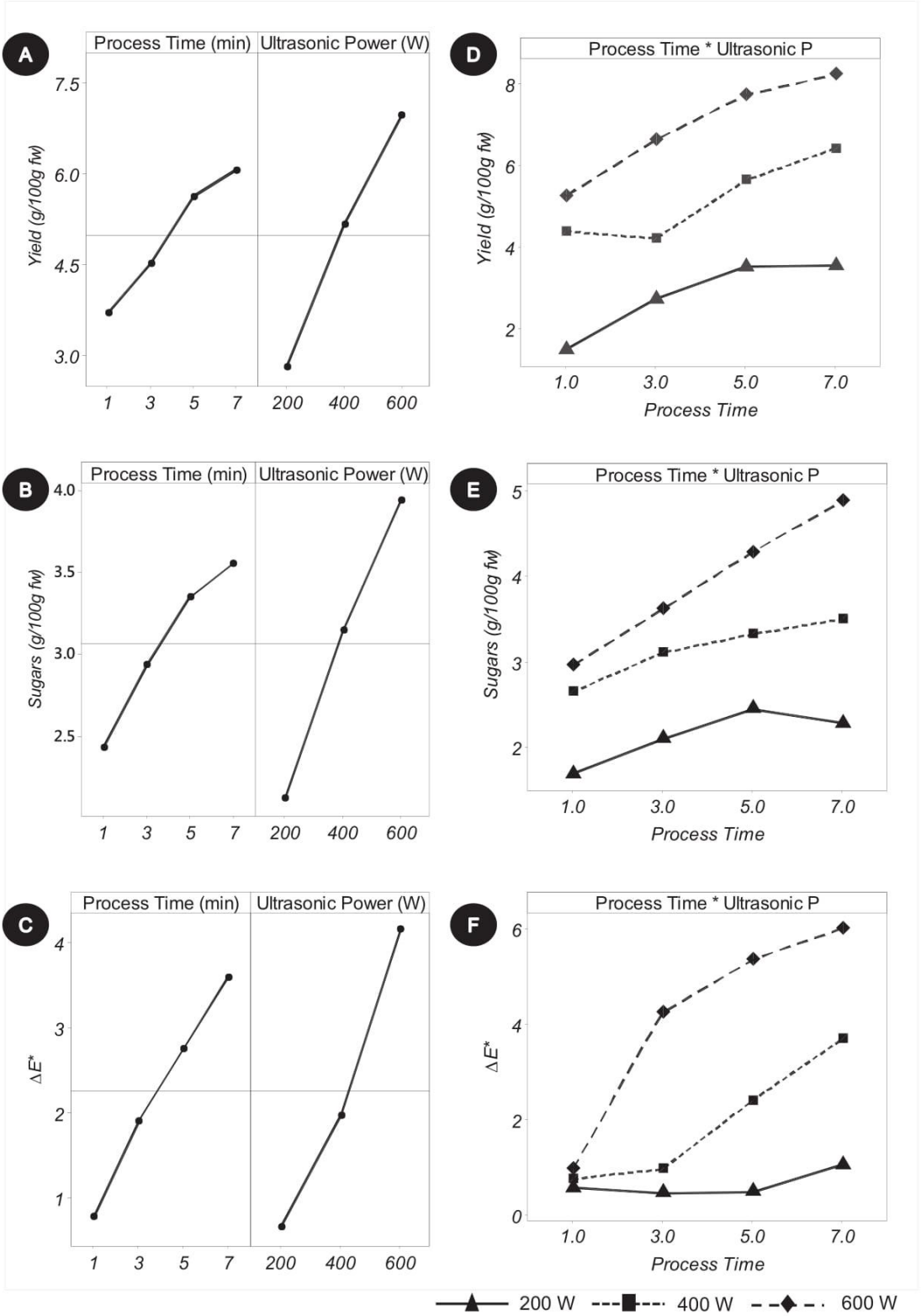

Fig. 1. Effect of the process time and ultrasonic power on: Yield (A); Sugar content (B); and ΔE^* (C); and effect of the interaction between process time and ultrasonic power on: Yield (D); Sugar content (E); and ΔE^* (F). *fw*, fresh weight.

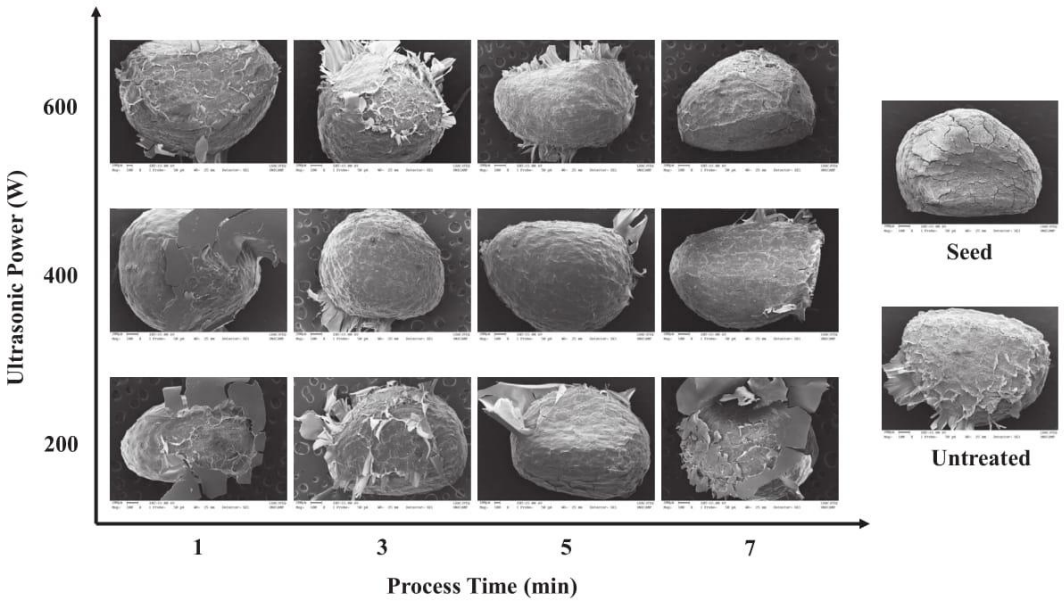

Fig. 2. Scanning electron micrograph (SEM) of intact mutamba seeds (Seed), seed after hydration with water for 20 h (untreated), and hydrated seed after HIUS treatment.

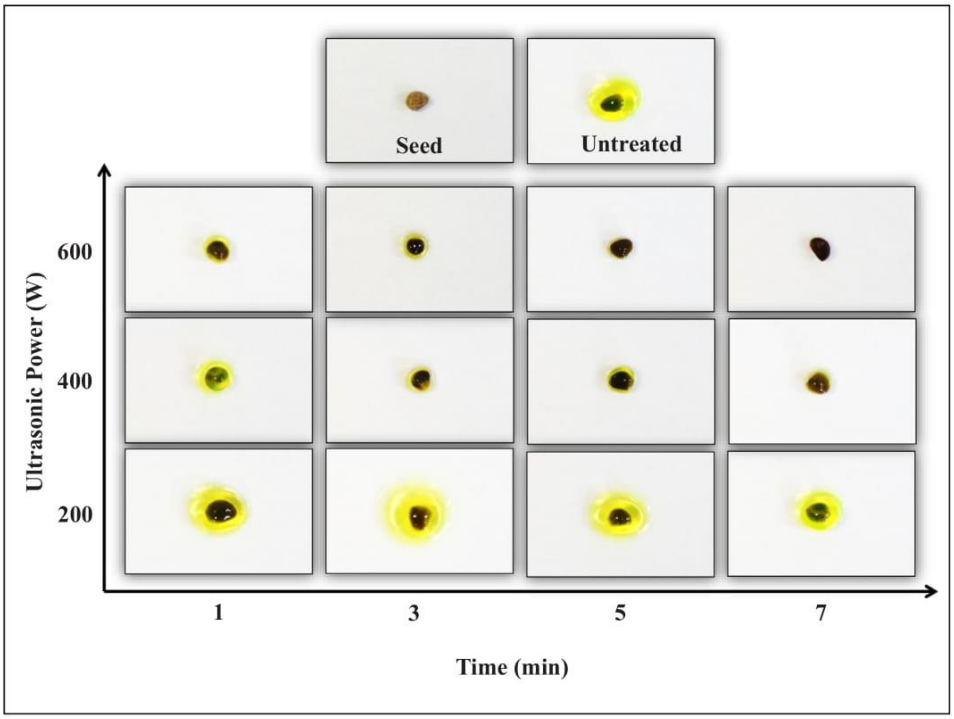

Fig. 3. Visualization of intact mutamba seed (Seed), seed after hydration with fluorescein aqueous solution (25 μg/mL, w/v) for 20 h (untreated), and hydrated seeds after HIUS treatment.

cavitation and its several physical effects, such as shock waves, microjets, turbulence, and shear forces, and increasing of pressure, which resulted in the effective and easy extraction of mucilage from mutamba seeds. The Figs. 2 and 3 show the visual observation of the impact of ultrasound process conditions intensification in the extraction of mucilage layer from mutamba seeds.

The scanning electron micrographs (Fig. 2) and the photographs (Fig. 3) of seeds before and after HIUS treatment showed that at 600 W/7 min (Energy density = 10,080 J/mL) the mucilage was completely removed from seed, demonstrating that the HIUS effectively extracted the MSM. However, using milder conditions (200 W) the extraction was not effective independently of the time employed (1–7 min), which can also be observed in the Fig. 1D and E. The intensification of HIUS process conditions gradually isolate the mucilage from seed.

Upon imbibition with water, the mutamba seeds liberate a viscous mucilage that envelops the seeds to form a gel-like capsule. The seed

mucilages are not easily extracted using convectional extraction methods, for example, magnetical stirring, and vigorous vortexing. Chia seed mucilage was extracted employing several conventional extraction techniques, but only the HIUS was able to totally remove the mucilage layer without altering the shape and the chemical composition of seed, which it was after used to extract ω–3 fatty acids [6]. Vigorous vortexing added of chemical agents (EDTA, ammonium oxalate, and diluted alkali and acids) was not effective in the mucilage extraction from *Arabidopsis thaliana* seeds. Furthermore, the use of chemical agents can promote loss of polysaccharide, and chemical structure changes [6,8]. In this sense, the development of a new extraction protocol using the HIUS could drive to the effectively recovery of mutamba seed mucilage without to alter its chemical properties [22].

Furthermore, the mucilages extraction using conventional procedures drive to high processing time, as they are not so effective as the HIUS in the recovery of the target compounds [22,23]. The mucilage from *Lepidium perfoliatum* L. seeds was extracted by magnetical stirring after 90 min [9]. Fabre et al. [7] found that 5 min of ultrasound treatment (400 W) was equivalent to 180 min of magnetical stirring treatment to obtain the same global yield of flaxseed mucilage extraction. Unlike conventional processes, the HIUS was able to extract MSM with just 7 min of process time. The HIUS, therefore, can be employed in the safer, faster and lower environmental impact extraction of mucilages from seeds.

The HIUS was able to recovery completely the mucilage layer around the mutamba seeds. However, what was the impact of ultrasound treatment on mucilage quality (colour) and structure? The following sections were driven to answer it.

3.1.2. Effect of the process parameters on colour

Table 1 shows the mean values of total colour-difference (ΔE^*), and the Fig. 1C and F the effect of HIUS process conditions on this response. The ultrasonic power (W), process time (min), and the interaction (W*min) significantly affected the colour of the MSM ($p < 0.0001$). The intensification of the both applied power and process time promoted the increase of ΔE^* (Fig. 1C and F), which indicate that high energy density can extract untargeted substances from the seeds, such as brown and yellow pigments from tegument and endosperm [9]. When the mucilage layer around the seeds is partially/completely removed the physical effects from phenomenon of acoustic cavitation then act on seed coat (Figs. 2 and 3). At milder HIUS process conditions (200 W/1 to 7 min) occurred lower extraction of mucilage layer, but using high energy density conditions (400 W or 600 W/1 to 7 min) the mucilage layer can be partially or completely removed, which justify the increase of ΔE^*. The MSM obtained at 600 W/7 min was brown/yellow coloured, whereas the MSM obtained at 200 W/1 min showed similar colour to mucilage extracted by gentle shaking (it showed non-brown colour, but turbid). It means that at higher applied power and process time, more brown/yellow pigment are extracted, and the final colour of the MSM changed.

Although the HIUS was able to extract completely the MSM, it also occurred the MSM colour change, which can affect its technological applications. This indicates that the HIUS extraction should be stopped before damaging the seed coat and, therefore, entering impurities to the hydrocolloid solution which darken it [10]. Alternatively, the MSM can be purified, *e.g.* by ethanol precipitation, which can partially remove the brown/yellow colour and impurities. The second one alternative is removing the seed coat pigments before the mucilage extraction [24]. The Fig. 4 shows the process steps of MSM extraction using HIUS (600 W/5 min). The freeze-dried MSM extracted at this condition showed a yellow colour, but this not restricts its use, since different mucilages have been used in food industrial applications at low concentration (0.05–1%) with little effect on product colour [25].

3.1.3. Effect of process parameters on ζ-potential

Table 1 shows the mean values of MSM ζ-potential. According of ANOVA ($\alpha = 0.05$), the ultrasonic power (W), process time (min), and the interaction (W*min) did not significantly affect the ζ-potential of the MSM suspensions ($p > 0.05$), demonstrating that the HIUS treatment did not affect the surface charge density and molecular interactions of the MSM. In this point, we used the same solution conditions (*e.g.*, hydrocolloid concentration, ionic strength and pH) to evaluate the ζ-potential, therefore it changes only could occur due to ultrasound treatment.

The surface charge density (ζ-potential) reflect the stability of the hydrocolloid system, since this value indicates the degree of electrostatic repulsion within the functional groups of the hydrocolloid. That is, high values of absolute ζ-potential trend to maintain the biopolymers stable without aggregation and flocculation. Hydrocolloid suspensions with ζ-potential higher than +30 mV and lower than −30 mV can be usually considered as stable [26,27]. The ζ-potential of MSM suspensions extracted by using HIUS ranged from −30.4 to −39.5 mV, which indicate that the MSM suspension is stable. The ζ-potential of MSM suspension was similar of flaxseed mucilage (–32 to –33 mV) [26]. The ζ-potential changes can also reflect hydrocolloid structural modification by degradation, hydrolysis, and oxidation [21]. As noted above, the intensification of HIUS process conditions did not change the MSM ζ-potential, maintaining the stability of MSM suspension, as well as its primary structure. This exploratory result was confirmed by FTIR spectrum and monosaccharide composition data as shown in the following Sections 3.1.4 and 3.1.5.

3.1.4. Effect of process parameters on FTIR spectrum

Fig. 5 shows the influence of the intensification of HIUS process conditions on MSM FTIR spectrum. There was no clear difference between the FTIR spectra of untreated and HIUS treated MSM. The same peak shape was detected in both samples; however, the signal intensity (relative absorbance) was different. The intensification of the HIUS process conditions led to the increase of the FTIR spectrum signal due to increasing of MSM global yield (Table 1). FTIR spectrometry has been extensively used as a tool to detect hydrocolloids structure, mainly functional groups. Therefore, the FTIR spectrum can reflect hydrocolloid structural modification [28]. The intensification of HIUS process conditions did not alter the FTIR spectrum, demonstrating that after HIUS treatment the primary structure of MSM was preserved; it corroborates with the ζ-potential results previously discussed.

The HIUS treatment (480 W/60 min at 25 °C; energy density = 86,400 J/mL) [29] altered the FTIR spectrum of apple pectin, whereas the FTIR bands of polysaccharides from mushroom were preserved after HIUS treatment (175 W/33 min at 57 °C; energy density = 13,860 J/mL) [30]. Certainly, the using of very high energy densities (*e.g.*, 86,400 J/mL) can affect the structure of polysaccharide during the extraction process. Moreover, the extraction medium (pH, temperature, chemical agents, etc.) can also affect the stability of polymer during the HIUS treatment [31]. Herein, the MSM extraction was carried out at milder conditions (600 W/7 min at 25 °C; energy density = 10,080 J/mL) than the above mentioned, which can justify the preservation of MSM primary structure [28]. In contrast to our results, the HIUS treatment (135 W/21 min; energy density ≈ 6530 J/mL) altered the FTIR spectrum of jujube seed polysaccharide [15], but the temperature was set to 52.5 °C, which can catalytic the structure modification by HIUS application. As noted above, the effect of HIUS on structure of polysaccharides is still uncertain because a large number of variables must be taken into account, such as HIUS process conditions (ultrasonic power and process time), extraction medium setting (pH, temperature, etc.), and the polysaccharide nature (*e.g.*, polysaccharides from different source, chemical structure, type of glycosidic bound, etc.) [28]. Therefore, the HIUS treatment could alter the structure of polysaccharides, but it depends of the process conditions and of the polysaccharide nature.

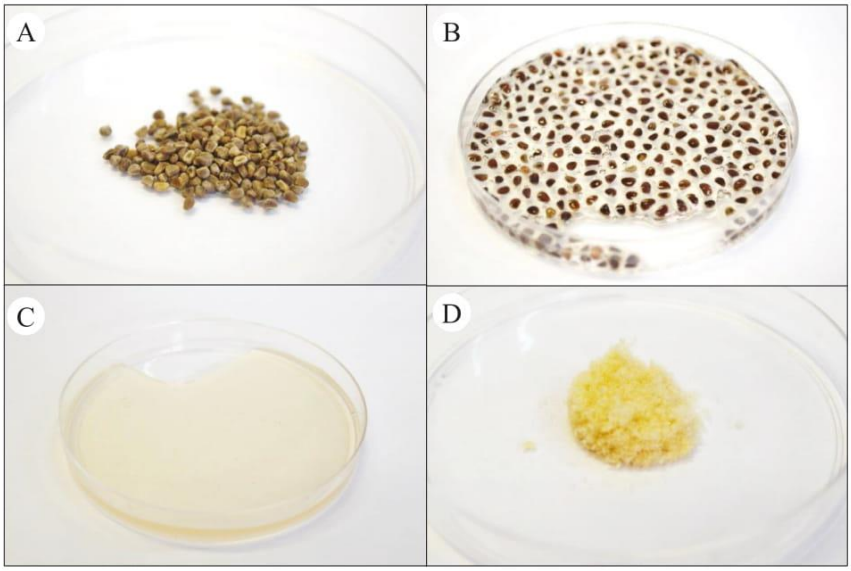

Fig. 4. Seeds of mutamba fruit (A), seeds after hydration with water for 20 h (B), mutamba gum solution after HIUS extraction (600 W/5 min) (C), and freeze-dried mutamba mucilage (D).

3.1.5. Effect of the process parameters on monosaccharides of mutamba seed mucilage

Table 2 shows the influence of HIUS treatment (600 W/7 min) on monosaccharides of MSM. According to paired Student's *t*-test the HIUS treatment did not affect the main monosaccharide composition ($p > 0.01$) of MSM, namely Gal, Rha, GalA and GlcA, whereas the minor monosaccharide residues (Ara, Xyl/Man and Glu) were affected ($p < 0.01$), which can indicate a very slight modification on side chain structure. However, the side chain structure from polysaccharide can change within the same gum/mucilage from different batch extraction, which can partially justify the variation of the minor monosaccharides of MSM [32]. Therefore, it is inappropriate to statement that the HIUS treatment altered the minor monosaccharide composition of MSM, but it is important to note herein that the main structure of MSM was preserved. This result corroborates with pervious findings, in which the monomeric structure of polysaccharide from mushrooms [23,28,30], and apple pectin [29] was not affected by HIUS treatment. Furthermore, the monosaccharide composition results are accordance with the ζ-potential and FTIR data previously discussed, which indicate that the primary structure of MSM was preserved after HIUS treatment even at the most intense process condition (600 W/7 min).

Table 2
Monosaccharide composition of mutamba mucilage extracted by gentle shaking (untreated) and HIUS (treated).

Compound	Untreated	Treated	p-value*
Galactose, *Gal*	32.7 ± 0.3	33.2 ± 0.6	0.297
Galacturonic Acid, *GalA*	21.2 ± 0.6	19.1 ± 0.4	0.022
Rhamnose, *Rha*	21.1 ± 0.1	20.6 ± 0.3	0.146
Glucuronic Acid, *GlcA*	17.5 ± 0.2	18.7 ± 0.8	0.155
Glucose, *Glu*	5.8 ± 0.2	8.0 ± 0.2	0.001
Arabinose, *Ara*	1.0 ± 0.1	0.4 ± 0.1	0.002
Xylose/Mannose, *Xyl/Man*	0.8 ± 0.1	0.1 ± 0.01	0.001

The contents of monosaccharides are presented as mean values (mol%) with standard deviation.
* p-value calculated using paired Student's *t*-test.

The identification of different monomeric units between HIUS treated and untreated MSM could indicate that non-target polymer with different structure was extracted. Therefore, the monomeric composition results also indicate the HIUS (600 W/7 min) was able to extract

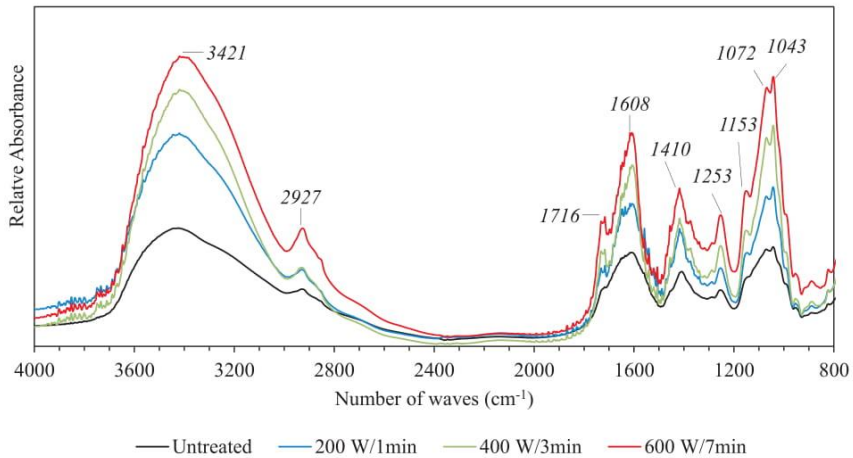

Fig. 5. Fourier Transform Infrared Spectroscopy (FTIR) spectra of mucilage extracted by gentle shaking (untreated) and after HIUS treatment.

Table 3
Moisture, protein and ash content, and mineral elements (mg/100 g dw) of mutamba seed mucilage extracted by HIUS at 600 W/7 min.

Component	Content
Moisture (%)	6.2 ± 0.1
Protein (%)	3.3 ± 0.4
Ash (%)	14.6 ± 0.4
Potassium, *K*	4528.1 ± 184
Calcium, *Ca*	577.0 ± 7.1
Magnesium, *Mg*	460.0 ± 7
Sodium, *Na*	30.4 ± 3
Iron, *Fe*	23.1 ± 0.2
Copper, *Cu*	5.0 ± 0.1
Zinc, *Zn*	3.0 ± 0.1
Manganese, *Mn*	2.7 ± 0.1

The contents of components are presented as mean values with standard deviation.

the mucilage around the seeds without extract polysaccharides from seed coat, since the main monomeric composition between treated and untreated was equal. Although the intensification of HIUS process conditions have changed the MSM colour (*see* Section 3.1.2), the applied energy density was not enough to extract non-target polysaccharides from seed coat.

3.2. Preliminary structure evaluation

To the best of our knowledge, the extraction and the structure of mucilage from mutamba seed have not been evaluated. Therefore, we briefly described herein the preliminary structure of MSM based on FTIR spectrum and monosaccharide residues data. However, the precise structure of MSM has yet to be evaluated using a depth analytical approach.

The FTIR spectrum of MSM (Fig. 5) showed two bands in 4000–2000 cm^{-1} region, being a broad band centred at 3421 cm^{-1} assigned to hydrogen bonded O–H stretching vibrations and the second one a weak signal at 2927 cm^{-1} due to C–H stretching vibrations; these two bands are characteristics of all polysaccharides [23]. The spectral signature of polysaccharides is found in 1200–950 cm^{-1} region. In detail, the bands at 1716 and 1153 cm^{-1} represent the bending vibration of C=O and C–O–C present in the pyranose ring. The bands at 1072 and 1043 cm^{-1} are assigned to C–O–C stretching of glycosidic bonds and C–O–H bending [33]. The peaks at 1608 and 1410 cm^{-1} represent the carboxylate ion stretches from the uronic acids [8]. Moreover, the C–O stretching vibration band at 1253 cm^{-1} may represent a higher uronic acid content [34]. The MSM showed spectral signature with characteristic peaks (1716, 1608, 1410, 1253, 1153, 1072, and 1043 cm^{-1}) of polysaccharides [35], such as rhamnogalacturonans [36], and mucilage from *Arabidopsis thaliana* seeds [8].

According to monosaccharide analysis, the MSM (Table 2) is a heteropolysaccharide and it was mainly composed of Gal (33.2%), Rha (20.6%), GalA (19.1%), GlcA (18.7%), and Glc (8.0%), whereas Ara (0.4%) and Xyl/Man (0.1%) were found at low amount. The Glc may be a remnant of soluble sugar from seed. The monosaccharide composition of MSM was similar to karaya gum, which is composed of 55–60% neutral monosaccharide residues, namely galactose and rhamnose, and 37–40% of uronic acid residues (galacturonic and glucuronic acids) [37,38]. The FTIR data are in agreement with the monosaccharide results, in which the FTIR spectrum of MSM showed a high content of uronic acids. The negatively charged carboxyl groups of GalA and GlcA can explain the negative MSM ζ-potential (−39 mV) [33], which can indicate an anionic characteristic.

The MSM showed low moisture content (6.2%) and high content of protein (3.3%) and total ash (14.6%) (Table 3). The MSM protein content was higher than flaxseed mucilage (0.89–1.63%) [27], but lower than durian seed mucilage (5.8%) [24]. The mineral analysis was performed because the high content of ash. We found potassium and calcium as the main minerals. The durian seed and guar gums also showed high amount of ash, 29.8% and 11.9%, but the main mineral was calcium [39]. The protein and ash content can be reduced by the purification step, which has been reported to improve the technological properties of mucilages [24]. However, the presence of protein in the MSM can contribute to its applications, such as emulsifier and carrier agents [40]. Moreover, the mineral content can affect the MSM suspension stability, which depends on how the functional groups of the main and side chain from polysaccharide interact with the ions [41].

4. Conclusion

A novel mucilage from mutamba seeds was recovered by HIUS. The process parameters evaluated (ultrasonic power and process time) had a significant effect on the mucilage recovery. Applying the HIUS, we were able to recovery completely the MSM at a short process time (7 min) without altering the primary structure of MSM. Although the HIUS process conditions intensification have changed the MSM colour, it can be easily overcome by a purification step to remove impurities. The HIUS treatment did not affect the mucilage suspension stability according to ζ-potential. Moreover, the functional groups from polysaccharide structure and the monomeric configuration was not affected by HIUS treatment even at applied high energy densities. We demonstrated herein that HIUS can be used to safe, fast and low environmental impact recovery of plant seed mucilages. Furthermore, the mutamba seed mucilage has great potential to be used as food and non-food functional ingredient due to its structural features.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2019.01.010.

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CHAPTER IV

RESEARCH ARTICLE

MUTAMBA SEED MUCILAGE AS A NOVEL EMULSIFIER: STABILIZATION MECHANISMS, KINETIC STABILITY AND VOLATILE COMPOUNDS RETENTION

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Mutamba seed mucilage as a novel emulsifier: Stabilization mechanisms, kinetic stability and volatile compounds retention

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ABSTRACT

We studied the use of a novel polysaccharide obtained from mutamba (*Guazuma ulmifolia* Lam.) seeds as an emulsifying agent. Orange peel oil-in-water emulsions stabilized by mutamba seed mucilage (MSM), gum acacia, and modified starches were produced by high-intensity ultrasound. The structural feature and interfacial tension data demonstrated the MSM has emulsifying property. MSM was able to stabilize an oil-in-water emulsion and retained effectively the volatile compounds from orange peel oil. Furthermore, the emulsion stabilized by MSM decreased the loss of limonene, myrcene, α-pinene, and linalool during the storage. MSM was able to increase the continuous phase viscosity, which improved the emulsion stability and delayed the volatile compounds release by decreasing the oil droplet motion. Additionally, the ability of MSM to adsorb rapidly into the oil-water interface aided to stabilize the emulsion. The emulsion stabilized by MSM showed better stability and control release than emulsions stabilized by gum acacia and modified starches. In conclusion, the MSM can be used as a natural emulsifying and thickening agent to prepare stable emulsions with high encapsulation efficiency of volatile compounds.

1. Introduction

An emulsion consists of two immiscible liquids (e.g.: oil and water), in which one of the substances is dispersed as small droplets into the other (continuous phase). The mixture of water and oil at high-speed homogenisation can form an emulsion, but the two phases can rapidly be separated because the contact between both substances is thermodynamically unfavourable. Therefore, it is possible to make emulsions kinetically stable over a period of time (from a few days to years) by using stabilizing agents, for example, the thickening and emulsifying agents (McClements, 2015).

Emulsifiers have been used by food and non-food industries to create metastable emulsion-based products. The emulsifying agent can reduce the interfacial tension between both immiscible phases, which enables the oil droplets disruption during the emulsion production. Furthermore, a protective coating is formed surrounding the spherical oil droplets, which prevent their aggregation due to repulsive forces, such as the steric and electrostatic repulsion. The most common emulsifiers used in the food industry are small molecule surfactants, phospholipids, proteins, and polysaccharides (McClements et al., 2017).

The emulsifying agents are critical ingredients to the proper performance of emulsion-based products and, consequently, they are widely used by industries. The food industry has been using synthetic emulsifiers, such as sorbitan esters, fatty alcohol ethoxylates, and sucrose esters due to their efficiency as stabilizing agent (McClements et al., 2017). However, it has been reported that the synthetic surfactants can increase the incidence of numerous allergic and autoimmune diseases (Csáki, 2011). Furthermore, the consumer's concern about human health and environmental issues has driven the factories to replace synthetic surfactants or animal-based ingredients by natural and sustainable emulsifying agents from plants (McClements et al., 2017), which represents a great challenge in the development of new food products.

The natural emulsifying agents, such as polysaccharides, proteins, phospholipids, and saponins have recently gained much attention, and a few currently reports have covered it (McClements et al., 2017; Ozturk and McClements, 2016; Soukoulis et al., 2018). The growing interest of the Food Engineering in natural ingredients to make 'clean-

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label' products has motivated researches to identify new sources of natural ingredients for food application (Ishii et al., 2018; Ozturk and McClements, 2016). In this approach, the mucilage from seeds are promising emulsifying and thickening agents to be used by food and non-food industries due to their colloidal structure properties. In addition to the technological aspects, the mucilaginous constituents of the seeds have showed healthy properties to human body due to its dietary fibre function (Soukoulis et al., 2018).

We have recently reported the extraction of a novel mucilage from mutamba (*Guazuma ulmifolia* Lam.) seeds (Pereira et al., 2019). The structural feature of mutamba seed mucilage (MSM) allow us to conceive an idea that this polysaccharide could be a novel and natural emulsifying agent to be used by food and non-food industries. In this regard, we evaluated herein the use of MSM as emulsifying agent in orange peel oil-in-water emulsion. The emulsion prepared with MSM was directly compared with other three biopolymers widely used by food industry, namely gum acacia, and Hi-cap 100 and Snow-Flake E6131, chemically modified starches. Emulsion stabilization mechanisms of these biopolymers were studied by ζ-potential and dynamic interfacial tension. The emulsions were subjected to droplet size and rheology measurements. Kinetic stability was evaluated by the near-infrared backscattering profile during 24 h. In addition, the encapsulation efficiency and release over time (0–168 h) of limonene, myrcene, α-pinene, and linalool were also investigated.

2. Material and methods

2.1. Oil and biopolymers

The oil-in-water emulsion was prepared using orange peel oil (Citrosuco, Araras, Brazil) as dispersed phase and ultra-pure water containing the biopolymer as continuous phase. The biopolymers used as emulsifiers were all polysaccharides, namely mutamba seed mucilage (MSM), gum acacia (GA) (Nexira Comercial Ltda., São Paulo, Brazil), and Hi-cap 100 (Hi-Cap) and Snow-Flake E6131 (SF), chemically modified starches (Ingredion Brasil Ingredientes Industriais Ltda., Mogi Guaçu, Brazil). MSM was extracted and purified as the procedure described in sections 2.2 and 2.3.

2.2. Plant material

The morphologically perfect and mature mutamba (*Guazuma ulmifolia* Lam.) fruits were collected in August 2017 in natural areas of the Cerrado Biome located in the municipality of Uberlândia (18°55′07″ south latitude, 48°16′38″ west longitude and 863 m altitude), Minas Gerais, Brazil. All fruits collected (1 kg) were transported from the collection site to the laboratory up to 48 h after collection. The fruits were manually opened with a knife and the seeds were easily separated from the pulp. The selected seeds (free of defects) were placed to vacuum packs, and stored in a desiccator until mucilage extraction. The Genetic Heritage Management Board (CGen) under number AA56F0A, following the Law n ° 13.123/2015 and its regulations, regimented the activity of access to Genetic Heritage.

2.3. Mutamba seed mucilage extraction

The mutamba seed mucilage was extracted as previously described by Pereira et al. (2019). Approximately 10 g of seeds were imbibed with 200 mL of deionized water (Millipore®), and they were kept at room temperature for 20 h to ensure complete hydration and swelling. The mucilage layer was extracted using a 13 mm diameter, 19 kHz ultrasonic probe (Unique, Desruptor, 800 W, Indaiatuba, Brazil) at 760 W per 15 min (Energy density equal to 3420 J/mL). The probe contact height with the medium was standardized to 30 mm, and the system was constantly agitated while ultrasonic waves were emitted. An ice bath was used to prevent overheating of the extraction medium. After ultrasound extraction, the viscous liquid (mucilage suspension) was separated from seeds with the aid of a plastic sieve with pore size of 10 mm. The mucilage suspension was centrifuged (2000 g, 10 min at 15 °C) to remove particulate matter. Sequentially, ethanol was carefully added to reach 80% (v/v) of concentration in the solution, and it was kept at 5 °C for 120 min. After that, the precipitated biopolymer was separated from the liquid with the aid of cheesecloth, and the biopolymer was dried at 40 °C for 12 h. The extraction yield was 4 g/100 g fw (fresh weight). The dried mutamba seed mucilage was placed to vacuum packs, and it was stored in a desiccator until emulsion preparation.

2.4. Mechanism of emulsion stabilization

The MSM, GA, Hi-Cap and SF were characterized by protein, ash and moisture content, pH, and ζ-potential. Moreover, the dynamic interfacial tension between the orange peel oil and the biopolymer suspensions was measured. These analyses were carried out aiming to understand how the MSM, GA, and modified starches can stabilize an oil-in-water emulsion.

2.4.1. Protein, ash and moisture content

The total protein content was measured by Biuret assay using bovine serum albumin from Sigma-Aldrich (St. Louis, USA) with purity ≥96% to construct the analytical curve (25–300 mg/mL) (Bradford, 1976). The total ash (n°940.26) and moisture (n°931.04) were measured according to the protocols described by the Association of Official Analytical Chemists, AOAC.

2.4.2. Surface charge density and pH

The native pH values of the biopolymer suspensions (1% w/v) were analysed three times at room temperature (25 ± 2 °C) using a pH meter. The surface charge density of the biopolymer suspensions (1% w/v) were determined six times at 25 ± 2 °C by measuring the ζ-potential using a microelectrophoresis chamber (Malvern Instruments, ZetaSizer Nano Z, Worcestershire, England). The biopolymer suspensions were diluted in deionized water (approximately 1:100 v/v) according to the optimal detection range of the equipment.

2.4.3. Dynamic interfacial tension

The dynamic interfacial tension between the orange peel oil and the biopolymer suspensions (1% w/v) was measured by ascending drop method using an optical tensiometer (Attension Theta, Biolin Scientific Inc., Gothenburg, Sweden). Briefly, an ascending drop with interfacial area of approximately 5 mm^2 was rapidly formed at the hook needle tip (a J form needle; gauge 22, d_{ext} = 0.64 mm; Biolin Scientific Inc., Gothenburg, Sweden) immersed in a cuvette containing 2 mL of biopolymer suspension (1%, w/v). Immediately, the image of the inverted pendent drop was acquired during 1200 s. The digitized shape profile of the drop was fitted into the Young–Laplace equation by computational methods to calculate the interfacial tension (Faour et al., 1996; Song and Springer, 1996). The control experiment using oil drop immersed in ultrapure water was also performed to assess the changes in absolute interfacial tension over the time (Berry et al., 2015). The measurements were performed three times for each biopolymer at 25 ± 2 °C.

2.5. Emulsion production

The oil-in-water (O/W) emulsion was prepared using orange peel oil as dispersed phase and ultra-pure water containing the biopolymer as continuous phase. The emulsion consisted of 0.5 g/100 g of orange peel oil, 98.5 g/100 g of ultra-pure water, and 1 g/100 g of biopolymer. The biopolymer was weighted in 50 mL plastic tubes with conical bottom containing water, and this biopolymer suspension was stored at room temperature for 24 h to ensure complete hydration and molecular reorganization. After that, the orange peel oil was slowly added into the

biopolymer suspension, and the coarse emulsion was immediately prepared with the aid of ultra-turrax (IKA, T25-Digital, Staufen, Germany) (6000 rpm;360 s; 25 °C). The coarse emulsion (25 g) was immediately submitted to ultrasound treatment (200 W; 240 s; 25 °C) using a 13 mm diameter, 19 kHz ultrasonic probe (Unique, Desruptor, 800 W, Indaiatuba, Brazil) to decrease the oil droplets size and to produce the emulsion. The probe contact height with the medium was standardized to 30 mm, and the system was constantly agitated during the applying ultrasound waves. An ice bath was used to prevent overheating of the extraction medium (Silva et al., 2015a. Polysaccharides tend to adsorb relatively slowly to the oil droplet surfaces during homogenisation as compared with protein and small surfactants (McClements et al., 2017; Ozturk and McClements, 2016), which justifies the time used herein to produce the emulsions. The emulsions were prepared two times, and they were evaluated immediately after their preparation by droplet size, rheological behavior, kinetic stability, and volatile compounds retention.

2.6. Emulsion characterization

2.6.1. Emulsion droplet size distribution

The droplet size distribution of freshly made emulsions was determined by light scattering technique using laser diffraction Mastersizer Hydro 2000 MU (Malvern Instruments Ltd., Malvern, UK). The samples were dispersed in distilled water prior to measurements according to the optimal obscuration range (10–20%). The size of the oil droplets was expressed as surface-weighted mean diameter (D_{32}), according to Equation (1), and as d_{10}, d_{50} and d_{90} that are the diameters at 10%, 50% and 90% of cumulative volume, respectively. The samples were analysed three times using the wet method with a refractive index of 1.52.

$$D_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (1)$$

where d_i is the mean diameter of the droplets and n_i is the number of droplets.

2.6.2. Optical microscopy

The microstructure of the freshly made emulsions was evaluated by placing 10 μL of freshly emulsion (without prior dilution) on a slide. The coverslips (22 × 22 mm), without sliding, were carefully placed to not induce coalescence of the oil droplets. An optical microscopy (Carl Zeiss, Zeiss Axio Scope A1, Gottingen, Germany) with a 20× objective lens and 10× eyepiece (magnification of 200x) was used to capture the imagens of emulsions.

2.6.3. Rheology

Rheological measurements were carried out in a Haake Mars III rheometer (Thermo Fisher Scientific, Karlsruhe, Germany) coupled with a double gap cylinder system (DG41). The gap between the cup and the rotor was set to 5.1 mm gap. The freshly made emulsion (6.5 mL) was carefully placed into the cup cylinder, and rotor cylinder was adjusted to the pre-setting gap. Before carrying out each measurement, the samples were subjected to the waiting time protocol (600 s) in order to eliminate the mechanical history and equilibrate the system to the temperature of 25 °C. After that, the samples were subjected to a controlled rate (CR) from 0.01 to 1000 s^{-1}, in which a particular value of shear rate was imposed for at least 15 s and at maximum 120 s. Within this time range, if the quotient $(\Delta\sigma/\sigma)/\Delta t$ was smaller than 0.01 s^{-1}, the measurement at the given shear rate was stopped. In this formula, $\Delta\sigma$ is the difference between the highest and the lowest shear stresses at the applied shear rate, and Δt the time during which the shear rate was maintained (Schramm, 2000).

The flow curve data was fitted to the power law model according to Equation (2). The parameters of *k* and *n* were estimated by non-linear regression using the Quasi-Newton method with convergence criteria of 10^{-4}. The model fit was evaluated based on the coefficient of determination (R^2) and the mean relative percentage of deviation modulus (*E*) according to Equation (3). Apparent viscosity was obtained at a shear rate of approximately 100 s^{-1}, which represents processes such as pumping, shaking and chewing. The data were statistically analysed using the STATISTICA software (Statsoft, version 12.0, Oklahoma, USA).

$$\sigma = k(\dot{\gamma})^n \quad (2)$$

where σ is the shear stress (Pa), k is the consistency index (Pa s^n), $\dot{\gamma}$ is the shear rate (s^{-1}) and n is the flow behavior index (dimensionless).

$$E = \frac{100}{N} \sum_{i=1}^{N} \frac{|m_i - m_p|}{m_i} \quad (3)$$

where m_i is the experimental value, m_p is the predicted value and N is the population of the experimental data.

2.6.4. Emulsion kinetic stability

The emulsions (20 mL) were placed into a cylindrical glass tube (internal diameter = 2.23 cm, height = 12.81 cm) sealed with a plastic cap, and they were stored at 25 °C for 24 h. The emulsion stability was observed by visual observation using a digital camera Nikon D3200 (Melville, USA) operating in the automatic mode. Digital imagens were taken at 0 and 24 h. The emulsion stability was measured by backscattering profiles using light backscatter scan analyzer (Formulaction, Turbiscan MA 2000; Toulouse, France). The backscattering analyses were carried out at 0 h, 1–6 h, and after 24 h of storage. The data were plotted as backscattering curves as a function of time. The data were also expressed as destabilization index (DSI) according to Equation (4). When the DSI value increases, the stability of the system decreases.

$$DSI = \sum_i \frac{\sum_h |scan_i(h) - scan_{i-1}(h)|}{H} \quad (4)$$

where $scan_i$ and $scan_{i-1}$ are the initial backscattering value and the backscattering value at a given time, respectively, h is a given height in the measuring cell, and H is the total sample height.

2.7. Retention of the volatile compounds by biopolymers

2.7.1. Volatile compounds analysis

The recovery of volatile compounds from freshly made emulsions was performed by liquid-liquid extraction using ethyl acetate (HPLC grade). Firstly, we broke the emulsions to improve the phase separation (oil and water) using the following procedure: the emulsion (500 μL) was placed into a 2 mL plastic microtube containing 500 μL of ethanol and 20 mg of NaCl, followed by agitation (40 s in vortex). The ethyl acetate (1000 μL) was added subsequently into the microtube to perform the volatile compounds recovery. This system was agitated (40 s in vortex) and centrifuged to accelerate the organic phase separation. The organic fraction was collected and the residual water was removed by adding sodium sulphate. The extraction procedure was carried out three times (Molina et al., 2015).

The organic fraction free of water (1 μL) was injected into a gas chromatography system (Agilent Technologies, HP-7890 GC-FID, Santa Clara, USA) coupled to a capillary column (J&W Scientific HP-5MS, 30 m lenght × 0.25 mm i.d. × 0.25 μm of film thickness) using the split mode (1:10). The carrier gas was helium at 1.0 mL/min, and the injector and detector temperature were set to 250 °C. The volatile compounds separation was performed using the following oven temperature programme: 80 °C (3 min), 80–240 °C at 20 °C/min, 240 °C (5 min). The volatile compounds were identified by comparing the retention times of the standards and the samples. All volatile compounds standard from Sigma-Aldrich (St. Louis, USA) with purity ≥95%, namely limonene, myrcene, α-pinene, and linalool were used to do it (Molina et al., 2015).

2.7.2. Encapsulation efficiency

Limonene, myrcene, α-pinene, and linalool were selected to be monitored because they are the main volatile compounds from orange peel oil (see Table S1 in Supplementary Material). Therefore, the chromatogram area of these compounds was used to calculate the encapsulation efficiency of volatile compounds according to Equation (5):

$$Encapsulation\ Efficiency\ (\%) = \frac{A_E}{A_O} \times 100 \quad (5)$$

where A_E is the area of the volatile compound effectively retained, A_O is the area of the volatile compound initially added to emulsion.

2.8. Release kinect study of volatile compounds

The freshly made emulsions (25 mL) were placed into 50 mL plastic tubes with conical bottom and these opened tubes were maintained at room temperature (25 ± 2 °C) for 168 h to evaluate the kinetic release of the volatile compounds. The tubes containing the emulsions were carefully homogenised (5 times) before the aliquot collect to perform the analysis. The volatile compounds area was measured as previously described (see Section 2.7.1). The compounds limonene, myrcene, α-pinene, and linalool were monitored and their chromatogram areas were used to calculate the remaining volatile compound according to Equation (6).

$$Remaining\ Volatile\ Compound = \frac{A_{t_1}}{A_{t_0}} \times 100 \quad (6)$$

where A_{t1} is the chromatogram area of the volatile compound at a given time (24–168 h), A_{t0} is the chromatogram area of the volatile compound of the freshly made emulsion (0 h).

2.9. Statistical analysis

The data were submitted to one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$). Statistical analysis of the data was carried out using the STATISTICA software (Statsoft, version 12.0, Oklahoma, USA). The data are presented as mean values with standard deviation of three measurements.

3. Results and discussion

The mucilaginous constituent of the mutamba seed (MSM) was extracted and it was used as emulsifying agent to prepare an orange peel oil-in-water emulsion. Firstly, we investigated the mechanism of emulsion stabilization by MSM. After that, the properties of the emulsion stabilized by MSM were compared with biopolymers widely used by food industry, namely GA, Hi-Cap, and SF to verify its effectiveness as emulsifier.

3.1. Mechanism of emulsion stabilization

Table 1 shows the protein, ash and moisture content of the biopolymers used as emulsifying agents. The MSM showed higher protein, ash, and moisture contents than GA, Hi-Cap and SF. The mucilages are hygroscopic materials that have shown water sorption higher than commercial gums, but it does not affects their stability during the storage (Soukoulis et al., 2018; Velázquez-Gutiérrez et al., 2015).

The effectiveness of polysaccharides as emulsifier depends on its structural feature, such as molecular weight, monosaccharide composition, surface charge, and presence of hydrophobic fraction. The polysaccharides primary consisting of polar monosaccharides are not effective emulsifiers. However, the presence of a hydrophobic fraction, such as proteins, in the polysaccharide backbone can provide it with a degree of hydrophobicity. For example, the emulsifying properties of gum acacia and pectin are attributed mainly to their hydrophilic anionic polysaccharide chains and hydrophobic polypeptide backbone (Williams et al., 2005); it means that the amphiphilic polysaccharide can adsorb to oil-water interfaces by its hydrophobic fraction and stabilize emulsions by steric (hydrophilic fraction) and electrostatic (electrostatic repulsion) mechanisms (McClements et al., 2017; Ozturk and McClements, 2016). Unlike GA and MSM, the Hi-Cap and SF do not have a natural hydrophobic fraction. However, these starches have been chemically modified by the addition of lipophilic component, octenyl succinic anhydride (OSA), which attributes emulsifying properties to these two modified starches (Dokić et al., 2012).

The presence of proteinaceous moiety in the MSM may contribute to its applications, such as emulsifier and carrier agents (Soukoulis et al., 2018). Moreover, the mineral content can affect the MSM suspension stability and its mechanical properties and, consequently, the emulsion stability. It depends on how the ions interact with the functional groups from polysaccharide and the oil-water interface (Pereira et al., 2019; Soukoulis et al., 2018). A current study demonstrated the MSM suspension is stable despite its high amount of ash, mainly potassium (4528.1 mg/100 g dw) (Pereira et al., 2019).

Table 1 also shows the surface charge density (ζ-Potential) and the native pH of the polysaccharides used as emulsifying agents. The MSM showed the highest value of pH (6.6) and the lowest ζ-Potential (−51.1) as compared with GA, Hi-Cap, and SF. The mucilage extracted from mustard seeds also showed ζ-Potential more negative than GA and citrus pectin (Wu et al., 2015). The MSM is composed of 50% neutral monosaccharide residues, namely galactose and rhamnose, and 37% of uronic acid residues (galacturonic and glucuronic acids). The negatively charged carboxyl groups of galacturonic and glucuronic acids can explain the negative MSM ζ-potential, which can indicate an anionic characteristic (Pereira et al., 2019).

The ζ-potential reflect the electrostatic repulsion between the functional groups of the hydrocolloid. Prospecting it to emulsion system, the negatively ζ-potential indicates that the electrostatic repulsion may contributes to stabilizing an emulsion (Silva et al., 2015b). The stability mechanism of polysaccharides (e.g.: gum acacia, and OSA starches) as emulsifying agent has been mainly attributed to steric phenomenon, which represents the macromolecules adsorption (hydrophobic fraction) on the oil surface. Therefore, amphiphilic polysaccharides form thick hydrophilic coatings around oil droplets which generates a strong long-range steric repulsion (Dokić et al., 2012; Jayme et al., 1999; McClements et al., 2017). Nevertheless, the strong negative ζ-potential of MSM may indicate that both steric and electrostatic mechanics (electrosteric) could occur to stabilize an emulsion (Jayme et al., 1999; Silva et al., 2015a). It should be further evaluated to discover the contribution of steric and electrostatic mechanism in oil-

Table 1
Characterization of biopolymers used as emulsifier/stabilizers.

Biopolymer	Moisture (g/100g)	Protein (g/100g)	Ash (g/100g)	pH	ζ-Potential (mV)
MSM	10.78 ± 0.58	1.28 ± 0.18	11.68 ± 0.1	6.6 ± 0.2[a]	−51.1 ± 2.5[a]
GA	4.52 ± 0.00*	1.05 ± 0.00*	4.67 ± 0.07*	5.4 ± 0.1[b]	−29.9 ± 2.5[b]
Hi-Cap	4.80 ± 0.00*	0.09 ± 0.00*	1.07 ± 0.04*	4.5 ± 0.2[c]	−14.6 ± 0.5[c]
SF	4.92 ± 0.00*	0.40 ± 0.00*	0.04 ± 0.04*	3.8 ± 0.1[d]	−17.1 ± 0.8[c]

The results are presented as the mean with standard deviation. Means with different lowercase letters (a, b, c, d) within a column were significantly different ($p < 0.05$) by Tukey's HSD test. *Data previously reported by Silva et al. (2015a).

in-water emulsion stabilized by MSM.

Certainly, the changes in pH affect the ζ-Potential of polysaccharides containing ionisable groups, but it does not necessarily affect the emulsion stability prepared with them (Wu et al., 2015). The polysaccharides, such as GA and OSA starches, can stabilize an emulsion mainly by steric mechanism (Dickinson, 2009; Jayme et al., 1999), whereas proteins can stabilize an emulsion by means of electrostatic repulsion. These different stabilization mechanisms indicate that the polysaccharides tend to be less affected by changes in pH and ionic strength than proteins (McClements et al., 2017; Wu et al., 2015). However, the MSM shows a high content of uronic acids, which indicates an anionic characteristic; consequently, the MSM may show a surface charge dependent on pH (Timilsena et al,. 2016).

An important property that defines a substance as emulsifying agent is its ability to adsorb rapidly into the oil-water interface. The oil and water phases are thermodynamically immiscible, which create an interface between the two phases with a great imbalance of molecular interactions, and consequently a high interfacial tension. The emulsifying agent can adsorb to its interface and interact with both phases, providing free energy to the system. It decreases the imbalance of molecular interactions and then the interfacial tension (McClements, 2015).

Fig. 1 shows the interfacial tension measurements between the orange peel oil and the biopolymer suspensions as a function of the time. The interfacial tension of the system containing MSM, GA, Hi-Cap, and SF decreased along the time but at different rates. The interfacial tension between orange peel oil and water showed a slight decrease, which can be explained by the presence of surface active components at low concentration from orange peel oil (Molina-Calle et al., 2015).

The decrease of the interfacial tension followed the decreasing order of SF > Hi-Cap > MSM > GA (Fig. 1). It indicates that the GA suspension took more time to diffuse to the interface, adsorb, and reorganise than MSM, Hi-Cap, and SF. Silva et al. (2015a) compared the interfacial tension between the annatto seed oil and GA, Hi-Cap, SF and WPI (whey protein isolate). They verified that GA showed the lowest rate of reduction during the time evaluated. The GA showed good surface properties but took more time to achieve a balance than MSM, Hi-Cap, and SF (Bouyer et al., 2011). It is very important to point out that MSM showed better surface properties than GA. The rapidly adsorption of emulsifying agent into the oil-in-water interface (as a consequence, the fast decrease of interfacial tension) can help produce small droplets by breakup at emulsion manufacturing, which is positively related to better emulsion stability (Jafari et al., 2012).

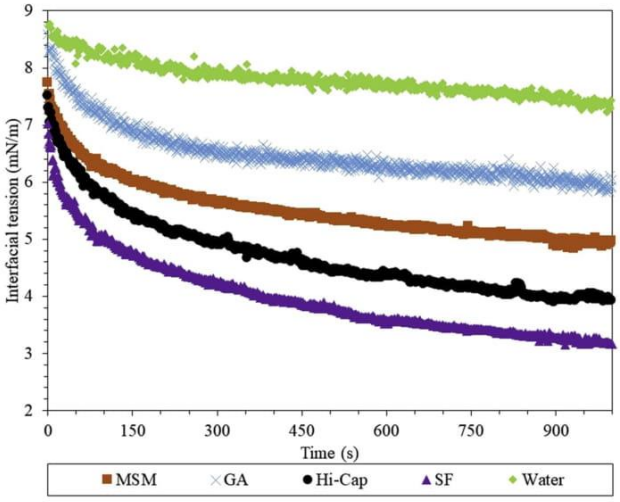

Fig. 1. Interfacial tension between the orange peel oil and the biopolymer suspensions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The literature data have shown that the presence of proteinaceous moiety in plant seed mucilages improves their capacity of decreasing the interfacial tension between oil and water (Naji-Tabasi and Razavi, 2016). As previously discussed, the MSM showed a great amount of protein. Therefore, the MSM's proteinaceous may contribute to its emulsifying property.

The MSM demonstrated potential to be used as emulsifying agent according to its structure feature and interfacial tension data. Moreover, the findings showed that the MSM could stabilize an emulsion by steric mechanism, although the electrostatic mechanism may contribute to it. Therefore, we produced an oil-in-water emulsion containing MSM and compared its properties with emulsions stabilized by emulsifiers commonly employed by food industries, namely GA and OSA starches. The following sections were driven to evaluate the effectiveness of MSM as emulsifying agent in oil-in-water emulsion system.

3.2. Emulsion production and characterization

3.2.1. Rheological properties

Table 2 shows the rheological properties of the orange peel oil-in-water emulsions. The emulsions stabilized by GA, Hi-Cap, and SF exhibited Newtonian behavior ($n \approx 1$), whereas the system containing MSM exhibited pseudoplastic fluid behavior ($n < 1$). These data are according to the flow curves presented in Fig. 2. The shear stress is proportional to the shear rate (Newtonian fluid) for the system containing GA and modified starches, while the emulsion stabilized by MSM showed a characteristic flow curve of a pseudoplastic fluid (Hashtjin and Abbasi, 2015). The pseudoplastic material manifests a decrease in its apparent viscosity with the increase of the shear rate (McClements, 2015).

The emulsion stabilized by MSM showed apparent viscosity (26.1 mPa.s) higher than emulsions containing GA and modified starches (1.3–1.8 mPa.s). The emulsions were produced with a low concentration of dispersed phase (< 30 g/100g), which can be considered as diluted emulsion ($\phi < 0.02$) (Pal, 2011). Therefore, it is expected that the oil droplets would not interfere in the emulsion rheology properties, mainly the system viscosity, and the properties of the system could be mainly determined by continuous phase (water containing the polysaccharide) (Quinzio et al., 2018). The mucilaginous constituents of seeds have shown significant thickening ability (Soukoulis et al., 2018). For example, the mucilage from chia seed can be used as thickening agent because it can increase the system's consistency (Capitani et al., 2015). MSM can act as thickening agent, which delays the collision between the oil droplets, and therefore could increase the emulsion stability (McClements, 2015).

MSM at 1 g/100 g significantly increased the system viscosity,

Table 2
Rheological characteristics (apparent viscosity, flow and consistency indices at 25 °C) of emulsions.

Biopolymer	Apparent viscosity at 92 s^{-1} (mPa.s)	Consistency index, k (mPa.s)	Flow behavior, n	R^2	E (%)
MSM	26.1 ± 0.4[a]	72.1 ± 2[a]	0.780 ± 0.003[a]	0.999	2.4
GA	1.8 ± 0.1[b]	2.5 ± 0.5[b]	0.959 ± 0.040[b]	0.999	4.6
Hi-Cap	1.4 ± 0.1[b]	1.9 ± 0.2[b]	0.948 ± 0.003[b]	0.999	4.4
SF	1.3 ± 0.2[b]	1.8 ± 0.4[b]	0.956 ± 0.023[b]	0.999	5.2

The results are presented as the mean with standard deviation. Means with different lowercase letters (a, b) within a column were significantly different ($p < 0.05$) by Tukey's HSD test. The flow curve data were fitted to the power law model (see Section 2.6.3). The model adequately represented the experimental data, as demonstrated by the high value of the coefficient of determination (R^2) and the low mean relative percentage deviation modulus (E).

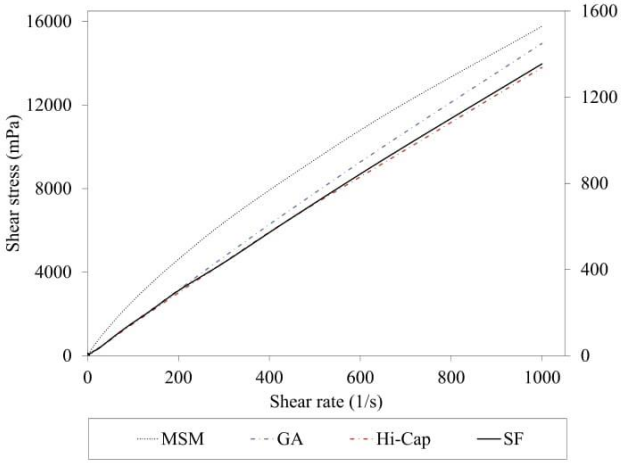

Fig. 2. Plots of shear rate vs shear stress of oil-in-water emulsions.

which indicates that this mucilage could be applied in food systems at lower concentrations (< 1 g/100 g) (Capitani et al., 2016). Furthermore, the MSM can act as a fat-replacing agent due to its high water absorption and viscosity development (Felisberto et al., 2015; Fernandes and Salas-Mellado, 2017).

3.2.2. Droplet size and distribution

Fig. 3 shows the droplet size distribution curves combined with optical micrograph of the orange peel oil-in-water emulsions. All emulsions (MSM, GA, Hi-Cap, and SF) presented bimodal distributions (a primary peak at smaller diameters, and a second peak at relatively high diameters). The emulsion stabilized by MSM showed a high volume of droplets with size ≥100 μm as compared with the GA, Hi-Cap and SF. It was confirmed by the optical micrographs, in which the MSM shows a heterogeneity droplet size, while the GA and modified starches showed a degree of uniformity (Jafari et al., 2012; Quinzio et al., 2018). Table 3 contains the mean droplet size (D_{32}) and cumulative diameter (d_{10}, d_{50} and d_{90}) of emulsions. The emulsion stabilized by MSM showed the largest D_{32} and d_{10}, d_{50} and d_{90}, whereas the GA and modified starches showed statistically equal values (p-value > 0.05) of D_{32} and

Table 3
Mean droplet size (D_{32}) and cumulative diameter (d_{10}, d_{50} and d_{90}) of emulsions.

Biopolymer	D_{32} (μm)	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)
MSM	4.67 ± 0.16^{a}	1.46 ± 0.04^{a}	29.03 ± 2^{a}	178.52 ± 7.45^{a}
GA	1.48 ± 0.02^{b}	0.86 ± 0.01^{b}	1.59 ± 0.02^{b}	3.56 ± 0.27^{b}
Hi-Cap	1.19 ± 0.01^{c}	0.70 ± 0.01^{c}	1.28 ± 0.01^{b}	2.65 ± 0.06^{b}
SF	1.28 ± 0.02^{c}	0.74 ± 0.01^{d}	1.36 ± 0.01^{b}	3.26 ± 0.32^{b}

The results are presented as the mean with standard deviation. Means with different lowercase letters (a, b, c, d) within a column were significantly different (p < 0.05) by Tukey's HSD test.

cumulative diameters, which corroborates with the data of Fig. 3. The oil-in-water emulsion stabilized by corn fibre gum showed bigger mean droplet size than GA and beet pectin independently on the polysaccharide concentration (1–5 g/100g) (Bai et al., 2017).

The droplet sizes of the dispersed phase affects the physical stability of an emulsion. According to the Stokes' law, the motion speed of a droplet is proportional to the square of its radius. It means that the bigger the droplet size, the more unstable the emulsion may be. Therefore, the destabilization process, for example, creaming and flocculation, can be delayed by reducing the droplets size (McClements, 2015).

Although the MSM have shown better surface activity than GA (see Section 3.1), which improves the breakup of emulsion droplets during the homogenisation step, the emulsion with MSM showed the biggest mean droplet size. The MSM highly increases the viscosity of the system, which hampers the mixing of the water and the oil phases, and the disruption of the droplets. Consequently, small droplet sizes cannot be obtained (Bai et al., 2017). The energy input by ultrasound device was not enough to produce fine emulsions stabilized by MSM (Jafari et al., 2012; Quinzio et al., 2018). Unlike the MSM, the GA, Hi-Cap, and SF did not thicken the system using 1 g/100 g. Therefore, the ultrasound energy input was sufficient to produce emulsions with D_{32} below 2 μm (Table 3) (Silva et al., 2015a).

3.2.3. Emulsion stability

The effectiveness of an emulsifying agent is evaluated by its capacity to form highly stable emulsion. Emulsion stability can be referred as the resistance of an emulsion to the changes in its properties over

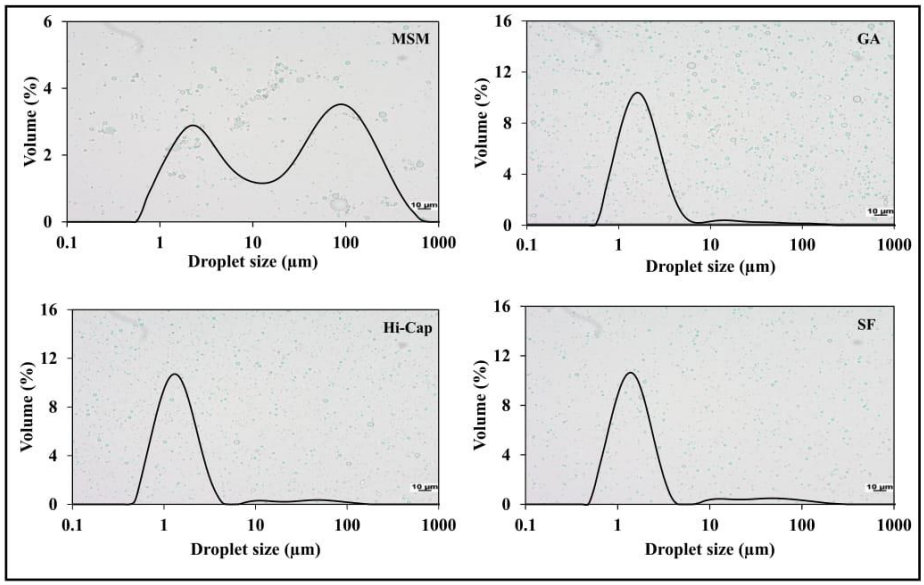

Fig. 3. Droplet size distribution curves and optical micrographs of the oil-in-water emulsions.

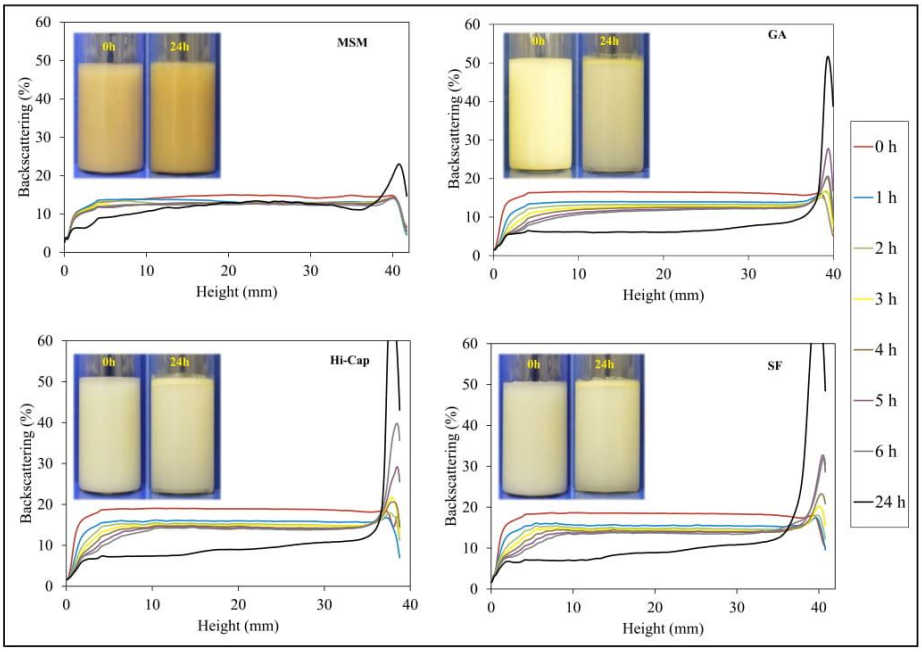

Fig. 4. Emulsion kinetic stability during the storage at room temperature (25 ± 2 °C). Backscattering profiles (0–24 h) of emulsions and imagens of measuring cells containing the fresh emulsions and the emulsions after 24 h of storage.

time (Kinect stability) (McClements, 2015). Fig. 4 shows the kinect stability (0–24 h) of emulsions produced with different polysaccharides as emulsifying agent. The difference between the backscattering profile of emulsions at 0 and 24 h of storage was less significant in emulsion stabilized by MSM and more apparent in the systems containing GA, and modified starches. The difference of backscattering profile over time indicates a destabilization process (phase separation), as well as the rate of phase separation in the emulsions with GA, Hi-Cap, and SF was higher than in the emulsion containing MSM.

The backscattering data indicate that the emulsions were destabilized by creaming due to the signal increase on the top of the measuring cell (cream layer) followed by a signal decrease on the bottom of the cell (clarification) (Silva et al., 2018). The imagens taken of the emulsions at 0 and 24 h of storage also showed this destabilization tendency (Fig. 4). We also evaluated the effectiveness of MSM as emulsifying agent by means of a destabilization index (DSI; see Section 2.6.4), as shown in Fig. 5. The data clearly showed that the emulsion stabilized by MSM was statistically ($p < 0.05$) more stable than the emulsions with GA, and modified starches.

The kinect stability of an emulsion is mainly affected by the oil droplets size, as well as the interaction between them. The larger droplet sizes generally accelerate the emulsion destabilization, such as creaming. Furthermore, the emulsion droplets are constantly moving and colliding, which can promote the droplet interaction and, consequently, flocculation and coalescence (McClements, 2015). It means that the emulsion system may be more stable where the droplets motion/contact is delayed by physical and chemical events, such as phenomenon' steric and electrostatic, and the increase of the system viscosity.

The emulsion stabilized by MSM showed the biggest droplet size (see Section 3.2.2). This emulsion was, however, more stable than emulsions with GA and modified starches due to MSM's high viscosity (26 mPa.s) at low concentration (1% w/v) (see Section.3.2.1), which helps delay the movement and agglomeration of the oil droplets. The mucilage from chia seeds and the hydrocolloid extracted from ora-pro-nobis leaves (*Pereskia aculeate* Miller) could also stabilize an oil-in-water emulsion due to the high viscosity of continuous phase (Capitani et al., 2016; Junqueira et al., 2018). Certainly, the viscosity of the system strongly decreased the destabilization process by creaming, but we cannot state that it led to the emulsion stability, because MSM rapidly adsorbed into the oil-water interface (see Section 3.1). Therefore, the MSM stabilized the orange peel oil-in-water emulsion by its both capacity to modulate the viscosity of the continuous phase and adsorb into oil-water interface (Avila-de la Rosa et al., 2015).

3.2.4. Volatile compounds retention

The encapsulation efficiency (EE, %) was evaluated immediately after the emulsions preparation. Table 4 shows the EE of the volatile compounds (VOC) from orange peel oil by emulsion systems containing different polysaccharides. The emulsion stabilized by GA presented the highest EE as compared with MSM, and modified starches. However, the emulsion with MSM showed EE statistically equal (p-value < 0.05) to Hi-Cap and higher than emulsion stabilized by SF. The VOC from orange peel oil, namely limonene, myrcene, α-pinene, and linalool were effectively retained by emulsion containing MSM.

We also evaluated the release of VOC from emulsions during the storage at room temperature (Fig. 6). The data clearly indicate that the emulsion stabilized by MSM showed the highest values of remaining compound during the emulsion storage, which reflects a slower VOC release. Therefore, the release rate of VOC from emulsions with GA, Hi-Cap, and SF was faster than emulsions stabilized by MSM. For example, the emulsion with MSM showed 79% of limonene remaining after 72 h of storage at room temperature, whereas the other emulsions showed values lower than 13% (Table 4). The emulsion stabilized by MSM effectively trapped the VOC from orange peel oil during the emulsion storage, which demonstrates it can delay and control the release of volatile compounds. The yogurt containing cress seed mucilage showed volatile release profile similar to yogurt with guar gum during a storage period of 15 days at 5 °C (Hassan et al., 2015).

Interestingly, the release behavior of linalool was different from limonene, myrcene, and α-pinene. This finding could be explained by the difference on chemical structure (e.g.: number of carbons, functional groups, etc.) and physicochemical properties (e.g.: volatility and polarity) of VOC, and how they interact with the surrounding

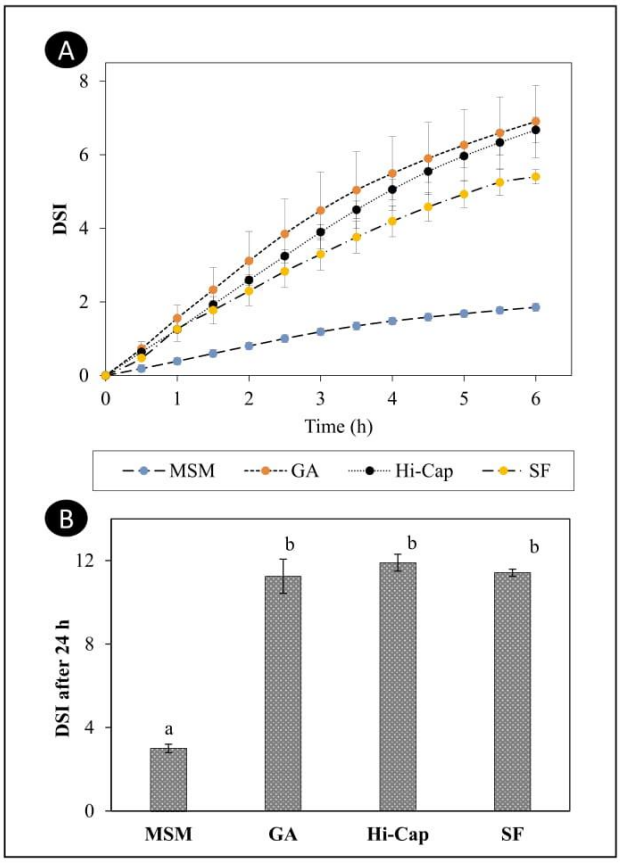

Fig. 5. Destabilization kinetic of emulsions during the storage at room temperature (25 ± 2 °C). A) Destabilization index (DSI) of emulsions for 6 h of storage at room temperature. B) DSI of emulsions after 24 h of storage at room temperature. When the DSI value increases, the stability of the system decreases. Means with different letters (a, b) were significantly different ($p < 0.05$) by Tukey's HSD test.

components from disperse and continuous phase (Benjamin et al., 2012; Marcuzzo et al., 2010; Van Ruth et al., 2002). Unlike limonene, myrcene and α-pinene, linalool is substituted by a hydroxyl group at position 3 (3,7-Dimethyl-1,6-octadien-3-ol). In addition to this structural difference, the linalool has a high flash point (75 °C) as compared with the others terpenes (approximately 40 °C). Therefore, the high flash point can explain the slow linalool release from emulsions. All emulsions showed a similar release behavior of linalool until 96 h of storage, but the emulsion stabilized by MSM showed a better linalool release control after this time (Fig. 6), which indicates that the MSM was able to trap it.

As previously discussed, the emulsion stabilized by MSM showed a high viscous system, which can delay the VOC release (see Section 3.2.1). The VOC diffusion is inversely proportional to viscosity according to the Stokes–Einstein equation. As a consequence, the VOC diffusion from the oil droplet to air phase is delayed by increasing the viscosity (Mao et al., 2017). Furthermore, the destabilization process strongly affect the VOC release (see Section 3.2.3). For example, the creaming instability in emulsions containing GA, Hi-Cap, and SF formed a cream layer on the top, which induced more VOC released to the headspace (Benjamin et al., 2012).

GA is the most used emulsifying agent by food industry to encapsulate volatile compounds due to the fact that it creates a strong protective film around the oil droplets. However, its high cost to food industry has motivated the development of ingredients to replace it (Dickinson, 2009; Jafari et al., 2012; Wu et al., 2015). In this context, MSM could be an alternative to replace GA, since this novel hydrocolloid showed emulsifying and encapsulating properties as effective as GA.

4. Conclusion

The structural feature and interfacial tension data demonstrated that the mutamba seed mucilage (MSM) has emulsifying property. The MSM can strongly increase the viscosity of the continuous phase, and its dispersion impart pseudopasticity. The oil-in-water emulsion containing MSM as emulsifying agent was more stable than emulsions produced with emulsifiers commonly used by food industry, namely gum acacia and modified starches. The emulsion stabilized by MSM effectively controlled the release of limonene, myrcene, α-pinene, and linalool while the emulsion with gum acacia and modified starches rapidly released them during the storage. The MSM can develop viscosity, which improved the emulsion stability and delayed the volatile compounds release by decreasing the droplet motion. Additionally, as MSM can rapidly adsorb into the oil-water interface, it helped stabilize the emulsion. Therefore, the MSM stabilized the orange peel oil-in-water emulsion by its both emulsifying and thickening properties. We demonstrated that a novel and natural polysaccharide extracted from mutamba seeds could be used as emulsifying and thickening agent by food industry.

Table 4
Encapsulation efficiency and remaining compound after 72 h of storage of emulsions.

Biopolymer	Encapsulation efficiency (%)			
	Limonene	Myrcene	α-Pinene	Linalool
MSM	95.66 ± 3.13^{b}	95.07 ± 3.15^{b}	94.96 ± 2.74^{b}	78.00 ± 6.29^{b}
GA	98.35 ± 0.16^{a}	99.59 ± 0.41^{a}	97.57 ± 0.18^{a}	99.48 ± 3.57^{a}
Hi-Cap	93.99 ± 3.89^{b}	94.67 ± 3.73^{b}	93.24 ± 3.82^{b}	85.20 ± 1.09^{b}
SF	86.31 ± 5.85^{c}	86.50 ± 6.83^{c}	85.20 ± 5.96^{c}	75.66 ± 6.35^{b}
	Remaining compound after 72 h of storage (%)			
MSM	79.26 ± 1.07^{a}	78.73 ± 0.95^{a}	81.87 ± 0.45^{a}	57.69 ± 2.03^{a}
GA	9.04 ± 0.32^{c}	9.33 ± 0.23^{c}	10.61 ± 0.29^{c}	49.65 ± 3.13^{b}
Hi-Cap	12.92 ± 1.07^{b}	12.97 ± 1.04^{b}	13.89 ± 0.09^{b}	52.58 ± 4.95^{b}
SF	12.75 ± 2.12^{b}	12.76 ± 2.12^{b}	14.00 ± 1.87^{b}	45.78 ± 5.08^{c}

The results are presented as the mean with standard deviation. Means with different lowercase letters (a, b, c) within a column were significantly different ($p < 0.05$) by Tukey's HSD test.

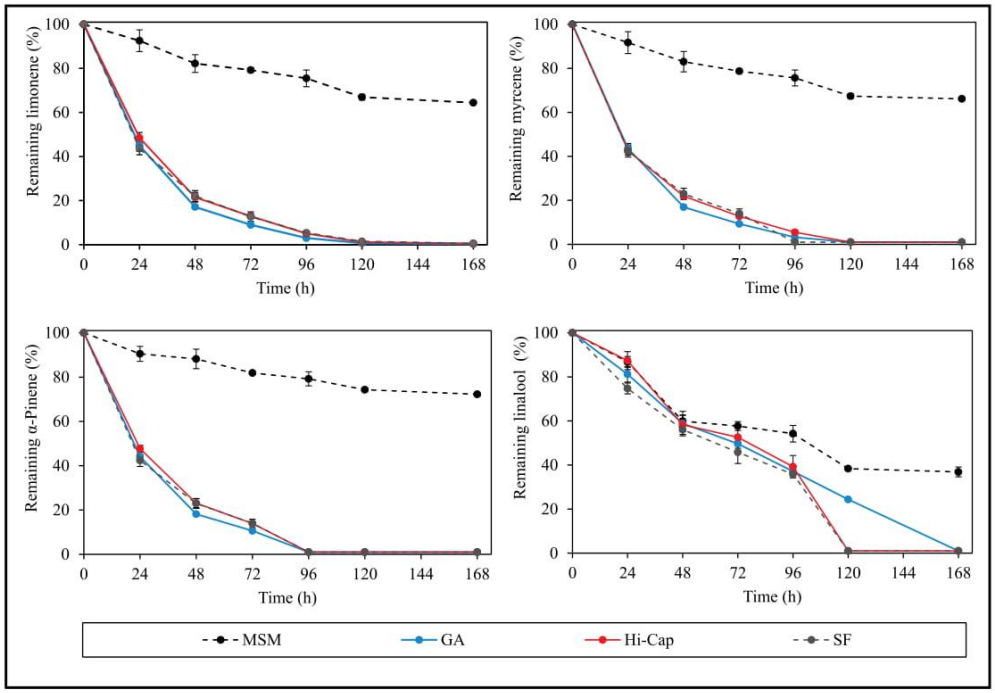

Fig. 6. Release kinetic of volatile compounds during the storage at room temperature (25 ± 2 °C).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2019.105190.

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GENERAL DISCUSSION

The traditional uses of mutamba (*Guazuma ulmifolia* Lam.) fruits as ingredient to homemade food and as medicine to treat diseases have motivated us to study its phytochemical composition in order to discover novel and natural bioactive and high added-value compounds. The first step of this thesis was to perform a literature search on phytochemical composition and biological properties of *G. ulmifolia* (**Chapter I**). We figure out the lack of studies about the composition of primary and secondary metabolites of mutamba fruit, which mays hinder it use by food technologists as well as food industry. Interestingly, the literature data have shown phenolic compounds as the main secondary metabolites from stem bark and leaves of *G. ulmifolia*, and additionally the bioactivities of these plant parts have been attributed to their phenolic compounds composition. In this regard, we investigated the phenolic compounds profile of mutamba fruit by liquid chromatography coupled to tandem mass spectrometry (LC-MS). Moreover, the physicochemical and proximate composition of this fruit were assessed (**Chapter II**). The data showed that mutamba fruit has great potential to be used by food and non-food industries because its low moisture content, high content of dietary fibre, and sweet pulp and attractive taste. Although mutamba fruit is not commonly consumed as fresh fruit due to its hardness, this fruit can be used to develop products, such as (but not limited to) flour, whole bread, and tea. Moreover, the LC-MS analysis showed mutamba fruit as a novel source of phenolic compounds, especially proanthocyanidins and aglycones and glycosylated flavonoids. Procyanidin C1 was the mains phenolic compound accounting 58% of the total phenolics content. The phytochemical profile of mutamba fruit matched with the stem bark and leaves of *G. ulmifolia* in which the proanthocyanidins, and aglycones and glycosylated phenolic compounds were reported as the main secondary metabolites. The scientists have proved the biological properties of mutamba stem bark and leaves claimed by traditional healers, such as antimicrobial, antiprotozoal, antioxidant, antidiarrheal activities, and cardioprotective effect. As previously mentioned, these biological properties have been linked to phenolic composition of *G. ulmifolia* botanical parts. Therefore, the mutamba fruit has potential as food and therapeutic plant-based (**Chapter II**).

The third and fourth steps of this thesis were dedicated to obtain a functional hydrocolloid from mutamba seeds (**Chapter III and IV**). Mutamba seeds liberate a viscous mucilage that envelops the seeds to form a gel-like capsule upon imbibition with water, like occur with chia seed and flaxseed. This mucilage is not easily removed from seed by using

convectional extraction techniques. Therefore, an extraction protocol by using high-intensity ultrasound (HIUS) was developed and the effect of HIUS process conditions (ultrasonic power and process time) on the yield recovery, quality and primary structure of the mutamba seed mucilage (MSM) was evaluated. The HIUS extraction system allows the full recovery of the mucilage surrounding the mutamba seeds without altering its quality and primary structure. It means that the nature of MSM was preserved and, consequently, its functionalities (**Chapter III**).

The preliminary structure evaluation demonstrated that the MSM is composed of 50% neutral monosaccharide residues, namely galactose and rhamnose, and 37% of uronic acid residues (galacturonic and glucuronic acids). MSM showed a negatively surface charge density (-39 mV) due to the negatively charged carboxyl groups of uronic acid residues. Moreover, this hydrocolloid showed a high content of protein (3.3%). The structural feature of MSM allow us to conceive an idea that this polysaccharide could be a novel and natural emulsifying agent to be used by food and non-food industries. In this regard, we investigated the use of MSM as emulsifying agent in orange peel oil-in-water emulsion. The oil-in-water emulsion containing MSM as emulsifying agent was more stable than emulsions produced with emulsifiers commonly used by food industry, namely gum acacia and OSA-modified starches. The emulsion stabilized by MSM effectively controlled the release of limonene, myrcene, α-pinene, and linalool while the emulsion with gum acacia and modified starches rapidly released them during the storage. The MSM can develop viscosity, which improved the emulsion stability and delayed the volatile compounds release by decreasing the droplet motion. Additionally, as MSM can rapidly adsorb into the oil-water interface, it helped stabilize the emulsion. We demonstrated that a novel and natural polysaccharide extracted from mutamba seeds could be used as emulsifying and thickening agent by food industry (**Chapter IV**).

GENERAL CONCLUSION AND PERSPECTIVES

The thesis's data clearly show that mutamba fruit has great potential to be explored by food and non-food industries as a source of bioactive and high-added value compounds, as well as in the product development. We demonstrated herein that mutamba fruit has high content of bioactive phenolic compounds, and its seeds liberate a mucilaginous substance that can be full recovered by using ultrasound technology. This hydrocolloid was structurally characterized and its emulsifying activity was experimentally proved by using an oil-in-water system.

This thesis brings new insights on phytochemical composition of mutamba fruit, which can support the next studies regarding its biological activities and technological properties. Finally, the execution of this thesis allowed us to discovering interesting things about mutamba fruit and, consequently, to look forward and to set up perspectives about it, namely:

- To evaluate the primary metabolites composition of mutamba fruit, such as mineral elements, fat acids, amino acids, mono-, di- and poly-saccharides (starch), soluble and insoluble fibre content, and physicochemical properties.
- To evaluate the phytochemical composition of mutamba fruit regarding the secondary metabolites that was not evaluated herein, such as carotenoids.
- To extract the phenolic compounds from mutamba fruit by using emergent technologies and safe solvents, such as ethanol and water or the mixture of both.
- To use the phenolic compounds from mutamba fruit as natural preservative for food, as well as to evaluate their antioxidant and antimicrobial activities.
- Scaling up the extraction protocol of phenolic compounds from mutamba fruit (pulp) and mucilage from mutamba seeds toward industrial process.
- To use the mutamba seed mucilage (MSM) as emulsifying agent for food, such as beverages containing essential oils, at low concentration (< 1g/100 g emulsion system).
- To evaluate the impact of mucilage concentration, proteinaceous impurities, pH, temperature, mono- and disaccharides addition, and ionic strength on MSM properties. Furthermore, the interaction between MSM and other polymers should be considered to improve its technological applications.

- To develop an integrated process aiming recovering phenolic compounds from mutamba pulp and mucilage and starch from mutamba seeds.
- To develop a mutamba fruit tea (pulp) and to evaluate its biological properties by using *in vitro* and *in vivo* models, such as antioxidant, antidiabetic, and effect on gastrointestinal disorders.
- To evaluate the healthy properties of mutamba fruit to human body due to its dietary fibre content.
- To use the mutamba fruit flour as ingredient in the development of bakery products.
- To develop functional products using mutamba fruit.

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ANNEXS

Annex 1. Accepted manuscript by Food Research International.

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Submissions with an Editorial Office Decision for Author Gustavo Araujo Pereira, Master

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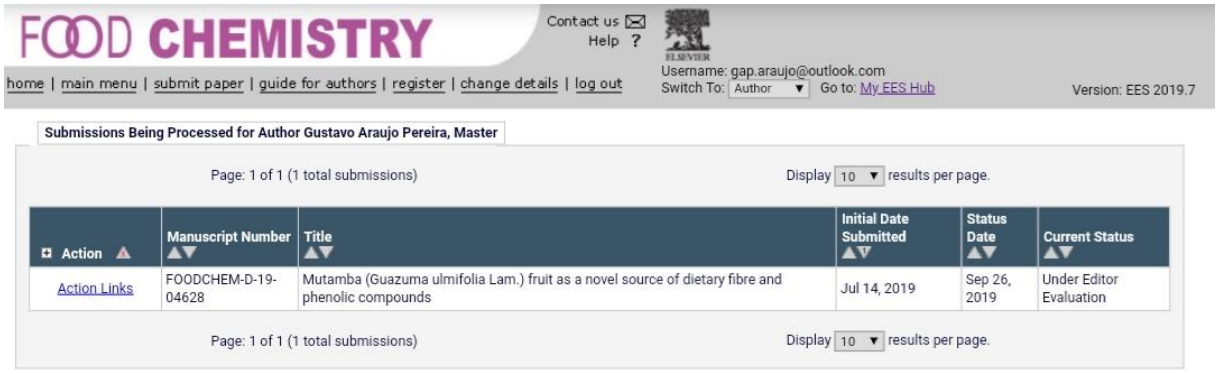
Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status	Date Final Disposition Set	Final Disposition
View Submission R1 View Decision Letter Send E-mail	FOODRES-D-19-03044	Phytochemicals and biological activities of mutamba (Guazuma ulmifolia Lam.): A review	Aug 21, 2019	Sep 28, 2019	Completed - Accept	Sep 28, 2019	Accept

Annex 2. Submitted manuscript to Food Chemistry.



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Action Links	FOODCHEM-D-19-04628	Mutamba (Guazuma ulmifolia Lam.) fruit as a novel source of dietary fibre and phenolic compounds	Jul 14, 2019	Sep 26, 2019	Under Editor Evaluation

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Annex 3: Declaration regarding access to the Brazilian genetic heritage

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AA56F0A

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AA56F0A**
Usuário:	**Gustavo Araujo Pereira**
CPF/CNPJ:	**099.197.436-07**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Guazuma ulmifolia
Muntingia calabura
Anacardium occidentale
Euterpe oleracea

Título da Atividade:	**Dos metabólitos primários até os secundários: caracterização química dos frutos calabura e mutamba e seus potenciais usos na industria alimentar**

Equipe

Gustavo Araujo Pereira	**UNICAMP**
Henrique Silvano Arruda	**UNICAMP**
Eric Keven silva	**UNICAMP**
Glaucia Maria Pastore	**UNICAMP**
Marcos Nogueira Eberlin	**UNICAMP**
Nayara Macêdo Peixoto Araujo	**UNICAMP**
Maria Angela de Almeida Meireles Patenate	**UNICAMP**
Damila Rodrigues de Morais	**UNICAMP**

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Situação do Cadastro:	**Concluído**

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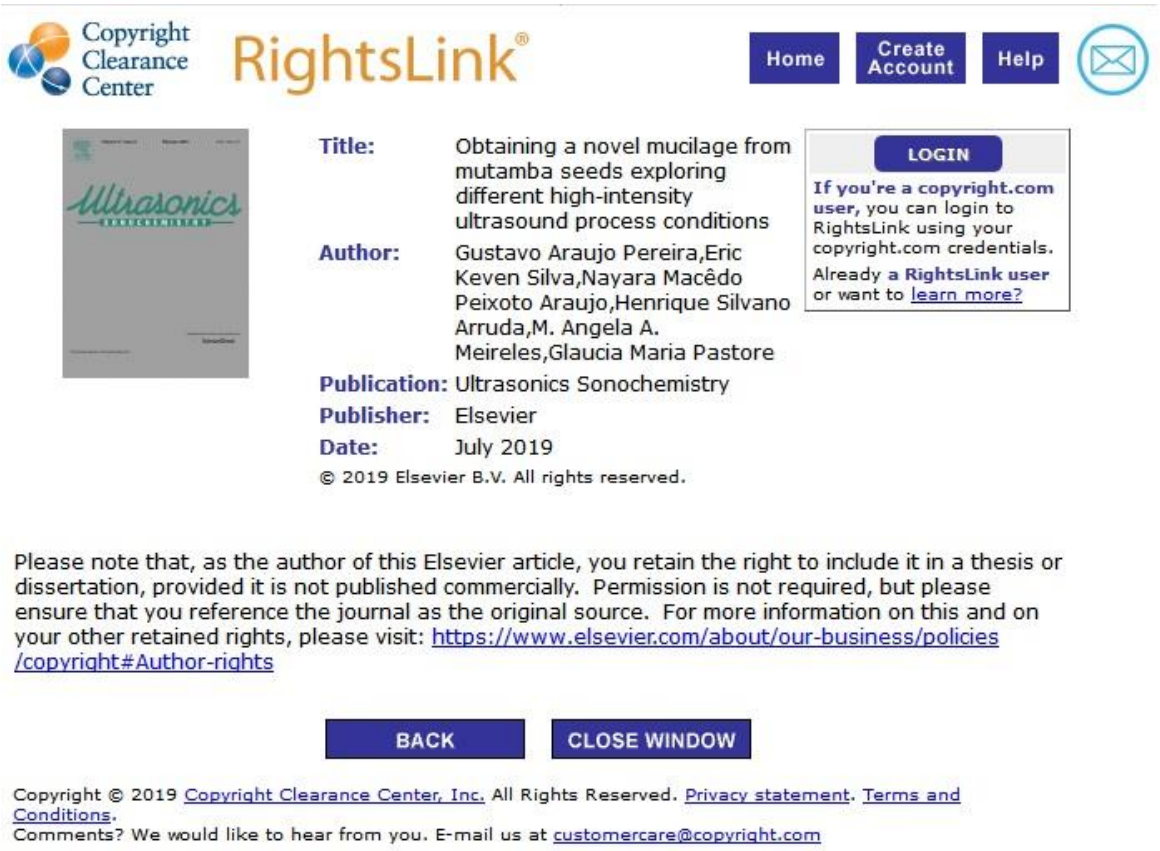
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

Annex 4: Licence to reuse the content of the research article published to Ultrasonics Sonochemistry in the doctoral thesis (Chapter III) and highlights.



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Title: Obtaining a novel mucilage from mutamba seeds exploring different high-intensity ultrasound process conditions

Author: Gustavo Araujo Pereira,Eric Keven Silva,Nayara Macêdo Peixoto Araujo,Henrique Silvano Arruda,M. Angela A. Meireles,Glaucia Maria Pastore

Publication: Ultrasonics Sonochemistry

Publisher: Elsevier

Date: July 2019

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Highlights

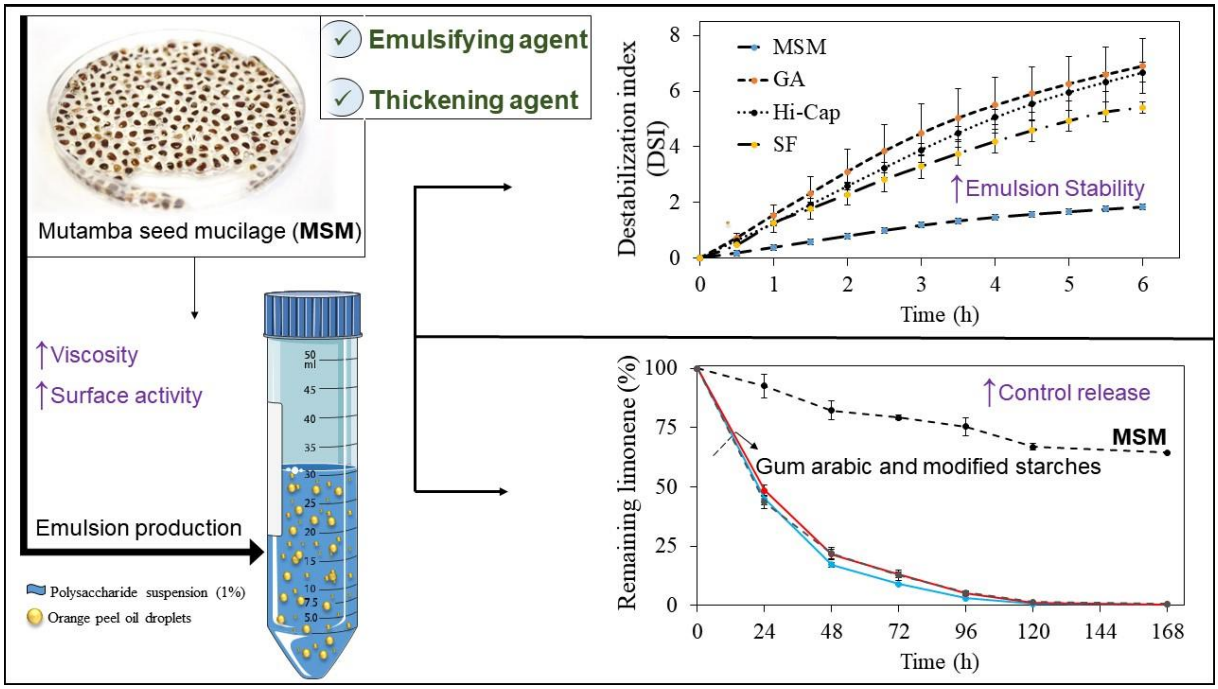
- High-intensity ultrasound (HIUS) process conditions intensification increase the mucilage recovery.
- HIUS totally removed the mucilage at a short process time (7 min).
- HIUS treatment did not affect the mucilage structure.
- Mutamba mucilage has great potential to be used as food and non-food functional ingredient.

Annex 5: Licence to reuse the content of the research article published to Food Hydrocolloids in the doctoral thesis (Chapter IV)

Annex 6: Highlights and graphical abstract of the research article published to Food Hydrocolloids (Chapter IV)

Highlights

- Mutamba seed mucilage (MSM) showed emulsifying and thickening properties.
- Emulsion stabilized by MSM was more stable than gum acacia, and modified starches.
- MSM showed better surface activity than gum acacia.
- Volatile compounds from orange peel oil were highly retained by MSM
- MSM was able to control effectively the release of volatile compounds.



Annex 7: Supplementary material of the research article published to Food Hydrocolloids (Chapter IV)

Supplementary Material

Mutamba seed mucilage as a novel emulsifier: stabilization mechanisms, kinetic stability and volatile compounds retention

Gustavo Araujo Pereira [a,*], Eric Keven Silva [b], Nayara Macêdo Peixoto Araujo [a], Henrique Silvano Arruda [a], M. Angela A. Meireles [b] and Glaucia Maria Pastore [a]

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Table 1. Identification of volatile compounds from orange peel oil using Headspace solid-phase microextraction (SPME) combined with gas chromatography–mass spectrometry detection (GC–MS) method.

Number	Compound	LRI	$LRI_{ref.}$	Relative area (%)
1	Limonene	1049	1038[a]	**91.31**
2	Myrcene	994	995[a]	**3.05**
3	α-Pinene	943	943[a]	**1.04**
4	Linalool	1103	1100[a]	**0.87**
5	Sabinene	982	981[b]	0.80
6	Decanal	1209	1210[a]	0.42
7	Octanal	1006	1007[b]	0.41
8	δ-3-Carene	1020	1025[a]	0.34
9	Geranial	1278	1283[d]	0.15
10	β-Ocimene	1054	1050[b]	0.15
11	α-Terpineol	1202	1209[c]	0.13
12	Citronellal	1158	1148[d]	0.11
13	α-Phellandrene	1014	1006[d]	0.11
14	Neral	1250	1242[d]	0.11
15	Terpinolene	1097	1089[b]	0.09
16	Nonanal	1107	1101[b]	0.09
17	Octanol	1072	1076[a]	0.09
18	Perilla aldehyde	1291	1290[e]	0.06
19	Eremophilene	1521	1504[i]	0.05
20	β-Pinene	988	982[a]	0.04

21	β-Terpineol	1078	1159[e]	0.04
22	Unknown	1231	-	0.04
23	Unknown	1119	-	0.04
24	*E*-Limonene oxide	1149	1147[e]	0.03
25	α-Copaene	1397	1391[e]	0.03
26	γ-Terpinene	1068	1060[b]	0.03
27	Carvone	1259	1251[c]	0.03
28	Unknown	1301	-	0.02
29	Geraniol	1235	1245[f]	0.02
30	Dodecanal	1413	1411[e]	0.02
31	Undecanal	1311	1305[d]	0.02
32	Unknown	1410	-	0.02
33	*Z*-limonene oxide	1146	1144[e]	0.02
34	Unknown	1456	-	0.02
35	α-Thujene	933	933[b]	0.02
36	Caryophyllene	1448	1442[a]	0.02
37	Unknown	1065	-	0.01
38	Unknown	1174	-	0.01
39	Unknown	1132	-	0.01
40	Unknown	1191	-	0.01
41	Unknown	1219	-	0.01
42	Germacrene D	1510	1499[e]	0.01
43	*E,E*-2,4-Decadienal	1325	1323[e]	0.01
44	Unknown	1225	-	0.01
45	*E*-2-Decenal	1268	1266[e]	0.01
46	Terpinen-4-ol	1186	1177[b]	0.01
47	α-Copaene	1367	1377[b]	0.01
48	Unknown	1198	-	0.01
49	δ-Cadinene	1546	1530[e]	0.01
50	Camphene	960	955[b]	0.01
51	Unknown	1422	-	0.01
52	Butanoic acid	800	790[g]	0.005
53	α-Terpinyl acetate	1361	1354[e]	0.004
54	Citronellyl acetate	1355	1348[e]	0.004
55	Unknown	1385	-	0.004
56	2-hexen-1-ol	854	867[c]	0.003
57	Unknown	1392	-	0.003
58	α-Humulene	1483	1474[h]	0.002

LRI, Linear retention indices relative to C7-C40 *n*-alkanes on the HP-5MS column.
$LRI_{ref.}$, Linear retention indices previously published: **a)** Zhao et al. (2006); **b)** Dehghan et al. (2007); **c)** Kim, Thuy, Shin, Baek, and Lee (2000); **d)** Samdja, Rondeau, and Sing (2005); **e)** Högnadóttir and Rouseff (2003); **f)** Shang, Yaoming, Deng, and Hu (2002); **g)** Pino, Mesa, Muñoz, Martí, and Marbot (2005); **h)** Yu, Kim, Kim, & Lee (2004); **i)** Oprean, Tamas, Sandulescu, and Roman (1998).

Methodology

The volatile compounds from orange peel oil were recovered by using a SPME fiber (Supelco PDMS/DVB, 10 mm in length × 65 μm layer). The fibre was activated according to the manufacturer's instructions. The extraction was performed as follows: 0.1 g of the orange peel oil were placed in sterile flasks with a plastic cap with a PTFE septum. The sample flaks were put under magnetic stirring at 30 °C for 30 min. After this time, the SPME fibre was exposed to the headspace of the solution to adsorb the compounds. The SPME fibre was removed after 30 min from the sample vial and introduced into the heated chromatograph injector (250 °C for 10 min) for desorption and analysis (Souza de Carvalho et al., 2014).

The volatile compounds were analysed using an Agilent HP-7890 GC system coupled to a mass spectrometer HP-5975C (Agilent Technologies, Santa Clara, USA). Volatile components were separated using a capillary column (J&W Scientific HP-5MS, 30 m lenght × 0.25 mm i.d. × 0.25 μm of film thickness). The oven temperature programme was as follows: 80 °C (3 min), 80–240 °C at 20 °C/min, 240 °C (5 min). The carrier gas was helium at 1.0 mL/min. The analysis was performed in the splitless mode and temperatures of the injector and detector were set at 250 °C. The mass spectrometer transfer line was set at a temperature of 250 °C, impact energy of 70 + eV and the acquisition mass range of *m/z* 35–500. The identification of the volatile compounds was made by comparing their mass spectra with National Institute of Standards and Technology (NIST) mass spectral library (70% similarity) and linear retention index (LRI). The LRI of the identified compounds were calculated using a series of *n*-alkanes (C_7–C_{40}) injected in the same SPME-GC-MS conditions, and the LRI values were compared with those reported in the literature. The proportion of each compound was estimated dividing its mean area by the total area of the chromatogram, and expressed as percentage (Souza de Carvalho et al., 2014).

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Annex 8: Research article published to Food Research International

Food Research International 108 (2018) 264–273

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Carbohydrates, volatile and phenolic compounds composition, and antioxidant activity of calabura (*Muntingia calabura* L.) fruit

Gustavo Araujo Pereira[a,*], Henrique Silvano Arruda[a], Damila Rodrigues de Morais[b], Marcos Nogueira Eberlin[b], Glaucia Maria Pastore[a]

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ARTICLE INFO

Keywords:
FODMAPs
β-Farnesene
Methyl salicylate
Cyanidin-3-*O*-glucoside
Gallic acid
Flavonoids
Berries

Chemical compounds studied in this article:
Catechin (PubChem CID: 73160)
Cyanidin-3-*O*-glucoside (PubChem CID: 441667)
Delphinidin-3-*O*-glucoside (PubChem CID: 443650)
Gallocatechin (PubChem CID: 65084)
Fructose (PubChem CID: 5984)
Gallic acid (PubChem CID: 370)
Glucose (PubChem CID: 79025)
Methyl salicylate (PubChem CID: 4133)
Quercetin (PubChem CID: 5280343)
β-Farnesene (PubChem CID: 5281517)

ABSTRACT

Soluble carbohydrates, volatile and phenolic compounds from calabura fruit as well as its antioxidant activity were assessed. The low amount of fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) and similar amount of glucose and fructose allow us to classify the calabura berry as low-FODMAPs. The terpenes β-Farnesene and dendrolasin identified by SPME-GC-MS were the major volatile components. UHPLC-MS/MS analysis revelled gallic acid (5325 μg/g dw) and cyanidin-3-*O*-glucoside (171 μg/g dw) as the main phenolic compounds, followed by gentisic acid, gallocatechin, caffeic acid and protocatechuic acid. In addition, gallic acid was found mainly in esterified (2883 μg/g dw) and insoluble-bound (2272 μg/g dw) forms. Free and glycosylated forms showed however the highest antioxidant activity due to occurrence of flavonoids (0.28–27 μg/g dw) in these fractions, such as catechin, gallocatechin, epigallocatechin, naringenin, and quercetin. These findings clearly suggest that calabura is a berry with low energy value and attractive colour and flavour that may contribute to the intake of several bioactive compounds with antioxidant activity. Furthermore, this berry have great potential for use in the food industry and as functional food.

1. Introduction

The species *Muntingia calabura* L. (see Fig. S1 in Supplementary material) belongs to Elaeocarpaceae family and it is the sole species within the genus *Muntingia*. This plant is native to Central America, but due to its good adaptation to soil and climate it has been widely grown in several countries of tropical climates, such as Brazil, China, India, Malaysia and Philippines (Mahmood et al., 2014). The calabura tree is also exotic and ornamental, and it grows fast even on poor soils. The first fruiting occurs one year after the planting and the fruiting occur throughout the year with peak from April to July.

Calabura is also a climacteric fruit that can ripen fully if they are harvested at completion of their growth period (Rahman, Solaiman, & Rahman, 2010). The fruit is small (average weight of 1.60 g) and red-coloured at mature stage, and shows a sweet and unique flavour, which attracts birds and also pleases the human taste. These ripe berries are very sweet due to high content of soluble solids (10.24°Brix) and low total titratable acidity (0.11 g citric acid per 100 g fruit, pH 5.64). Besides being very appreciated for its flavour and color, the fruit has been reported to contribute to the intake of carbohydrates (14.64%), proteins (2.64%), lipids (2.34%), fibers (1.75%) and minerals (1.28%), with low total energy value (Pereira, Tomé, Arruda, Fragiorge, & Ribeiro, 2016). In addition, it contains high content of soluble phenolic compounds, mainly phenolic acids and flavonoids (Lin et al., 2017; Rotta, Haminiuk, Maldaner, & Visentainer, 2017). Extracts rich in phenolic compounds from calabura fruits have been found to exhibit bioactivities such as

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0963-9969/

antimicrobial activity against food borne pathogens (Sibi, Kaushik, Dhananjaya, Ravikumar, & Mallesha, 2013), antioxidant properties suppressing human LDL (Low Density Lipoprotein) oxidation (Lin, Chen, Chang, Chen, & Yang, 2017), and anti-inflammatory activity in carrageenan rat paw edema model (Gomathi, Anusuya, & Manian, 2013) and lipopolysaccharide-induced pro-inflammatory mediators in macrophages (Lin, Chang, et al., 2017).

The calabura fruits are eaten fresh and they are mostly consumed as homemade preparations, but these berries could have great potential for use in the food industry and as functional food due to its physicochemical properties and high content of antioxidant compounds (Lin, Chen, et al., 2017; Pereira et al., 2016). However, there is little information available in the literature about chemical composition of this berry; and the use of fruits in food science and industry depends mainly of its composition. Although there have been recent studies on the soluble phenolic compounds from calabura (Lin, Chang, et al., 2017; Lin, Chen, et al., 2017; Rotta et al., 2017), other fractions of phenolic compounds were not investigated, which can result in the underestimation of phenolic compounds content and consequently the actual biological properties of calabura fruits. In this approach, we evaluated the composition of volatile organic compounds, functional sugars and oligosaccharides, the individual anthocyanins content, and the soluble (free, esterified and glycosylated) and insoluble-bound forms of non-coloured phenolic compounds from calabura fruits. Colorimetric and fluorometric assays were also used to assess the phytochemicals and antioxidant activity of these berries. The aim of this study was therefore to investigate the functional components of calabura berry to help future studies about its potential functionality and uses in food industry.

2. Material and methods

2.1. Chemicals and reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazil), AAPH (2,2′-azobis(2-methylamidinopropane)-dihydrochloride), ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt), fluorescein sodium salt, sodium carbonate, and Folin–Ciocalteu reagent were purchased from Sigma–Aldrich (St. Louis, USA). Sugars (glucose, fructose and sucrose), polyols (xylitol, mannitol, and sorbitol), galactooligosaccharides (GOS; raffinose, stachyose, and verbascose), and maltooligosaccharides (MOS; maltotriose to maltoheptaose) were purchased from Sigma-Aldrich (St. Louis, USA), whereas the fructooligosaccharides (FOS; 1-kestose, nystose, and 1F-β-fructofuranosylnystose) were purchased from Wako (Wako Pure Chemicals Industries, Osaka, Japan). All phenolic compounds standards with purity ≥96% (gallic acid, gallocatechin, protocatechuic acid, epigallocatechin, catechin, chlorogenic acid, 4-hydroxybenzoic acid, epicatechin, caffeic acid, vanillic acid, gentisic acid, *p*-coumaric acid, sinapic acid, ferulic acid, rutin, quercetin, naringenin, cyanin, delphinidin-3-*O*-glucoside, cyaniding-3-*O*-glucoside, and methyl salicylate) were purchased from Sigma-Aldrich (St. Louis, USA). The saturated alkanes standard (C_7-C_{40}) was purchased from Supelco (Bellefonte, USA). Only HPLC grade solvents were used for preparing mobile phases. Ultrapure water (18 MΩ cm^{-1}) obtained from a Milli-Q water purification system (Millipore, Bedford, USA) was used.

2.2. Volatile constituents of calabura fruits by SPME and GC–MS

2.2.1. Harvest of the fruits

Ripe calabura fruits (red-coloured peel) were collected in July 2016 from five calabura trees located in Campinas-SP, Brazil. The fruits (10 fruits per tree in each day) were harvested at different date (once a week, totalling four days), and the analysis of volatile compounds from calabura fruits was carried out in triplicate on the same day of fruit harvest.

2.2.2. Solid phase microextraction (SPME)

SPME fiber (Supelco PDMS/DVB, 10 mm in length × 65 μm layer) was used. The fiber was activated according to the manufacturer's instructions. For each extraction, 5 g of the fruit homogenized and 15 mL of distilled water were placed in sterile flasks with a plastic cap with a PTFE septum. The sample flaks were put under magnetic stirring at 30 °C for 30 min. After this time, the SPME fiber was exposed to the headspace of the solution to adsorb the compounds. After 30 min, the SPME fiber was removed from the sample vial and introduced into the heated chromatograph injector (250 °C for 10 min) for desorption and analysis (Souza de Carvalho et al., 2014).

2.2.3. Gas chromatography-mass spectrometry (GC–MS)

The volatile compounds were analysed using an Agilent HP-7890 GC system coupled to a mass spectrometer HP-5975C (Agilent Technologies, Santa Clara, USA). Volatile components were separated using a capillary column (J&W Scientific HP-5MS, 30 m lenght × 0.25 mm i.d. × 0.25 μm of film thickness). The oven temperature programme was as follows: 40 °C (5 min), 40–120 °C at 5 °C/min, 120 °C (5 min), 120–180 °C at 3 °C/min, 180–240 °C at 10 °C/min, 240 °C (5 min). The carrier gas was helium at 1.0 mL/min. The analysis was performed in the splitless mode and temperatures of the injector and detector were set at 250 °C. The mass spectrometer transfer line was set at a temperature of 250 °C, impact energy of 70 + eV and the acquisition mass range of *m/z* 35–500. The identification of the volatile compounds was made by comparing their mass spectra with National Institute of Standards and Technology (NIST) mass spectral library (90% similarity) and linear retention index (LRI). The LRI of the identified compounds were calculated using a series of *n*-alkanes (C_7–C_{40}) injected in the same SPME-GC-MS conditions, and the LRI values were compared with those reported in the literature. The proportion of each compound was estimated dividing its mean area by the total area of the chromatogram, and expressed as percentage (Souza de Carvalho et al., 2014).

2.3. Plant material and sample preparation for carbohydrates, phenolic compounds and antioxidant analyses

Calabura fruits with full physiological maturity (red-coloured peel) were collected from June to July 2016. The collection of fruits was performed from five calabura trees located in Campinas-SP, Brazil. To avoid variation in the calabura composition, all fruits collected (1 kg) were mixed as a single lot. The fruits collected were immediately washed with distilled water to remove surface dirt, frozen at −20 °C, and thus all fruits frozen (1 kg) were freeze-dried. The average moisture content of the calabura fruit was 75.00 ± 1.6%. The freeze-dried material was powdered using a knife grinder and stored at freezer (−20 °C) until analysis.

2.4. Chromatographic analysis of sugars and oligosaccharides

Freeze-dried fruit was mixed with ultrapure water (1,10, w/v) with the aid of Ultra-Turrax Homogenizer at 11000 rpm for 30 s at room temperature. After the centrifugation (4000g, 5 min, 5 °C) the supernatant was filtered with a 0.22 μm filter and used for the analysis of sugars and oligosaccharides as described by Pereira, Arruda, Molina, and Pastore (2017), with some modifications.

High performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) system model DIONEX ICS-5000 (Thermo Fisher Scientific, Waltham, USA) was used. The flow-rate was 1.0 mL/min, the column temperature was kept at 30 °C and the injection volume was 25 μL. Two different chromatographic column were used, one for the analysis of galactoligo-, mono- and disaccharides and polyols (Carbopac PA1 (250 × 4 mm, 10 μm particle size)), and the second one for the fructo- and maltooligosaccharides (CarboPac PA100 (250 × 4 mm, 8.5 μm particle size)). Isocratic mobile

phase (0.12 M NaOH) was used for sugars and galactooligosaccharides analysis. On the other hand, in the analysis of fructo- and maltooligosaccharides the mobile phase consisted of 0.1 M NaOH (A) and 0.5 M sodium acetate containing 0.1 M NaOH (B). The gradient was performed as follows: 0–2 min, 3% B; 2–18 min, 3–40% B; 18–25 min, 100% B; and 25–30 min, 3% B. The content of individual compounds was expressed as mg/g dw (dry weight). Analytical parameters of identification and quantification are displayed in Table S2 in the Supplementary material.

The sugars and oligosaccharides were identified in sample by comparing the retention times of the standards and the samples. The identity of the individual peaks was further confirmed by co-injection of samples spiked with commercial standards (co-chromatography). Calibration curves were constructed with commercial standards (0.4–20 μg/mL) to quantify the sugar/oligosaccharides in the extracts. Stock solutions of each standard compound (1 mg/mL) were prepared in deionized water and stored. For injection into column, samples and standards were diluted in deionized water.

2.5. Phenolic compounds

2.5.1. Extraction and purification of anthocyanins

Freeze-dried fruit was homogenized with methanol 0.20% HCl (1,15, w/v) and left in ultrasonic bath for 10 min at 20 °C. The mixture was centrifuged (4000*g*, 15 min, 5 °C). The supernatant was collected and the residue was re-submitted to extraction protocol. This procedure was repeated three times and the supernatants were combined. The supernatant was concentrated under vacuum in a rotary evaporator (35 °C) to total evaporation of the methanol. The extract was re-suspended in ultrapure water 0.20% HCL, and this aqueous extract was washed five times with *diethyl ether:ethyl acetate* (1,1, v/v) to remove free phenolic compounds with low molecular weight (Revilla, José-María Ryan, & Martín-Ortega, 1998).

Pre-conditioned Amberlite XAD-7 resin was used to obtain extract rich in anthocyanins (ERA). The aqueous extract was mixed with Amberlite XAD-7 (1,5, v/w) and the mixture was shaken at 160 rpm for 40 min at room temperature. The resin was filtered, washed with ultrapure water and anthocyanins were desorbed with MeOH 0.20% HCl (Jiménez-Aspee et al., 2016). The ERA was evaporated under reduced pressure at 35 °C, re-suspended in water 0.20% HCl (pH < 3.0), filtered with a 0.22 μm filter and used for the colorimetric/fluorometric assays and UHPLC-MS/MS analysis.

2.5.2. Extraction of non-coloured soluble and insoluble-bound phenolic compounds

Soluble and insoluble-bound phenolic compounds from the calabura fruit were extracted and fractionated according to the method described by Wang et al. (2016), with some modifications (Arruda, Pereira, de Morais, Eberlin, & Pastore, 2018).

2.5.2.1. Extraction of soluble phenolic compounds. Freeze-dried fruit (1 g) was homogenized with 15 mL of a mixture of methanol-acetone-water (7:7:6, v/v/v) and left in ultrasonic bath for 30 min at room temperature. The mixture was centrifuged (4000*g*, 15 min, 5 °C). The supernatant was collected and the residue was re-submitted to extraction protocol. This procedure was repeated three times and the supernatants were combined. The solid residue was stored for further extraction of insoluble-bound phenolic compounds. The supernatant was evaporated under vacuum at 35 °C and the remaining aqueous phase was acidified to pH 2 using 6 M HCl, and centrifuged (4000*g*, 5 min, 5 °C) to remove precipitates. Following this treatment, the aqueous phase was extracted three times with an equal volume of hexane to remove interfering lipids. This aqueous phase was used for the fractionation of soluble phenolic compounds.

2.5.2.2. Fractionation of soluble phenolic compounds. The free phenolic compounds (F1) were extracted three times with diethyl ether-ethyl acetate (1:1, v/v) at a solvent to aqueous phase ratio of 1:1 (v/v). The remaining aqueous phase after the F1 extraction was hydrolysed with 4 M NaOH containing 10 mM EDTA and 1% ascorbic acid at 150 rpm for 4 h at room temperature to release esterified phenolics. The pH of the hydrolysate was adjusted to 2 using 6 M HCl and the phenolic compounds (F2, esterified) released from soluble esters were extracted using the procedure as described for F1. The aqueous phase remained after the F2 separation was hydrolysed with 5 mL 6 M HCl at 150 rpm for 60 min at 75 °C to release glycosylated phenolics. Phenolic compounds (F3, glycosylated) released from soluble glycosides were extracted as described for free phenolics (F1).

2.5.2.3. Hydrolysis and extraction of insoluble-bound phenolic compounds. The solid residue from soluble phenolics extraction was hydrolysed with 4 M NaOH containing 10 mM EDTA and 1% ascorbic acid at a solid to solvent ratio of 1:20 (w/v) at 150 rpm for 4 h at room temperature to release insoluble-bound phenolics. The pH of the mixture was adjusted to 2 using 6 M HCl and centrifuged (4000*g*, 5 min, 5 °C). The supernatant was extracted 3 times with an equal volume of hexane to remove interfering lipids. Phenolic compounds (F4, insoluble-bound) released from insoluble-bound phenolics were extracted as described for free phenolics (F1).

Each organic phenolic fractions (F1, F2, F3 and F4), obtained as described above, were dehydrated with anhydrous sodium sulfate, and evaporated to dryness under vacuum at 35 °C. The dry residues were dissolved into 5 mL of methanol, and these solutions were used for colorimetric/fluorometric assays and UHPLC-MS/MS analysis.

2.5.3. Phenolic compounds of calabura fruits by UHPLC-MS/MS

Analysis of phenolic compounds in ERA and non-coloured phenolic fractions (F1 to F4) were conducted using an ultra-high performance liquid chromatography triple quadrupole mass spectrometer (UHPLC-MS/MS) system model LCMS-8040 (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source.

Stock solutions of each standard compound (1 mg/mL) were prepared in methanol and stored. For injection into column, samples and standard were diluted in water 0.1% formic acid to improve the stability of phenolic compounds. Calibration curves were constructed (20–1000 ng/mL for non-coloured phenolic compounds, and 20–600 ng/mL for anthocyanins) to quantify the phenolics in the extracts. Results were expressed as μg/g dw (Morais et al., 2015).

Anthocyanins were analysed in the positive ion mode, whereas the analysis of non-coloured phenolic compounds were performed in the negative ion mode. For each phenolic standard, the precursor ions were determined in full scan experiments and product ions were chosen by MS/MS experiments. The analyses were conducted by multiple reaction monitoring (MRM) using two of the most selective transitions for each standard compound. The settings of the mass spectrometer were optimized for each transition (see Table S3 in Supplementary material). ESI source parameters were as following: capillary voltage, 3.5 kV; heat block temperature, 300 °C; desolvation line temperature, 250 °C; drying gas flow (N_2), 20 L/min; nebulizing gas flow (N_2), 3 L/min; collision induced dissociation gas pressure (Ar), 224 kPa.

The chromatographic separation was performed on a Shimpack XR-ODS III column (2.2 μm, 2.0 mm i.d., and 150 mm) at 40 °C using binary mobile phase at flow rate of 0.4 mL/min. Solvent A was ultrapure water 0.1% formic acid and solvent B was methanol. The autosampler temperature was maintained at 10 °C and the injection volume was 10 μL. The gradient elution for anthocyanins was as follows: 0–3.0 min, 5–100% B; 3.0–3.5 min, 100% B; 3.5–4.0 min, 100–5% B; and 4.0–8.0 min, 5% B. On the other hand, the gradient elution for non-coloured phenolics was as follows: 0–1.0 min, 5%B; 1.0–4.0 min, 5–60% B; 4.0–7.0 min, 60–70% B, 7.0–10.0 min, 70–100% B; 10.0–10.5 min, 100% B; 10.5–11.0 min, 5% B; and 11.0–15.0 min, 5% B (Bataglion, da Silva, Eberlin, & Koolen, 2015).

2.6. Colorimetric and fluorometric assays

ERA and the non-coloured phenolic fractions (F1 to F4) were used to colorimetric and fluorometric assays. Total phenolic compounds (TPC) were determined using the Folin–Ciocalteu assay according to Arruda, Pereira, and Pastore (2017). The results were expressed as gallic acid equivalents (mg GAE/g dw). Total anthocyanins (Total Acys) was determined using the method as described by Lao and Giusti (2016). Condensed tannins or proanthocyanidins content (CT) were determined following the procedure described by Broadhurst and Jones (1978), and the results were expressed as catechin equivalents (mg CE/g dw). Antioxidant activity was determined by the DPPH, TEAC (Trolox Equivalent Antioxidant Capacity) and ORAC (Oxygen Radical Absorbance Capacity) assays, following the procedures described by Leite-Legatti et al. (2012). The results were expressed as Trolox equivalents (μmol TE/g dw). All colorimetric assays were performed on a spectrophotometer (Beckman, model DU600, Indianapolis, USA), whereas the ORAC fluorometric assay was performed on a NovoStar Microplate reader (New Brunswick Scientific Classic Series, model C76, Edison, EUA).

2.7. Statistical analysis

The data were submitted to one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$). All determinations were carried out in triplicate, and the values were reported as the mean ± standard deviation. All statistical analyses were performed using the STATISTICA software (Statsoft, Oklahoma, USA) version 12.0.

3. Results and discussion

3.1. Volatile constituents of calabura fruits

There is an important distinction between volatile analysis and aroma analysis, since odor active compounds may occur at very low concentrations (Bicas et al., 2011). In the recent study we focus therefore on volatile analysis of calabura fruits using solid phase microextraction (SPME) and GC–MS. Terpenes, alcohols and esters comprised 57.9%, 3.7% and 2.7%, respectively, of volatile compounds found in calabura fruits. Fig. 1 shows the sesquiterpene β-Farnesene (28.7%) and the aromatic monoterpene dendrolasin (15.4%) were the major volatile constituents from calabura, followed by β-himachalene (3.9%), α-curcumene (3.7%), limonene (3.1%), 3-hexen-1-ol (2.8%) and methyl salicylate (MeSA, 2.7%), among others with area lower than 2.5%.

In 1996, Wong and co-authors reported the volatile constituents from calabura fruits using vacuum and steam distillation combined with solvent extraction. From the 42–56 compounds identified by GC–MS, MeSA (20.9%), 3-hydroxybutanone (20.2%) and 3-hexen-1-ol (18.2%) were the main compounds detected in vaccum distillation, whereas in steam distillation the most abundant were MeSA (30.6%), followed by benzyl alcohol (7.8%), furfural (6.9%) and (*E,E*)-farnesol (5.9%). Alcohols and esters were the major constituents and MeSA was the most abundant component (Wong, Chee, & Er, 1996). In the present study, we also detected MeSA, but β-Farnesene and dendrolasin were the most volatile component identified in the headspace and terpenes were the major class of volatile compounds. Dendrolasin is structurally similar to (*E,E*)-farnesol, so it is possible that the two compounds share a common origin (Moniodis et al., 2017).

Other parts of the plant were analysed and we found that MeSA was the most abundant constituent in no-infected tissues of fruit peel, leaves, flowers and bark of calabura tree (see Fig. S4 in Supplementary material). MeSA and other volatile compounds (β-Farnesene) may play a role in the resistance of the calabura tree to many diseases and herbivores attacks (Forouhar et al., 2005; Mahmood et al., 2014; Wong et al., 1996). The extraction and quantification of MeSA in tissue of different parts of calabura tree was not the aim of this study, but we performed this investigation to verify if the MeSA amount in calabura tree would show toxic or bioactivity effect in human body (see Table S5 in Supplementary material).

3.2. Sugars and oligosaccharides of calabura fruits

The calabura fruits showed high content of glucose, fructose and sucrose, and low content of 1-kestose, maltopentaose, maltohexaose and maltoheptaose. Only trace content of the nystose, 1^F-β-fructofuranosylnystose, maltotriose and maltotetraose were detected (≤LOQ), whereas raffinose, stachyose and verbascose (galactooligosaccharides, GOS), and xylitol, mannitol and sorbitol (polyols) were not detected. Table 1 shows the individual and total contents of sugars and oligosaccharides in calabura fruits.

The total content of sugars in calabura fruits (7.52 g/100 g fw, fresh weight) was similar to cherry (7.26 g/100 g fw), blackberry (6.8 g/100 g fw) and raspberry (6.58 g/100 g fw), lower than the content in grape (22.71 g/100 g fw), blueberry (17.29 g/100 g fw), mulberry (11.61 g/100 g fw) and black currant (9.85 g/100 g fw), and higher than strawberry (3.62 g/100 g fw).The calabura showed however total content of FOS (0.01 g/100 g fw) lower than the content of the fruits mentioned above (Jovanovic-Malinovska, Kuzmanova, & Winkelhausen, 2014).

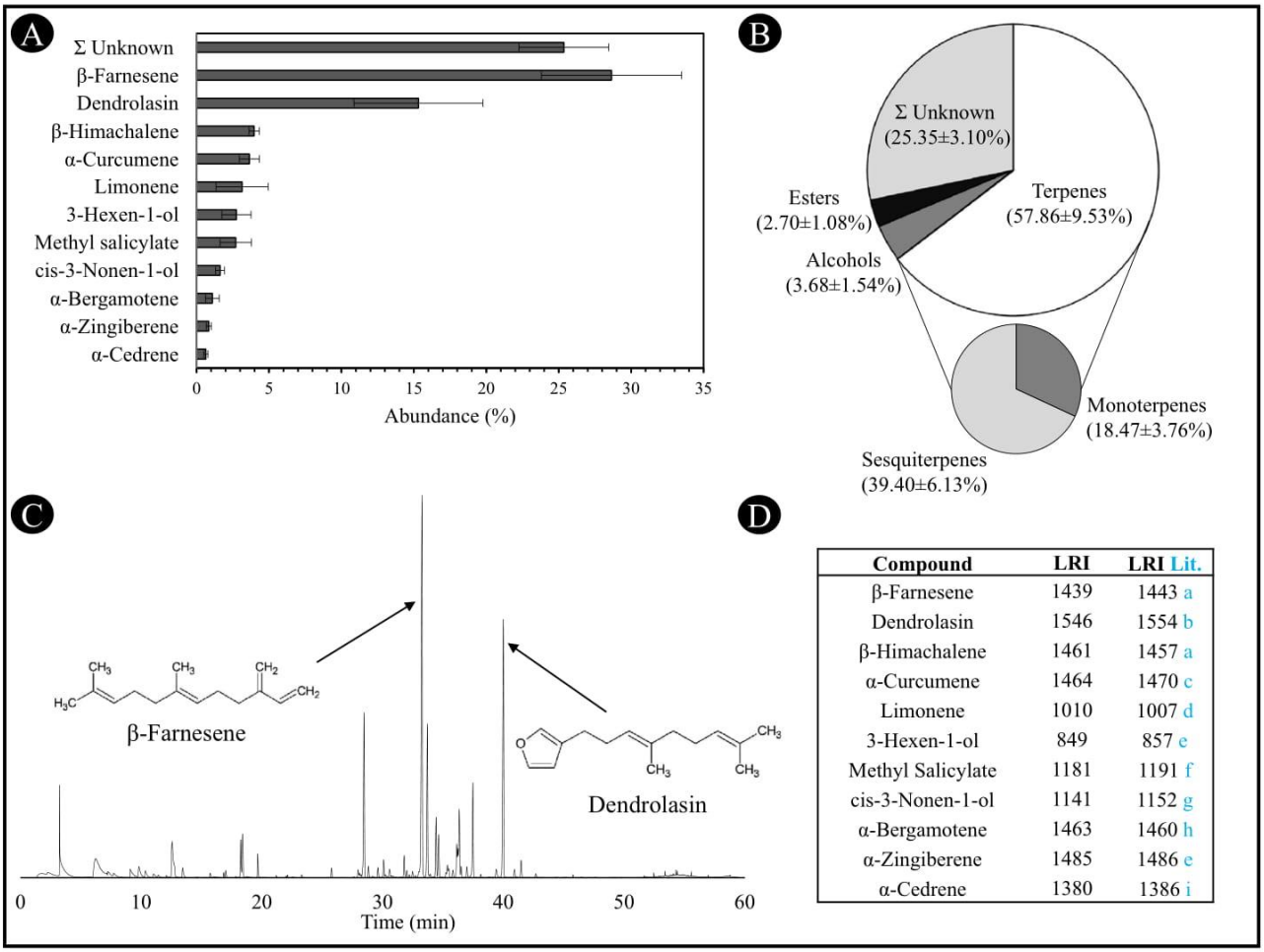
The results showed calabura fruit could be inserted in low-FODMAPs diets due to low content of fermentable oligosaccharides, such as FOS and GOS, and similar amounts of glucose and fructose (1:1). The content of fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) in calabura fruits was similar to blueberry and raspberry, which are classified as low-FODMAPs fruits. FODMAPS are carbohydrates with low molecular weight, such as (but not limited to) fructose in excess of glucose, FOS, GOS and polyols, that can be poorly absorbed by the small intestine. These compounds then arrive into the large intestine and can cause abdominal symptoms and disturbed stool output in many people, especially those with Irritable Bowel Syndrome (IBS). Low-FODMAPs diet can improve functional gastrointestinal symptoms in people with IBS, such as bloating, abdominal discomfort or pain, and altered bowel habit (Muir et al., 2009; Varney et al., 2017).

3.3. Anthocyanins of calabura fruits

As Fig. 2 shows, substantial quantitative and qualitative information can be obtained from the spectral characteristics of anthocyanins. The spectra of ERA (380–700 nm) was very similar to cyanindin-3-*O*-glucoside (C3G) and delphinidin-3-*O*-glucoside, which are the most common anthocyanins in nature. The mass spectrometry Full-Scan(+) (*m/z* 270-1000) of ERA showed that the ion of *m/z* 449[M]$^+$ was the most abundant; and its MS/MS fragmentation provides the ion of *m/z* 287[M-162]$^+$, which is a transition characteristic of C3G. The chromatography separation coupled to ESI-MS/MS also showed high amount of C3G in the calabura fruits extract. Delphinidin-3-*O*-glucoside was also identified, but in lower amount. Our findings clearly demonstrate therefore that C3G (97% of anthocyanins fraction) was the main anthocyanin of calabura fruit.

C3G is the main anthocyanin in most edible parts of several plants. Cyanidin glycosides were identified in some berries of the genus *Rubus* including blackberry. For example, C3G represents about 85–95% of the anthocyanin fraction in blackberries, followed by small amounts of other anthocyanins (Wu et al., 2006).

The total content of anthocyanins (4.4 mg/100 g fw, Table 2) in calabura fruits was similar to that found in nectarine, peach, and apple, and lower than that of blackberry, blueberry, black and red currant, raspberry and strawberry (Wu et al., 2006). Anthocyanins are found mainly in the peel of calabura, as in grape and plum, whereas in other fruits such as strawberry and raspberry the anthocyanins are distributed in the whole fruit (Pereira et al., 2016).



Compound	LRI	LRI Lit.
β-Farnesene	1439	1443 a
Dendrolasin	1546	1554 b
β-Himachalene	1461	1457 a
α-Curcumene	1464	1470 c
Limonene	1010	1007 d
3-Hexen-1-ol	849	857 e
Methyl Salicylate	1181	1191 f
cis-3-Nonen-1-ol	1141	1152 g
α-Bergamotene	1463	1460 h
α-Zingiberene	1485	1486 e
α-Cedrene	1380	1386 i

Fig. 1. Volatile compounds identified from calabura (*Muntingia calabura* L.) fruit using Headspace solid-phase microextraction (SPME) combined with gas chromatography–mass spectrometry detection (GC–MS) method. A) The average abundance (%) of each compound. B) The average abundance (%) of each class of volatile organic compounds. C) The chromatogram contained β-Farnesene and dendrolasin as main volatile compounds from calabura fruit. D) Linear retention indices (LRI) relative to C7-C40 *n*-alkanes on the HP-5MS column; LRI Lit.: a) Adams (2001), b) Marongiu, Porcedda, Porta, and Reverchon (2001), c) Sati and Mathela (2005), d) Qiao et al. (2008), e) Chokeprasert, Charles, Sue, and Huang (2007), f) Aligiannis, Kalpoutzakis, Kyriakopoulou, Mitaku, and Chinou (2004), g) Zhao et al. (2006), h) Elmore, Erbahadir, and Mottram (1997), and i) Basta, Tzakou, and Couladis (2006). As expected, the analysis of fruits harvested in different date promoted the larger standard deviation of volatile compounds values (%), but similar compounds, although not in the same proportion, were found.

Table 1
Sugars and oligosaccharides contents in the calabura (*Muntingia calabura* L.) fruits.

Sugar/oligosaccharide	Concentration in fruit (mg/g dw)
Glucose	119.82 ± 1.03
Fructose	133.33 ± 1.10
Sucrose	47.77 ± 0.34
Total Mono- and Disaccharides	300.92 ± 2.33
1-Kestose	0.53 ± 0.04
Total fructooligosaccharides (FOS)	0.53 ± 0.04
Maltopentaose	0.45 ± 0.04
Maltohexaose	0.50 ± 0.05
Maltoheptaose	0.19 ± 0.01
Total maltooligosaccharides (MOS)	1.14 ± 0.09
Total oligosaccharides	1.66 ± 0.11

The *in vitro* antioxidant properties of anthocyanins from calabura fruit was measured using different assays, that is, TPC, DPPH, TEAC and ORAC (Table 2). Anthocyanins from calabura fruits showed high antioxidant activity due to the content of C3G and delphinidin-3-*O*-glucoside. The results were similar to those anthocyanins from potent antioxidant berries, such as blackberry, blueberry, strawberry and raspberry (Paredes-López, Cervantes-Ceja, Vigna-Pérez, & Hernández-Pérez, 2010). Anthocyanins, phenolic acids and other bioactive compounds (*e.g.*: vitamins and minerals) are mainly responsible for berries health benefits and are also associated with their antioxidant activity (Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). It has also been shown that the intake of fruits rich anthocyanins possess several beneficial effects to human health (Paredes-López et al., 2010; Pascual-Teresa & Sanchez-Ballesta, 2008). In particular, *in vivo* reports suggest that C3G and its derivates possess different bioactivities and therefore they have potential beneficial effects in various human pathologies (Galvano et al., 2004).

3.4. Total phenolic content and antioxidant activity of non-coloured phenolic fractions from calabura fruits

The phenolic compounds can be found in the free, esterified, glycosylated and insoluble-bound forms in plant-based foods. The free phenolics were the most studied fraction in the past years, but

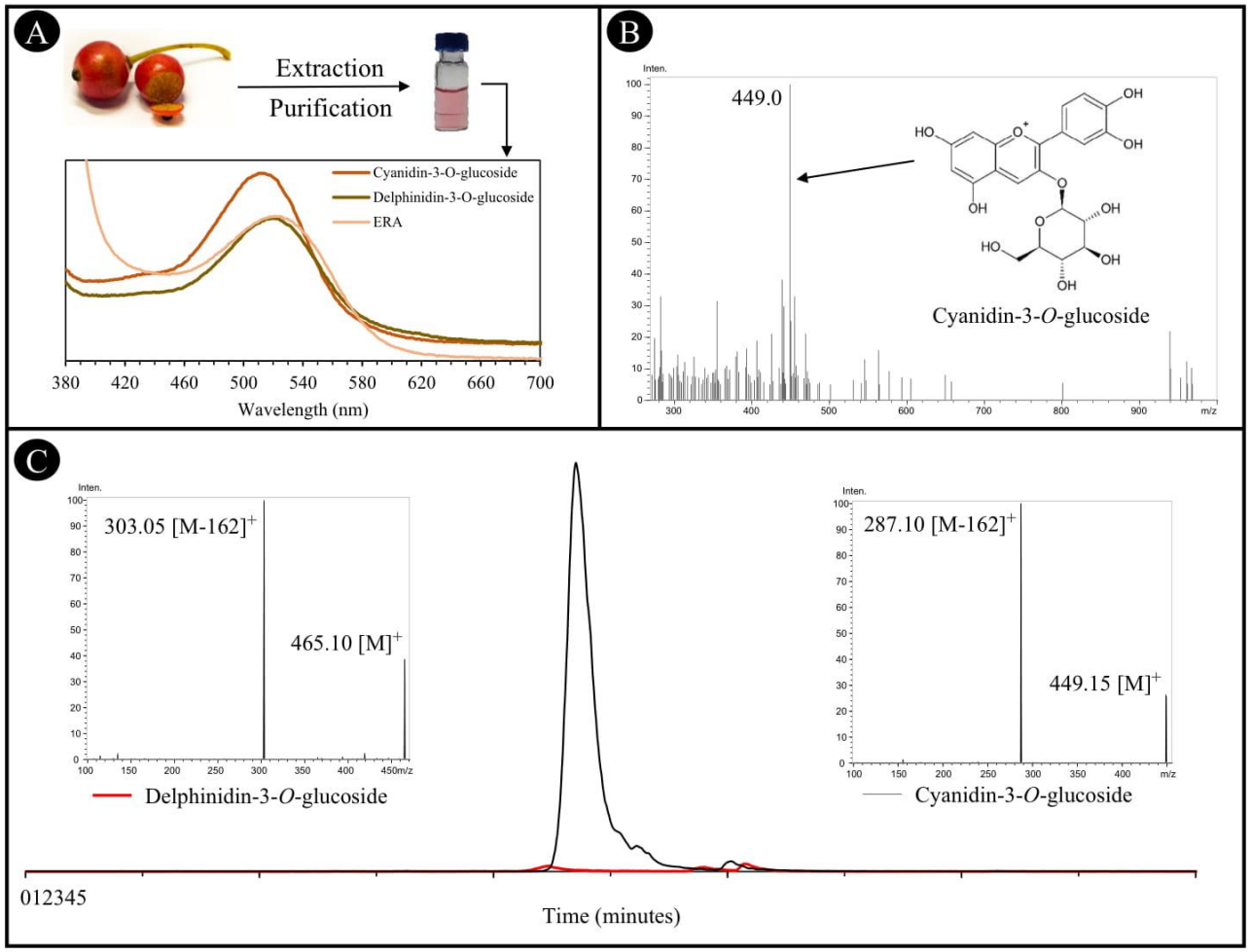


Fig. 2. Anthocyanins in calabura (*Muntingia calabura* L.) fruit. A) Visible absorption spectra (380–700 nm) of aqueous solution (pH 3) of cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and ERA (extract rich in anthocyanins); B) Full-Scan(+) (270–1000 *m/z*) of ERA, and C) chromatogram showing the main anthocyanins found in ERA and their MS/MS fragmentation.

Table 2
Total phenolic compounds (TPC), total anthocyanins content (Total Acys) and antioxidant activity, and individual anthocyanins content in extract rich in anthocyanins (ERA) from calabura (*Muntingia calabura* L.) fruits.

Assay	ERA
TPC (mg GAE/g dw)	15.21 ± 0.23
Total Acys (μg/g dw)[a]	30.20 ± 0.19
DPPH (μmol/g dw)	228.59 ± 4.65
TEAC (μmol/g dw)	143.59 ± 2.48
ORAC (μmol/g dw)	67.26 ± 2.82
Cyanidin-3-*O*-glucoside (μg/g dw)[b]	171.3 ± 2.19
Delphinidin-3-*O*-glucoside (μg/g dw)[b]	5.33 ± 0.19
Total Acys (μg/g dw)[b]	176.36 ± 2.27

Total Acys, a) Total Anthocyanin Method (Lao & Giusti, 2016) and b) Quantification of anthocyanins using UHPLC–MS/MS method.

nowadays other phenolic forms have been extensively investigated because they are abundant and also highly contribute to the plant-based foods bioactivities, such as antioxidant properties. Clearly, the identification and quantification of soluble and insoluble forms are very important to found the actual functional composition of plants, which also provide scientific support to studies about the effect of plant-based foods on human health (Shahidi & Yeo, 2016).

We extracted and fractioned the soluble and insoluble-bound phenolic compounds from calabura fruits. Four fractions were obtained as follows: free (F1), esterified (F2), glycosylated (F3) and insoluble-found (F4) forms. Colorimetric and fluorometric assays were used to assessed the phytochemical (TPC and CT) and antioxidant activity (DPPH, TEAC and ORAC) of these four fractions. Table 3 summarizes the total phenolic compounds (TPC), condensed tannins (CT) and antioxidant activity of different phenolics fractions of calabura fruits.

TPC in the different phenolic fractions of calabura fruits followed the decreasing order of free > esterified > glycosylated > insoluble-bound, whereas glycosylated and esterified fractions showed similar CT (proanthocyanidins), followed by insoluble-bound and free phenolic fractions. The TPC in the free form were also higher than that of bound form in cranberry, strawberry, apple, red grape and grapefruit (Sun, Chu, Wu, & Liu, 2002). The sum of free, esterified, glycosylated, and bound phenolics (total TPC) of calabura fruits was similar to those of cranberry genotypes (0.18–61 mg/g dw) (Abeywickrama, Debnath, Ambigaipalan, & Shahidi, 2016), pomegranate peels (9.38–21.18 mg/g dw) (Ambigaipalan, de Camargo, & Shahidi, 2016), and araticum pulp (20.49 mg/g dw) (Arruda et al., 2018). The TPC of calabura fruits was also similar to those found in a previous study (1.66–33.96 mg/g dw) (Lin, Chang, et al., 2017; Rotta et al., 2017) which investigated only the soluble phenolic compounds. However, these authors evaluated crude

Table 3
Total phenolic compounds (TPC), condensed tannins (CT) and antioxidant activity of different phenolics fractions of calabura (*Muntingia calabura* L.) fruits.

Assay	Free	Esterified	Glycosylated	Insoluble-bound	Total
TPC (mg GAE/g dw)	6.88 ± 0.02a	6.23 ± 0.08b	5.17 ± 0.01c	4.00 ± 0.03d	22.28 ± 0.10
CT (mg CE/g dw)	0.65 ± 0.02c	1.91 ± 0.04a	1.95 ± 0.04a	1.68 ± 0.02b	6.19 ± 0.08
DPPH (μmol TE/g dw)	95.39 ± 2.90a	81.71 ± 2.14b	41.67 ± 0.21d	47.90 ± 1.33c	266.67 ± 3.09
TEAC (μmol TE/g dw)	91.04 ± 0.89a	87.53 ± 0.22b	51.84 ± 0.55d	70.74 ± 031c	301.14 ± 1.55
ORAC (μmol TE/g dw)	39.63 ± 1.43c	41.46 ± 1.56c	75.62 ± 1.61a	49.36 ± 1.87b	206.07 ± 2.62

Data presented as mean ± standard deviation for the triplicate determination (n = 3). Means with different lowercase letters (a, b, c, d) within a row were significantly different ($p < 0.05$).
Total Flavonoid Content (TFC) was not detected in all fractions.

extracts from calabura fruits that contain other compounds significantly reactive with Folin-Ciocalteu reagent, such as vitamins, amino acids, proteins, carbohydrates, organic acids, inorganic ions and metal complexes, which can lead to overestimation of TPC values (Everette et al., 2010).

The antioxidant activity measured by ORAC assay of calabura fractions followed a different trend compared to TPC and CT (Table 3), but the DPPH and ABTS antioxidant assay followed a similar trend as TPC. The free fraction of calabura fruits showed the highest DPPH and ABTS antioxidant activity, followed by esterified, insoluble-bound, and glycosylated, whereas the ORAC assay showed glycosylated fraction as most antioxidant fraction, followed by insoluble-bound, esterified and free. The antioxidant activity assessed by DPPH, ABTS and ORAC assays was similar to well-known berry fruits; for example, bilberry, blackberry, blueberry, cranberry, raspberry, redcurrant, jabuticaba and strawberry (Einbond, Reynertson, Luo, Basile, & Kennelly, 2004; Paredes-López et al., 2010). Rotta et al. (2017) and Pereira et al. (2016) reported that the antioxidant activity was higher in peel than in pulp with seeds of the calabura fruits due to the highest content of vitamin C, anthocyanins and other phenolic compounds in the peel.

3.5. Non-coloured phenolics of calabura fruits

The individual soluble phenolic compounds of the concentrated ethanolic extract from calabura fruits (Lin, Chang, et al., 2017) and its peel (Rotta et al., 2017) was recently reported, but other fractions of phenolic compounds were not investigated, such as glycosylated, esterified and insoluble-bound forms. This failure can result in the underestimation of phenolic compounds content and consequently the actual biological properties of the fruits. We decided therefore to evaluated for the first time the phenolic compounds in different phenolic fractions from the calabura fruits. Table 4 summarizes the concentration of identified phenolic compounds in four phenolic fractions (free, esterified, glycosylated and insoluble-bound) of calabura fruits.

In general, phenolic acids, namely, hydroxybenzoic and hydroxycinnamic acids were the most abundant in all phenolic fractions. Only in the free fraction the flavonoids mainly flavanols gave significantly contribution in the phenolic composition. Gallic acid was predominant in all fractions. Free and glycosylated were the fractions that showed the major diversity of phenolic compounds, but the esterified and insoluble-bound fractions showed the highest content of phenolic compounds due to their high amount of gallic acid. Gallocatechin, epigallocatechin, catechin, quercetin, naringenin and chlorogenic acid were identified in the free fraction, but catechin and quercetin were the only flavonoids identified in glycosylated fraction.

From 14 identified phenolic compounds, gallic acid was the most abundant in calabura fruits accounting 97% of total phenolic composition. In agreement with our findings, previous reports described phenolic acids mainly gallic acid as the main phenolics extracted from calabura (Lin, Chang, et al., 2017) and its peel (Rotta et al., 2017). Ayoub et al. (2016) also reported that gallic acid and its derivative gave the major phenolic acid contribution in berry seed meals.

The phenolic acids of calabura fruits are mainly in the esterified and insoluble-bound forms, and the flavonoids were found in free and glycosylated forms. The high content of hydroxybenzoic acids in esterified fraction can be due to the release of gallic acid from hydrolysed tannins, namely gallotannins and ellagitannins. Berries, such as blackberries, strawberries and raspberries, generally contain remarkable amounts of hydrolysed tannins, mainly ellagitannins (Smeriglio, Barreca, Bellocco, & Trombetta, 2017). The gallic acid from calabura can also be released from the cell wall matrix (insoluble-bound form) (Shahidi & Yeo, 2016). The phenolic acids from pomegranate peel were also found mainly in the insoluble-bound forms in which gallic acid was the major phenolic acid (Ambigaipalan et al., 2016). Arruda et al. (2018) showed insoluble-bound phenolics accounted 40% of the phenolic composition in araticum pulp, and that these insoluble phenolics contribute significantly to the antioxidant activity of araticum pulp.

Table 4 shows that the total content of non-coloured phenolic compounds that is the sum of flavonoids (non-anthocyanins) and phenolic acids in calabura fruits (549.89 mg/100 g dw) was higher than seedless berries commonly consumed in Finland, such as blueberry, cloudberry, cranberry and bilberry (Häkkinen et al., 1999). The authors also found phenolic acids as main phenolic compounds in blueberry, bilberry, white currant, cloudberry and rowanberry. It is known that berry fruits are rich sources of phenolic compounds, such as phenolic acids, flavonoids, stilbenes, and tannins, and these bioactive compounds have been associated with their health properties (Paredes-López et al., 2010). Calabura fruits can be an important source of antioxidant phenolics to human diet, since these berries showed high amount of soluble and insoluble-bound phenolic compounds.

The antioxidant activity measured by DPPH, TEAC and ORAC assays of calabura fractions (Table 3) followed a different trend compared to their individual profile of phenolic compounds. Total content of non-coloured phenolic compounds in the different phenolic fractions of calabura fruits followed the decreasing order of esterified > insoluble-bound > glycosylated > free (Table 4). The free and glycosylated fractions that showed the lowest values of phenolics were however the most antioxidant in DPPH and TEAC, and ORAC assay, respectively. This contrasting trend can be partially explained due to difference of the phenolic profiles between the fractions, for example, only the free and glycosylated fractions showed flavonoids in their composition (Fig. 3). The ferulic, gentisic, vanilic and 4-hydroxybenzoic acids were also identified only in the glycosylated fraction, whereas only in the free fraction, a trace amount of anthocyanins was identified, which can contribute minimally to antioxidant activity of this fraction. Rice-Evans, Miller, and Paganga (1996) reported an relationship between structure and antioxidant activity of flavonoids and phenolic acids, showing that in general, flavonoids such as quercetin and epigallocatechin were more antioxidant than phenolic acids, for example gallic acid. The occurrence of flavonoids in free and glycosylated fractions even at lower amount can therefore improve the antioxidant activity of these fractions, showing that flavanols and flavonols are also important antioxidant compounds in calabura fruits. Flavonoids can also act synergistically with phenolic acids, which can increase the total antioxidant activity (Rice-Evans et al., 1996).

The consumption of the calabura fruits would contribute to intake of

Table 4
Concentration of identified phenolic compounds in four phenolic fractions (free, esterified, glycosylated and insoluble-bound) of calabura (*Muntingia calabura* L.) fruits.

Polyphenol Sub-class	Compound	Concentration in fruit (μg/g dw)				
		Free	Esterified	Glycosylated	Insoluble-bound	Σ (Free to Insoluble-bound)
Flavanols	Gallocatechin	27.64 ± 0.78B	n.d.	n.d.	n.d.	27.64 ± 0.78B
	Epigallocatechin	3.39 ± 0.18C	n.d.	n.d.	n.d.	3.39 ± 0.18B
	Catechin	2.06 ± 0.13D	n.d.	< LOQ	n.d.	2.06 ± 0.13B
	Epicatechin	n.d.	n.d.	n.d.	n.d.	–
	Σ Flavanols	33.09 ± 0.66 [2]	–	–	–	33.09 ± 0.66 [2]
Flavanones	Naringenin	0.29 ± 0.02G [4]	n.d.	n.d.	n.d.	0.29 ± 0.02B [2]
Flavonols	Rutin	n.d.	n.d.	n.d.	n.d.	–
	Quercetin	0.28 ± 0.02bG	n.d.	0.52 ± 0.03aC	n.d.	0.81 ± 0.05B
	Σ Flavonols	0.28 ± 0.02b [4]	–	0.52 ± 0.03a [2]	–	0.81 ± 0.05 [2]
Σ Flavonoids		33.65 ± 0.66b	n.d.	0.52 ± 0.03a	n.d.	34.18 ± 0.63
Hydroxybenzoic acids	Gallic acid	34.75 ± 0.07dA	2883.66 ± 123.14aA	134.65 ± 9.90cA	2272.51 ± 123.26bA	5325.58 ± 180.03A
	Protocatechuic acid	n.d.	6.04 ± 0.49aB	1.07 ± 0.09cC	4.33 ± 0.11bB	11.44 ± 0.31B
	4-Hydroxybenzoic acid	< LOQ	< LOQ	1.50 ± 0.12C	< LOQ	1.50 ± 0.12B
	Vanillic acid	< LOQ	n.d.	8.80 ± 0.34C	n.d.	8.80 ± 0.34B
	Gentisic acid	1.19 ± 0.12bE	n.d.	85.64 ± 6.97aB	n.d.	86.83 ± 7.07B
	Σ Hydroxybenzoic acids	35.94 ± 0.11d [1]	2889.70 ± 123.26a [1]	231.68 ± 16.21c [1]	2276.84 ± 123.16b [1]	5434.16 ± 183.91 [1]
Hydroxycinnamic acids	Chlorogenic acid	0.30 ± 0.03G	n.d.	n.d.	n.d.	0.30 ± 0.03B
	Caffeic acid	n.d.	n.d.	0.32 ± 0.01bC	21.11 ± 1.21aB	21.44 ± 1.20B
	p-Coumaric acid	0.98 ± 0.02cEF	3.92 ± 0.30aB	0.55 ± 0.03cC	3.00 ± 0.19bB	8.45 ± 0.16B
	Sinapic acid	n.d.	n.d.	n.d.	n.d.	–
	Ferulic acid	n.d.	n.d.	0.33 ± 0.04C	< LOQ	0.33 ± 0.04B
	Σ Hydroxycinnamic acids	1.29 ± 0.03c [3]	3.92 ± 0.30b [2]	1.21 ± 0.02c [2]	24.11 ± 1.29a [2]	30.53 ± 1.00 [2]
Σ Phenolic acids		37.22 ± 0.14d	2893.62 ± 123.5a	232.89 ± 16.23c	2300.95 ± 124.14b	5464.68 ± 184.11
Σ (flavonoids + phenolic acids)		70.88 ± 0.79d	2893.62 ± 123.53a	233.41 ± 16.20c	2300.95 ± 124.14b	5498.86 ± 184.57

n.d., not detected; < LOQ, below the limit of quantification.
Data presented as mean ± standard deviation for the triplicate determination (n = 3). Means of concentration of identified phenolic compounds with different lowercase letters (a, b, c, d) within a row were significantly different ($p < 0.05$). Means of concentration of identified phenolic compounds with different uppercase letters within a column (A, B, C, D, E, F, G) were significantly different ($p < 0.05$). Means of concentration of polyphenol sub-class with different number superscript within a column ([1, 2, 3, 4, 5]) were significantly different ($p < 0.05$).

bioactive compounds, such as cyanidin-3-*O*-glucoside, gallic acid, flavanols and flavonols. These berries showed high antioxidant activity due to the occurrence of anthocyanins, esterified and insoluble-bound phenolic acids, and free and glycosylated flavonoids. Naturally, the presence of bioactive compounds in calabura fruit is not a guarantee of biological effects, we point to the importance of determining the efficiency of these compounds *in vivo*. These studies could confirm our expectation by assess the absorption, metabolism, tissue distribution, and excretion of these bioactive compounds, as well as their effect on biomarkers (Hassimotto, Genovese, & Lajolo, 2008).

4. Conclusion

The low amount of fermentable oligosaccharides, such as FOS and GOS, and similar amounts of glucose and fructose (1:1) allow us to classify the calabura fruits as low-FODMAPs. The SPME-GC-MS analysis showed the terpenes β-Farnesene and dendrolasin as the major volatile compounds from calabura fruits. UHPLC-MS/MS revelled gallic acid

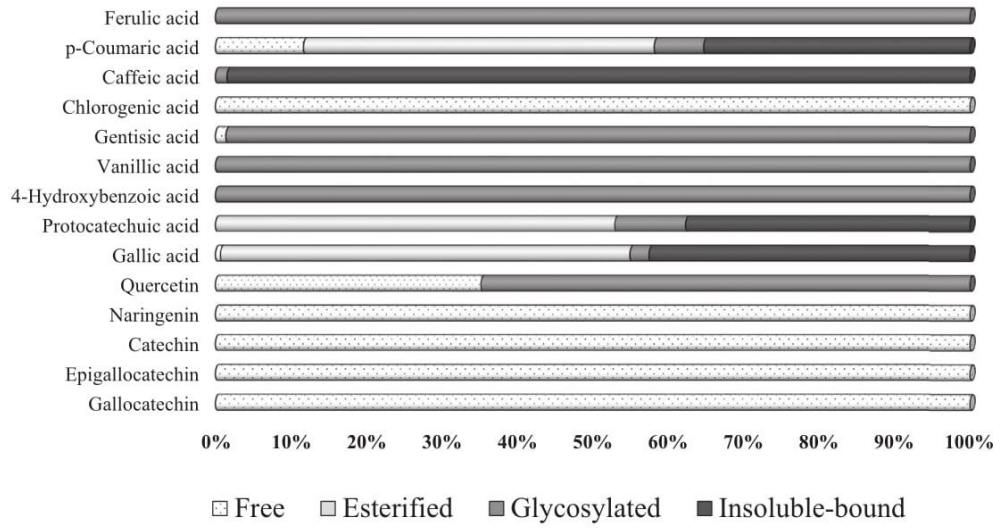


Fig. 3. Individual amount express in percentage of phenolic compounds identified in different phenolics fractions of calabura fruits.

and cyanidin-3-*O*-glucoside as the main phenolic compounds from calabura fruits. Phenolic acids made the highest contribution for phenolics in calabura fruits, being present mainly in the insoluble-bound and esterified forms. However, the free and glycosylated forms showed the highest antioxidant activities due to occurrence of flavonoids in these fractions, such as catechin, gallocatechin, epigallocatechin, naringenin, and quercetin. The insoluble-bound phenolics accounted for 42% of total phenolic composition of calabura. Therefore the simple evaluation of soluble phenolic compounds might lead to underestimation of phenolic compounds content and consequently the actual biological properties of these berries. These findings clearly suggest that calabura fruits would contribute to intake of bioactive compounds. Furthermore, this berry shows great potential for use in the food industry and as functional food due to its physicochemical properties, chemical composition and high content of phenolic compounds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.03.046.

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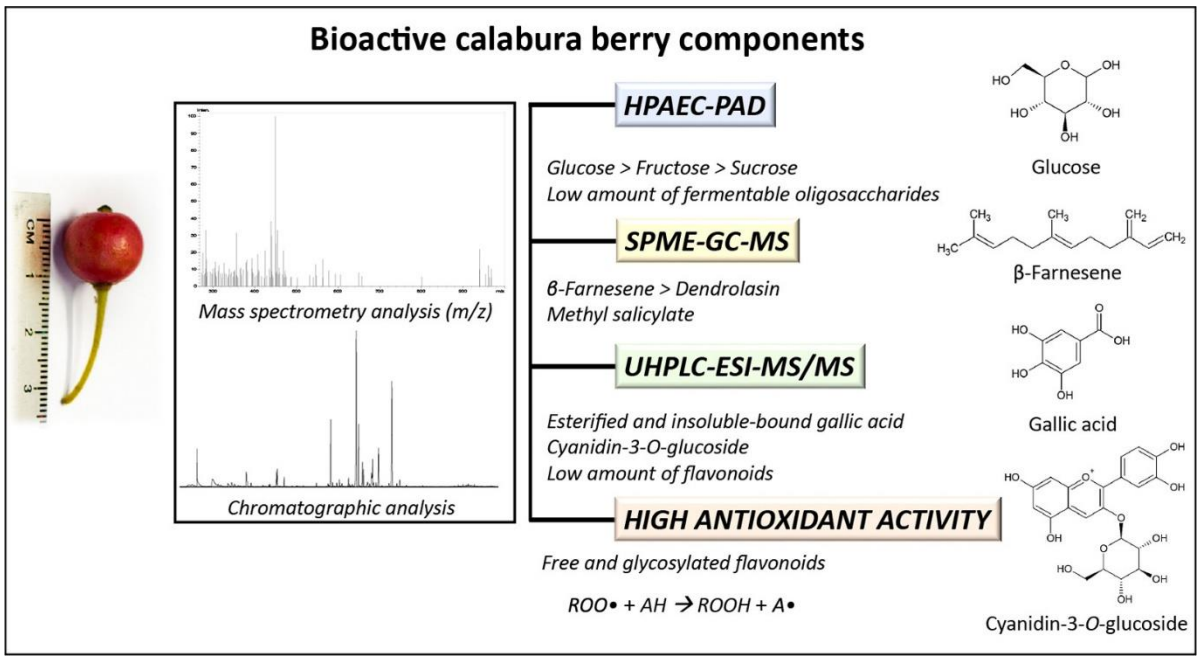
Annex 9: Highlights and graphical abstract of the research article published to Food Research International (Annex 8)

Highlights

- Calabura can be classified as Low-FODMAPs fruit.
- Insoluble-bound and esterified phenolics were the major contributors to total phenolics.
- Cyanidin-3-*O*-glucoside is the main antioxidant anthocyanin of calabura fruits.
- Gallic acid is the most abundant phenolic compound in calabura fruits.
- Low levels of flavonoids from calabura fruit showed high antioxidant activity.



Annex 10: Supplementary material of the research article published to Food Research International (Annex 8)

Supplementary Material

Carbohydrates, volatile and phenolic compounds composition, and antioxidant activity of calabura (*Muntingia calabura* L.) fruit

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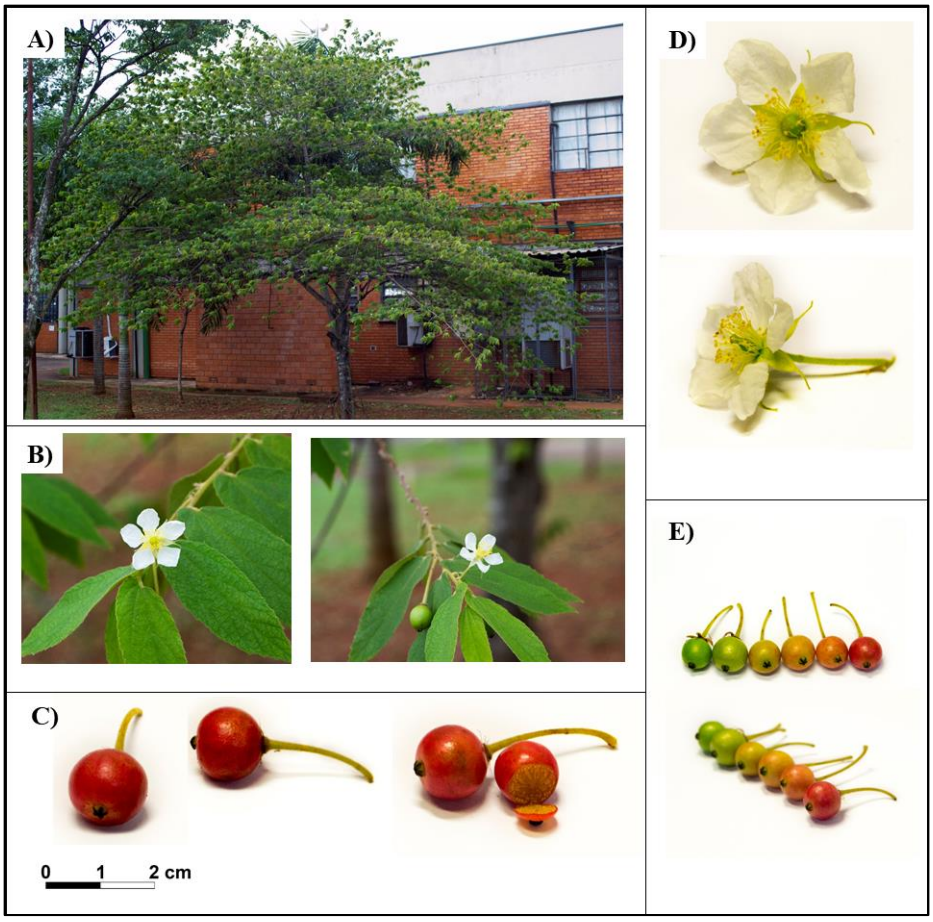


Supplement S1 (Figure S1). A) Tree of calabura (*Muntingia calabura* L.), B) Flowers, leaves and unripe fruit of calabura, C) Ripe fruits of calabura, D) Flowers of calabura, and E) colour changes during fruit maturation (From right to left: green fruit, bright green fruit, yellowish green fruit, reddish yellow fruit and red fruit).

Supplement S2 (Table S2). Analytical parameters of quantification (retention time (r.t.), linearity (R^2), slope, intercept, limit of detection (LOD) and limit of quantification (LOQ)) for sugars and oligosaccharides analysed by HPAEC-PAD.

Compound	r.t. (min)	R^2	Slope	Intercept	LOD (ng/mL)	LOQ (ng/mL)
Xylitol	2.00	0.9997	3.3596	0.0843	39.692	132.306
Mannitol	2.42	0.9998	3.1535	-0.4368	62.692	208.974
Sorbitol	2.70	0.9982	1.4731	-0.739	140.520	468.400
Glucose	4.68	0.9828	2.5749	-1.259	178.260	594.198
Fructose	5.30	0.9942	1.5756	-0.2915	341.387	1137.950
Sucrose	8.90	0.9973	1.0750	-0.0837	163.959	546.529
Raffinose	15.32	0.9965	1.3326	-0.1566	74.514	248.380
Stachyose	17.33	0.9998	1.7110	-0.1118	96.567	321.891
Maltose	19.63	0.9979	0.9639	-0.1592	110.845	369.485
Verbascose	22.10	0.9998	1.7927	-0.2188	158.211	527.371
1-kestose	8.03	0.9994	1.8383	0.0455	65.697	218.989
Nystose	10.05	0.9992	2.0701	0.4717	14.200	47.332
Maltotriose	10.92	0.9998	1.5634	0.1072	39.905	133.018
1F-β-fructofuranosylnystose	11.88	0.9989	2.0468	0.5956	80.246	267.488
Maltotretaose	12.85	0.9947	1.4542	0.1097	449.830	1499.434
Maltopentaose	14.38	0.9996	1.6941	-0.0849	103.950	346.500
Maltohexaose	15.72	0.9996	1.5578	-0.1313	99.693	332.308
Maltoheptaose	16.92	0.9995	1.2939	-0.0459	97.638	325.459

The limit of detection (LOD) and limit of quantification (LOQ) of each analyte was defined according to LOD = 3 s/S and LOQ = 10 s/S, respectively, where s is the standard deviation of the regression line equation, and S is the calibration curve slope.

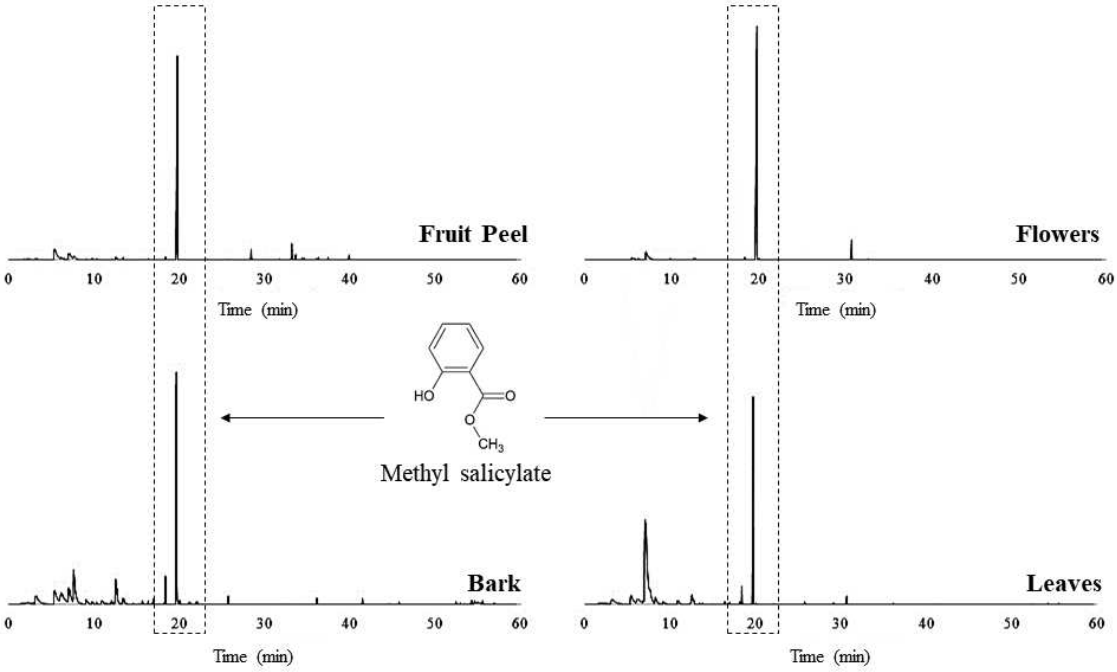
Supplement S3 (Table S3). Mass spectrometry parameters for MRM transitions (Q1 Pre Bias voltage, Q3 Pre Bias voltage and collision energy (CE)) and analytical parameters of quantification (retention time (r.t.), linearity (R^2), slope, intercept, limit of detection (LOD) and limit of quantification (LOQ)) for the compounds analysed by UHPLC-MS/MS.

Compound	Transitions (m/z)*	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)	r.t. (min)	R^2	Slope	Intercept	LOD (ng/mL)	LOQ (ng/mL)
Gallic acid	169.00>125.05	30	17	19	2.81	0.9978	121.6600	27.6716	0.41	1.35
	169.00>78.95	30	21	29						
Gallocatechin	305.00>125.15	20	20	22	3.34	0.9993	241.7230	-66.0853	0.19	0.64
	305.00>165.05	20	17	26						
Protocatechuic acid	153.00>109.00	30	15	17	3.71	0.9967	407.1180	214.7370	0.40	1.32
	153.00>108.15	30	30	18						
Epigallocatechin	305.00>125.20	14	20	21	3.92	0.9986	252.4700	-113.4150	0.16	0.53
	305.00>179.10	14	17	10						
Catechin	289.10>151.00	18	22	24	4.00	0.9979	27.9498	-2.2498	0.89	2.97
	289.10>123.00	19	27	28						
Chlorogenic acid	353.10>191.15	16	20	18	4.24	0.9981	834.2690	362.9680	0.19	0.63
	353.10>85.15	16	39	27						
4-Hydroxybenzoic acid	137.00>93.10	26	16	14	4.35	0.9903	1430.5600	1416.2000	0.26	0.85
	137.00>65.05	26	28	23						
Epicatechin	289.10>244.90	21	13	24	4.39	0.9984	56.0846	-30.6813	1.33	4.43
	289.10>108.90	13	23	15						
Caffeic acid	179.00>134.15	12	26	30	4.50	0.9943	178.8970	98.8710	0.39	1.29
	179.00>89.30	12	34	28						
Vanillic acid	167.05>152.05	11	14	27	4.54	0.9954	31.9106	6.9804	0.38	1.27
	167.05>108.30	11	17	21						

Compound	Transitions (m/z)*	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)	r.t. (min)	R^2	Slope	Intercept	LOD (ng/mL)	LOQ (ng/mL)
Gentisic acid	153.00>108.10	29	15	19	5.59	0.9925	74.4750	-39.3319	0.46	1.54
	153.00>109.10	29	23	17						
p-Coumaric acid	163.10>119.10	10	14	21	4.91	0.9877	1785.2000	1999.7400	0.18	0.59
	163.10>93.10	10	34	14						
Sinapic acid	223.00>208.05	25	14	20	4.95	0.9985	385.2260	78.9865	0.31	1.03
	223.00>164.00	25	16	28						
Ferulic acid	193.10>134.00	12	17	21	5.00	0.9985	315.2540	106.9520	0.51	1.70
	193.10>178.00	12	11	30						
Rutin	609.00>301.05	28	35	28	5.08	0.9998	314.6330	35.1150	0.20	0.66
	609.00>299.95	28	48	27						
Quercetin	301.00>151.00	20	23	24	5.88	0.9969	913.9250	187.1070	0.05	0.18
	301.00>178.96	20	19	30						
Naringenin	270.90>151.00	18	17	29	5.99	0.9944	1125.2200	768.3400	0.42	1.41
	270.90>119.10	18	29	18						
Cyanin	611.00>287.10	-28	-40	-22	2.22	0.9998	1651.4800	-140.9770	0.483	1.610
	611.00>449.20	-28	-21	-23						
Delphinidin 3-*O*-glucoside	465.00>303.05	-21	-19	-23	2.23	0.9944	2066.9800	-1124.2000	0.656	2.187
	465.00>229.10	-21	-49	-26						
Cyanidin 3-*O*-glucoside	448.90>287.20	-20	-21	-21	2.35	0.9992	5332.2200	-1232.9000	0.490	1.632
	448.90>137.10	-20	-53	-26						
Methyl salicylate	152.80>120.95	-15	-14	-14	3.77	0.998	48676.7	9632.57	0.056	0.188
	152.80>64.95	-10	-28	-29						

* First transition used for quantitation. Second transition used for identification.

The limit of detection (LOD) and limit of quantification (LOQ) of each analyte was defined according to LOD = 3 s/S and LOQ = 10 s/S, respectively, where s is the standard deviation of the regression line equation, and S is the calibration curve slope.



Supplement S4 (Figure S4). Volatile compounds found in calabura (*Muntingia calabura* L.) fruit peel, flowers, bark and leaves using Headspace solid-phase microextraction (SPME) combined with gas chromatography–mass spectrometry detection (GC-MS) method. Highlight to methyl salicylate (MeSA) that was the main compound found in headspace of no-infected tissues of leaves, flowers, bark and fruit peel of calabura tree. The samples collection and the extraction and analysis of volatile compounds were performed as described in section 2.2 of the manuscript.

Supplement S5 (Table S5). Concentration of methyl salicylate (MeSA) in different parts of calabura (*Muntingia calabura* L.) tree

Fraction	MeSA (µg/100 g fw)
Fruit	0.17
Fruit Peel	2.56
Flowers	0.85
Leaves	0.20
Bark	< LOQ

Samples were collected, pulverized in liquid nitrogen, and frozen at -80 °C until required. Frozen samples were homogenized with ice-cold methanol (1:5, w/v) and left in ultrasonic bath for 10 min at 5 °C. The mixture was centrifuged (4000 g, 15 min, 5 °C). The supernatants were collected and the residues were re-submitted to extraction protocol. This procedure was repeated two times and the supernatants were combined (Riet et al., 2016). The supernatants were used for UHPLC-MS/MS analysis as described in section 2.5.3 of the manuscript. MeSA was analysed in the positive ion mode. Calibration curve was constructed from 20 to 1200 ng/mL. The chromatography gradient elution for MeSA analysis was as follows: 0-3.0 min, 5-100% B; 3.0-3.5 min, 100% B; 3.5-4.0 min, 100-5% B; and 4.0-8.0min, 5% B (solvent A was ultrapure water 0.1% formic acid and solvent B was methanol). The amount of MeSA was very low to have toxic or anti-inflammatory effect in human body after intake of different parts of calabura tree (Davis, 2007; Duthie et al., 2011).

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Annex 11: Licence to reuse the content of the research article published to Food Research International in the doctoral thesis (Annex 8)

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Title: Carbohydrates, volatile and phenolic compounds composition, and antioxidant activity of calabura (Muntingia calabura L.) fruit

Author: Gustavo Araujo Pereira,Henrique Silvano Arruda,Damila Rodrigues de Morais,Marcos Nogueira Eberlin,Glaucia Maria Pastore

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Annex 12: Research article published to Brazilian Journal of Food Research

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ISSN: 2448-3184

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Modification and validation of Folin-Ciocalteu assay for faster and safer analysis of total phenolic content in food samples

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ABSTRACT

Folin-Ciocalteu (F-C) is a routine assay in several laboratories around the world and has been widely employed for total phenolic content (TPC) quantification from food samples. F-C assay was modified to obtain a system reaction safer, as well as decrease the use of reagents, energy expenditure and time-consuming. After evaluating alternatives solvents and supports, and the effect of the binomial time/temperature, the modified F-C assay was as follows: 40% ethanol as solvent, 20 minutes at room temperature (25±2°C) using 50% F-C reagent and 5% sodium carbonate solution. The modified F-C assay was linear in 2.5-50.0 µg/mL range to gallic acid. Limit of detection and limit of quantification were 0.195 and 0.591 µg/mL, respectively. Intra-day and inter-day precision (relative standard deviation (RSD) 0.06-3.28%) and accuracy (93.28-104.28%) were also demonstrated. The assay was robust for F-C reagent (45-55%) and sodium carbonate (4.5-5.5%) changes. The modified assay was employed to analyse food samples containing phenolic compounds and the results corroborated with conventional assay. The modified F-C assay demonstrated to be reproducible, robust, fast, easy, inexpensive, safe and reliable for quantify phenolic compounds in food samples. The employment of ethanol in F-C assay decreases the environmental impact and, consequently, makes the analysis safer than conventional F-C assay. Furthermore, the modified F-C assay is conducted under milder conditions (time/temperature), which is particularly helpful for numerous analyses. Thus, the modified and validated F-C assay can be used as a routine assay in quality control and chemical profiling for natural product extracts and foods.

KEYWORDS: bioactive compounds; extraction; food analysis; food control; food quality; phenolic compounds.

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INTRODUCTION

Phenolic compounds are the secondary metabolites found in all plant organs. They are widespread constituents of plant foods and beverages, and therefore they are an important part of the human diet (DAI & MUMPER, 2010). Chemically, phenolic compounds possess at least one aromatic ring containing one or more hydroxyl substituents and may be associated with carbohydrates, lipids, organic acids, amines and cell wall components. The structure of phenolic compounds ranges from simple molecules (phenolic acids) up to polymers (proanthocyanidins) (IGNAT *et* al., 2011). In general, phenolic compounds are excellent antioxidant and antimicrobial agents and have been described as food preservatives, as well as the consumption of fruits rich in (poly)phenols can prevent, slow or reverse the development of diseases, such as cancer, cardiovascular diseases and neurological disorders (VALDÉS *et al.*, 2015).

The interest in phenolic compounds from foods has progressively increased and spectrophotometric analytical methods, such as Folin-Ciocalteu (F-C) assay, have become a routine in a number of laboratories around the world due to their simplicity and low cost. The F-C assay is widely used for measuring total phenolic content (TPC) and provides simple and fast screening of TPC from food samples (DAI & MUMPER, 2010; IGNAT *et al*., 2011; KHODDAMI *et al*., 2013).

The F-C assay is a colorimetric method based on the reduction of Folin-Ciocalteu reagent (a mixture of sodium molybdate, sodium tungstate, and other reagents) through single electron transfer from phenol to complexed Mo (VI) **(**Mo (VI) + e → Mo (V)**)**. In alkaline medium (pH ~10 adjusted by a sodium carbonate solution), the dissociation of a phenolic proton leads to a phenolate anion that is able to reduce the F-C reagent. The result of the reaction between phenolic compounds and the F-C reagent is the development of blue complex, which presents wavelength of maximum absorption close to 760 nm (EVERETTE *et al.*, 2010; HUANG *et al*., 2005; SÁNCHEZ-RANGEL *et al.*, 2013).

The F-C reagent is significantly reactive toward other food compounds besides phenols, such as vitamins, amino acids, proteins, carbohydrates, organic acids, inorganic ions and metal complexes (EVERETTE *et al.*, 2010). However, different methodologies have been proposed to improve the specificity of the F-C assay (CASTRO-ALVES & CORDENUNSI, 2015; SÁNCHEZ-RANGEL *et al.*, 2013). Likewise, different modifications were suggested over the past years, for example, to eliminate interferences due to fine solids formation (CICCO *et al.*, 2009; CICCO & LATTANZIO, 2011) and to decrease the time-consuming of the F-C assay (AINSWORTH & GILLESPIE, 2007; MAGALHÃES *et al.*, 2010). Furthermore, the effect of the basification step on results was demonstrated (CHEN *et al*., 2015).

Certain limitations of the conventional F-C assay were overcome. However, there are still two main problems: i) the employment of toxic solvent (methanol) and ii) the heating of the reaction medium. In this way, we evaluated the use of alternative solvents (water and ethanol) and supports (glass or polypropylene microtube) and the effect of time/temperature on the development of the blue complex. The modified F-C assay was validated according to guidelines established by the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH Guidelines, 2017). In this approach, the aim of this work was to improve the F-C assay to obtain a system

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reaction safer, as well as decrease the reagents, energy and time-consuming of conventional F-C assay.

MATERIAL AND METHODS

APPARATUS

All of the spectrophotometric measurements were performed with a UV-Vis spectrophotometer (Beckman, model DU600, CA, USA).

CHEMICALS AND REAGENTS

All chemicals were analytical-reagent grade and the water was ultrapure (18 MΩ cm^{-1}) obtained from a Milli-Q water purification system (Millipore, Bedford, USA). The chemicals included methanol, ethanol, Folin-Ciocalteu reagent (Dinâmica, Diadema, Brazil), anhydrous sodium carbonate (Synth, Diadema, Brazil), and gallic acid with purity > 95% (Sigma-Aldrich, St. Louis, MO, USA).

GALLIC ACID STOCK SOLUTIONS PREPARATION

Gallic acid was chosen as standard phenol because of its abundance in food matrices (DANESHFAR *et al*., 2008). Gallic acid stock solutions (100 µg/mL) were prepared by dissolving 2.5 mg of the standard in ultrapure water, 40% methanol (v/v) or 40% ethanol (v/v) in a 25 mL volumetric flask.

EFFECT OF THE SOLVENT ON THE FOLIN-CIOCALTEU ASSAY

For assessing the effect of the solvent on the F-C assay, three solvents (ultrapure water, 40% methanol (v/v) and 40% ethanol (v/v)) were used to prepare the gallic acid standard solutions. The influence of the solvent on the development of the blue complex was performed through measuring of absorbance at 760 nm (maximum wavelength absorption), related to mixtures containing 100 µL of 10, 30 and 50 µg/mL gallic acid, 100 µL of 50% F-C reagent (v/v), and 800 µL of 5% sodium carbonate (w/v), after 20 minutes of incubation at 40 °C using a water bath shaker. Sealed glass test tubes were used to perform the reactions. The absorbance values obtained for each studied solvent were evaluated statistically by analysis of variance using one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$).

EFFECT OF THE REACTION SUPPORTS ON THE FOLIN-CIOCALTEU ASSAY

Glass test tubes and polypropylene microtubes were used to evaluate the effect of the reaction supports on the F-C assay. The influence of the reaction support on the development of the blue complex was performed through measuring the absorbance at 760 nm, related to mixtures containing 100 µL of 10, 30 and 50 µg/mL gallic acid prepared in 40% ethanol (v/v), 100 µL of 50% F-C

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reagent (v/v), and 800 µL of 5% sodium carbonate (w/v), after 20 minutes of incubation at 40 °C. The statistical significance of differences between the reaction supports was evaluated by the Student's independent-samples t test ($p \leq 0.05$).

DETERMINATION OF THE OPTIMUM REACTION TIME AND TEMPERATURE

In order to establish the lower reaction time at which the development of the blue complex can be considered maximum, the time-behaviour of absorbance curve at 760 nm, related to a mixture containing 100 µL of 30 µg/mL gallic acid prepared in 40% ethanol (v/v), 100 µL of 50% F-C reagent (v/v), and 800 µL of 5% sodium carbonate (w/v), was monitored in a period of 60 minutes at 25, 30 and 40 °C. The absorbance values of the reaction temperatures for each reaction time were observed and evaluated statistically by analysis of variance using one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$).

ANALYTICAL ASSAY VALIDATION

The TPC assay was modified and optimized based on F-C assay given by Ainsworth and Gillespie (2007), Cicco *et al.* (2009) and Cicco and Lattanzio (2011). The analytical assay was conducted as follows: 100 µL of properly diluted samples, calibration solutions or blank were pipetted into separate polypropylene microtubes. Then 100 µL of 50% F-C reagent (v/v) and 800 µL of 5% sodium carbonate (w/v) were added to each. The material was mixed and incubated at room temperature for 20 minutes. The absorbance was measured at 760 nm against a blank on a UV-Vis spectrophotometer. 40% ethanol (v/v) was used for samples, calibration solutions and blank preparation. The 50% F-C reagent (v/v) and 5% sodium carbonate (w/v) was prepared in deionized water.

The validation of the analytical assay was conducted on the basis on the guidelines established by the ICH (International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) (ICH Guidelines, 2017). For assessing the validation parameters, the assays were performed according to the experimental conditions previously mentioned.

For the linearity study, an eleven-point calibration curve was constructed using different concentrations of gallic acid stock solutions (range of 2.5-50.0 µg/mL). These ranges let to cover absorbance values up to 1. The absorbance at 760 nm was plotted versus the gallic acid concentrations to produce a calibration curve. The verification of the outliers' absence was made by Grubbs' test while data homoscedasticity was checked by Cochran's C test. Linear regression analysis was performed by analysis of variance (ANOVA) to check the statistical significance of regression equation and linearity deviation. All measurements were carried out as three genuine replicates.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the Equations 1 and 2.

$$LOD\ (\mu g/mL) = \frac{3.3\sigma}{S} \quad \text{(Equation 1)}$$

$$LOQ\ (\mu g/mL) = \frac{10\sigma}{S} \quad \text{(Equation 2)}$$

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Where σ is the standard deviation of the blank and S is the slope of the calibration curve.

The modified F-C assay precision was determined by repeatability (intra-day) and reproducibility (inter-day) assays. The repeatability was assessed using three concentrations of gallic acid (10, 30 and 50 µg/mL) analysed three times within the same day, whereas the reproducibility was assessed using the above mentioned concentrations of gallic acid analysed on three successive days. Precision was expressed as relative standard deviation (RSD) of three replicates. A RSD over 5% were considered unacceptable.

The robustness of the modified F-C assay was determined by analysing the effect of the sodium carbonate (Na_2CO_3) (4.5, 5.0 and 5.5% (w/v)) and F-C reagent (45, 50 and 55% (v/v)) concentrations on the stability of the reaction using three concentrations of gallic acid (10, 30 and 50 µg/mL). All measurements were performed in three replicates and the results were expressed as relative standard deviation (RSD). A RSD over 5% were considered unacceptable.

The accuracy of the modified F-C assay was evaluated using different food samples spiked with gallic acid solution (10 µg/mL). Accuracy was measured by means of the recovery percentage, which was calculated from Equation 3.

$$Recovery\ (\%) = \frac{A_{Spiked\ Sample} - A_{Standard}}{A_{Sample}} \times 100 \qquad \text{(Equation 3)}$$

Where $A_{Spiked\ Sample}$ = absorbance of the sample after addition of the standard (10 µg/mL gallic acid); $A_{Standard}$ = absorbance of the standard solution (10 µg/mL gallic acid); and A_{Sample} = absorbance of the sample without addition of the standard.

All measurements were performed in three replicates. The assay was considered accurate if the recovery percentages were between 85% and 115%.

APPLICATION OF THE MODIFIED FOLIN-CIOCALTEU ASSAY TO FOOD EXTRACTS

In order to demonstrate the applicability of the modified F-C assay, the TPC quantification in common food sample-derived extracts was carried out.

The food samples (apple, banana, grape, pineapple, cabbage, lettuce, carrot, tomato, broccoli, soybean, peanut, flaxseed, bean and popcorn) used for the assay validation were purchased from the local market of Campinas (São Paulo, Brazil). The food samples were washed with tap water to remove surface dirt. The edible parts of food samples (10 g) were extracted in 100 mL of 50% ethanol (v/v) with the aid of Ultra-Turrax Homogenizer at 11,000 rpm for 30 seconds (PEREIRA *et al.*, 2017). Only the grains and seeds were previously immersed overnight in 50% ethanol (v/v) at 5 °C. Once the extraction was completed, the supernatant was separated from the insoluble solids by centrifugation at 3,801 *g* for 11 minutes at 5 °C (Hettich Zentrifugen, model Rotanta 460R, Germany). The supernatant was used for the TPC analysis.

The TPC determination in the food samples by the modified F-C assay was performed according to the experimental conditions previously mentioned (see Section Analytical Assay Validation). The absorbance values were interpolated in the calibration curve to calculate the TPC. The results were expressed as mg gallic acid equivalents per 100 g of fruit fresh weight (g GAE/100 g fw). TPC was also

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determined by means of conventional F-C assay (ROESLER *et al.*, 2007). All measurements were carried out in triplicate.

STATISTICAL ANALYSIS

All statistical analyses were performed at a significance level of 5% ($p \leq 0.05$) using STATISTICA software (Statsoft, Oklahoma, USA) version 12.0. The data are reported as mean values with standard deviation of three measurements.

RESULTS AND DISCUSSION

OPTIMIZATION OF REACTION CONDITIONS

Solvent used to prepare the standard solutions and samples, reaction support, and reaction time were considered and analysed in order to establish the optimal reaction conditions to the present analytical assay.

The solvent affects the performance of F-C assay due to phenolic compounds solubility and fine solids formation. Table 1 presents the effect of the solvents on the F-C assay. The use of methanol and ethanol as solvent did not affect the absorbance, whereas water decreased the response. In the low gallic acid concentration (10 µg/mL) the absorbance was statistically equal to all solvents tested. However, when the standard concentration increased (30-50 µg/mL) the response decreased only to water. Phenolics are compounds of low polarity and therefore, present higher solubility in methanol (76.2 polarity) and ethanol (65.4 polarity) than water (100 polarity) (SMALLWOOD, 1996). For example, the relative solubility of gallic acid in the solvents was found as methanol > ethanol > water > ethyl acetate (DANESHFAR *et al.*, 2008). According to the above-mentioned comments, the ethanol can be employed as substitute of methanol in F-C assay. Methanol has similar physical properties to ethanol; however, it is toxic and therefore, ethanol is preferred solvent in most applications (KERTON & MARRIOTT, 2013). The employment of ethanol in F-C assay decreases the environmental impact, furthermore, it makes the analysis safer than conventional F-C assay.

Table 1. Effect of the solvents on the Folin-Ciocalteu assay.

Gallic acid concentration (µg/mL)	Absorbance at 760 nm		
	Water	40% Ethanol	40% Methanol
10	$0.1438^{A} \pm 0.0035$	$0.1473^{A} \pm 0.0041$	$0.1476^{A} \pm 0.0047$
30	$0.4091^{B} \pm 0.0060$	$0.4322^{A} \pm 0.0027$	$0.4292^{A} \pm 0.0051$
50	$0.6636^{B} \pm 0.0048$	$0.7063^{A} \pm 0.0090$	$0.7079^{A} \pm 0.0057$

NOTE: Data are presented as mean with standard deviation of three measurements (n=3). Values on the same line indicated with the same capital letter do not differ according to Tukey's test ($p>0.05$).

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Fine solids can be formed throughout the F-C assay due to alcohol and sodium carbonate concentrations in the final mixture and this affects the TPC results. The formation of particles is delayed and slowed down with decreasing alcohol concentration and can be further prevented when a carbonate solution at 5% (w/v) is used (CICCO & LATTANZIO, 2011), which justify the use of alcohol solutions at 40% (v/v) in the present study.

For reaction supports, there was no statistically significant difference ($p>0.05$) between glass test tubes and polypropylene microtubes (Table 2). The use of microtubes presents the advantage to make the assay faster and safer than conventional F-C assay because glass test tubes require careful handling.

Table 2. Effect of the reaction supports on the Folin-Ciocalteu assay.

Gallic acid concentration (µg/mL)	Absorbance at 760 nm		Student's t test	
	Glass test tubes	Polypropylene microtubes	*t* value	*p*-value
10	0.1473 ± 0.0041	0.1467 ± 0.0057	0.1573	0.8827^{ns}
30	0.4322 ± 0.0027	0.4342 ± 0.0070	-0.4618	0.6682^{ns}
50	0.7063 ± 0.0090	0.7063 ± 0.0105	0.0042	0.9969^{ns}

NOTE: Data are presented as mean with standard deviation of three measurements (n=3). ns: not significant according to Student's t test ($p>0.05$).

In order to find optimal reaction time, 30 µg/mL gallic acid solutions were tested with the modified F-C assay. The absorbance of the blue complex was monitored in a period of 60 minutes at 25, 30 and 40 °C (Figure 1). These data showed that the F-C reaction was stable during the period analysed for all studied temperatures, since after 20 minutes the absorbance increased less than 5% of the value at 5 minutes, and did not decrease between 20 and 60 minutes. Furthermore, it could be observed that the reaction temperature did not affect the formation rate and stability of blue complex, once the mean absorbance values of the reaction temperatures for each reaction time were statistically equal ($p>0.05$). These results suggested the optimum reaction condition was 20 minutes at room temperature. The optimal reaction time of 20 minutes (at room temperature) of the modified F-C assay was lower than the protocol time established by other conventional F-C assays available: 120 minutes (AINSWORTH & GILLESPIE, 2007; BOBO-GARCÍA *et al.*, 2015).

ASSAY VALIDATION

The linearity of standard calibration curve was evaluated by linear regression analysis. The standard calibration curve was calculated by the least squares regression method to calculate the calibration equation and the determination coefficient (R^2). Figure 2 shows the standard calibration curve of the modified F-C assay. A linear correlation was found between absorbance of the blue complex at 760 nm and concentration of gallic acid in the range 2.5-50.0 µg/mL. The determination coefficient obtained from the linear regression was 0.9997, indicating excellent linear correlation between the data. Furthermore, the linear regression of the gallic acid calibration curve were statistically analysed and the

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results obtained are shown in Table 3. No significant outliers were detected in any of the groups of absorbance for each concentration level of gallic acid by Grubbs' test. Data were homoscedastic by Cochran's C test, indicating that data variances were homogeneous. The regression equation was statistically significant and it did not exhibit linearity deviation by analysis of variance (ANOVA) in the range evaluated. The slope and intercept of linear regression were statistically different from zero. These data indicates a good fit of regression model. Therefore, the obtained regression equation can be satisfactorily used to estimate the TPC in unknown samples.

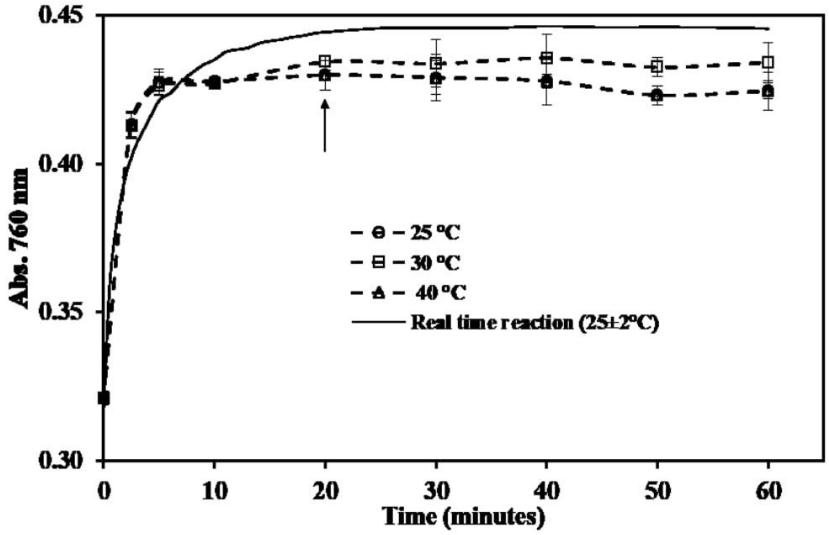

Figure 1 - Time required to complete the Folin-Ciocalteu reaction at different temperatures. The absorbance (Abs.), data referred to mixtures contain 30 µg/mL gallic acid, represent means of triplicates (n=3).

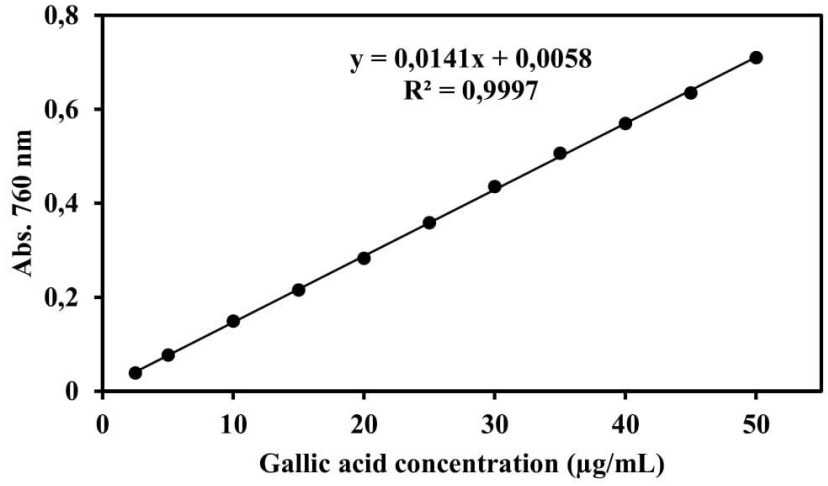

Figure 2 - Standard calibration curve for modified Folin-Ciocalteu Micro-assay. Markers correspond to the means of triplicates (*n*=3).

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From the slope of the calibration curve, LOD and LOQ were established. The LOD and LOQ for the modified F-C assay were 0.195 and 0.591 µg GAE/mL, respectively (Table 3). The LOD and LOQ found herein were lower than the values obtained in other TPC assays available in the literature (MITIC *et al.*, 2014; STOICESCU *et al.*, 2012), which indicates that the actual modified F-C assay shows high sensitivity. The modified spectrophotometric assay was even more sensitive than microplate assays (BOBO-GARCÍA *et al.*, 2015; PUEYO & CALVO, 2009).

Table 3. Statistical data for the linear regression of the gallic acid calibration curve for modified Folin-Ciocalteu assay.

Parameter	Gallic acid calibration curve
Linear range (µg/mL)	2.5-50.0
Slope (SE)[a]	0.0141 (0.00007)
t value	197.26
p-value	<0.0001
Intercept (SE)[a]	0.0058 (0.00212)
t value	2.73
p-value	0.0123
Determination coefficient (R^2)	0.9997
Linear Regression (Analysis of variance)	
F value	38,912.28
p-value	<0.0001
Lack of fit (Linearity deviation test)	
F value	1.44
p-value	0.2295
Cochran's C test (Homoscedasticity test)	
C value	0.33
p-value	0.2099
Grubbs' test (Outlier test)	No significant outliers were detected in any of the groups
LOD[a] (µg/mL)	0.195
LOQ[a] (µg/mL)	0.591

SE: standard error; LOD: limit of detection; LOQ: limit of quantitation.

The precision of the modified F-C assay was evaluated by measuring repeatability (intra-day) and reproducibility (inter-day) of the measurement for three replicates at three different concentrations and expressed in terms of RSD. As can be seen in Table 4, the RSD values were ≤0.88% and ≤3.28% for repeatability and reproducibility, respectively. These results show that the proposed assay is adequately precise.

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Table 4. Intra-day (repeatability) and inter-day (reproducibility) precision of the modified Folin-Ciocalteu assay.

Gallic acid concentration (µg/mL)	Intra-day precision (*n*=3)		Inter-day precision (3 days)	
	Absorbance at 760 nm	RSD (%)	Absorbance at 760 nm	RSD (%)
10	0.1493 ± 0.0010	0.68	0.1500 ± 0.0049	3.28
30	0.4313 ± 0.0038	0.88	0.4302 ± 0.0020	0.47
50	0.7095 ± 0.0004	0.06	0.7088 ± 0.0004	0.06

NOTE: Data are presented as mean with standard deviation of three measurements (*n*=3).
RSD: relative standard deviation.

The robustness of the modified F-C assay was examined by evaluating the influence of small variations in the assay variables (sodium carbonate and F-C reagent concentrations) on its analytical performance and expressed in terms of RSD. The results presented in Table 5 revealed that small variations in the sodium carbonate (4.5-5.5%, w/v) or F-C reagent (45-55%, v/v) concentrations did not induce considerable changes in absorbance values at 760 nm, in which the RSD values were ≤2.95% and ≤3.48% for sodium carbonate and F-C reagent concentrations, respectively. The proposed assay is sufficiently robust under the described experimental conditions.

Table 5. Effect of the sodium carbonate (Na_2CO_3) and Folin-Ciocalteu reagent concentrations on the robustness of the modified Folin-Ciocalteu assay.

Gallic acid concentration (µg/mL)	Sodium carbonate (Na_2CO_3) concentration (4.5-5.5%, w/v)		Folin-Ciocalteu reagent concentration (45-55%, v/v)	
	Absorbance at 760 nm	RSD (%)	Absorbance at 760 nm	RSD (%)
10	0.1441 ± 0.0042	2.95	0.1432 ± 0.0050	3.48
30	0.4265 ± 0.0018	0.42	0.4283 ± 0.0048	1.11
50	0.7067 ± 0.0050	0.71	0.7099 ± 0.0029	0.40

NOTE: Data are presented as mean with standard deviation of three measurements (*n*=3).
RSD: relative standard deviation.

The accuracy of the modified F-C assay was determined by means of recovery test. Different food samples were spiked with gallic acid solution (10 µg/mL) and then analysed using the proposed assay. The results presented in Table 6 show good recovery percentages (93.28-104.28%) and small RSD values (≤4.02%), which indicates excellent accuracy of the proposed assay to TPC quantification in food samples.

APPLICATION OF THE MODIFIED FOLIN-CIOCALTEU ASSAY TO FOOD SAMPLES

In order to demonstrate the performance of the present assay, real food samples were submitted to quantification process. The results were compared with those obtained from conventional F-C assay (Table 6). In this study, the RSD values and error percentages were used as an indicator of the suitability of the modified F-C assay. TPC results obtained by means of modified F-C assay demonstrated to be reproducible and comparable with those from conventional F-C assay, once RSD values and error percentages between the assays were ≤6.85%

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and ≤9.23%, respectively (Table 6). Taking into consideration that conventional assays are not necessarily absolute nor accurate, RSD values and error percentages lower than 10% are quite satisfactory. In addition, the modified F-C assay is safer and has low environmental impact than the conventional F-C assay because the described procedure does not need heating, requires less organic solvent, employs GRAS solvent (ethanol) and generates less wastes (reduction in the reaction volume). Although the LC-MS/MS methods and others more advanced techniques are more selective and sensitive, the F-C assay is considered more convenient for routine analyses to be fast and low cost. Moreover, the proposed F-C assay herein showed advantages, such as the shorter analysis time (20 minutes of reaction), easier procedure (requires less manipulation) and lower cost (employment of cheaper reagents), besides the afore-mentioned advantages. Therefore, the usefulness of proposed assay is evident in routine natural product and food chemistry studies.

Table 6. Accuracy of the modified Folin-Ciocalteu assay by recovery test and TPC in food samples using conventional and modified Folin-Ciocalteu assays.

Sample	Accuracy		TPC (mg GAE/100 g fw)			
	Recovery (%)[a]	RSD (%)[b]	Conventional assay	Modified assay	RSD (%)	Error (%)
Apple	101.40 ± 1.80	1.07	79.90 ± 2.36	77.72 ± 0.28	1.95	-2.72
Banana	100.55 ± 1.74	1.73	34.12 ± 0.16	33.47 ± 0.23	1.35	-1.89
Grape	97.56 ± 0.09	0.09	47.82 ± 0.59	47.20 ± 0.08	0.92	-1.30
Pineapple	97.74 ± 0.81	0.83	30.05 ± 0.37	27.28 ± 0.62	6.85	-9.23
Cabbage	98.21 ± 0.02	0.02	217.21 ± 4.42	216.41 ± 6.64	0.26	-0.37
Lettuce	99.91 ± 0.76	0.76	41.06 ± 0.31	41.49 ± 1.04	0.72	1.03
Carrot	104.28 ± 0.88	0.84	20.44 ± 0.87	20.04 ± 0.20	1.40	-1.96
Tomato	99.51 ± 0.99	1.00	19.87 ± 0.66	18.83 ±0.01	3.81	-5.25
Broccoli	98.33 ± 1.09	1.11	113.21 ± 1.88	110.93 ± 1.00	1.44	-2.02
Soybean	99.89 ± 1.16	1.16	129.23 ± 1.19	132.73 ± 0.92	1.87	2.68
Peanut	93.28 ± 3.75	4.02	304.40 ± 4.81	303.74 ± 2.25	0.15	-0.22
Flaxseed	98.52 ± 1.90	1.93	266.32 ± 1.93	249.31 ± 5.53	4.67	-6.39
Bean	96.49 ± 0.51	0.53	112.28 ± 1.99	111.93 ± 1.84	0.22	-0.31
Popcorn	96.08 ± 0.46	0.47	58.22 ± 0.15	54.77 ± 0.25	4.32	-5.93
Overall	98.70 ± 2.62	2.65				

NOTE: Data are presented as mean with standard deviation of three measurements (n=3).
RSD: relative standard deviation.

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CONCLUSION

A spectrophotometric assay was modified and validated for the TPC determination using F-C reagent. This spectrophotometric assay demonstrated to be precise, robust and accurate using a simple, inexpensive, sensitive, easy, and fast procedure. Furthermore, the modified F-C assay involved simple and safe experimental conditions; once it is free from extreme experimental conditions such as heating and time-consuming reaction, beyond to employ GRAS solvent (ethanol). The modified F-C assay also exhibited comparable analytical performance with conventional assay for TPC quantification and proved to provide adequate analytical results on food extracts as representative real samples. Therefore, the proposed assay can be used as a routine assay in quality control and chemical profiling for natural product extracts and foods.

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Modificação e validação do ensaio de Folin-Ciocalteu para análise mais rápida e segura do conteúdo total de compostos fenólicos em alimentos

RESUMO

O Folin-Ciocalteu (F-C) é um ensaio de rotina em vários laboratórios do mundo e tem sido amplamente empregado para a quantificação do conteúdo total de compostos fenólicos (TPC) a partir de alimentos. No presente estudo, o ensaio de F-C foi modificado para obter um sistema reacional mais seguro, bem como diminuir o uso de reagentes, o gasto de energia e o tempo de análise. Após avaliar o uso alternativo de solventes e suportes para a reação, e o efeito do binômio tempo/temperatura, o ensaio F-C modificado foi definido como segue: etanol 40% (v/v) como solvente, tempo de reação de 20 minutos a temperatura ambiente (25±2 °C) empregando reagente F-C 50% (v/v) e solução de carbonato de sódio 5% (p/v). O ensaio de F-C modificado foi linear no intervalo de 2,5-50,0 µg/mL de ácido gálico. O limite de detecção e limite de quantificação apresentaram valores de 0,195 e 0,591 µg/mL, respectivamente. A repetitividade, reprodutibilidade (desvio padrão relativo (RSD) 0,06-3,28%) e acurácia (93,28-104,28%) do método modificado também foram demonstradas. O ensaio foi robusto para as alterações na concentração do reagente F-C (45-55%) e carbonato de sódio (4,5-5,5%). O ensaio modificado foi empregado para analisar amostras de alimentos contendo compostos fenólicos e os resultados corroboraram com o ensaio convencional. O ensaio F-C modificado demonstrou ser reprodutível, robusto, rápido, fácil, barato, seguro e confiável para quantificar compostos fenólicos em alimentos. O emprego do etanol no ensaio F-C diminui o impacto ambiental e, consequentemente, torna a análise mais segura do que o ensaio convencional. Além disso, o ensaio F-C modificado é conduzido em condições mais brandas (tempo/temperatura), o que é particularmente útil para um grande volume de análises. Assim, o ensaio F-C modificado e validado pode ser usado como ensaio de rotina no controle de qualidade e no perfil químico de extratos de produtos naturais e alimentos.

PALAVRAS-CHAVE: compostos bioativos; extração; análise de alimentos; controle de qualidade; compostos fenólicos.

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