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CENTRO DE CIÊNCIAS AGRÁRIAS

Programa de Pós-Graduação em Ciência de Alimentos

OBTENÇÃO DE EXTRATOS E FRAÇÕES DE FOLHAS DE UMA NOVA VARIEDADE DE *STEVIA* REBAUDIANA, FORTICAÇÃO DA ATIVIDADE ANTIOXIDANTE E ANTIDIABÉTICA DE *WHEY PROTEIN* ISOLADO COM FRAÇÃO DE STEVIA RICA EM COMPOSTOS FENÓLICOS.

PAULA GIMENEZ MILANI FERNANDES

Maringá

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Tese apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para a obtenção do título de doutor em Ciência de Alimentos.

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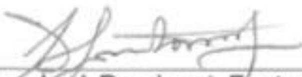
PAULA GIMENEZ MILANI FERNANDES

"OBTENÇÃO DE EXTRATOS E FRAÇÕES DE FOLHAS DE UMA NOVA VARIEDADE DE STEVIA REBAUDIANA, E FORTIFICAÇÃO DA ATIVIDADE ANTIOXIDANTE E ANTIDIABÉTICA DE WHEY PROTEIN ISOLADO COM FRAÇÃO DE STEVIA RICA EM COMPOSTOS FENÓLICOS".

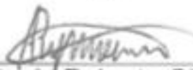
Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

Prof. Dr. Jurandir Fernando Comar

Profa. Dra. Maria Ida Bonini Ravanelli Speziali



Prof. Dr. José Domingos Fontana



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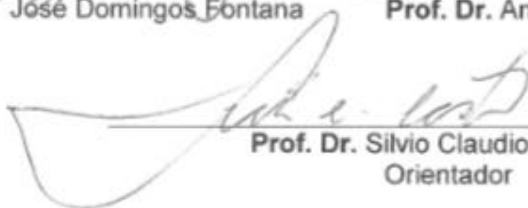

Prof. Dr. Silvio Claudio da Costa
Orientador

Orientador: Silvio Claudio da Costa

Co-Orientadora: Cecília Edna Mareze da Costa

BIOGRAFIA

Paula Gimenez Milani nasceu em 30 de julho de 1988 na cidade Martinópolis, no estado de São Paulo, Brasil. Concluiu o Ensino Médio, no ano 2005, na cidade de Tupã, estado de São Paulo, no Colégio Seletivo. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá, concluída em 2011. Possui mestrado em Ciência de Alimentos, pela Universidade Estadual de Maringá, concluído em 2014. Atualmente faz parte do corpo docente do departamento de bioquímica da Universidade Estadual de Maringá. Tem experiência, participando de projetos de iniciação científica, nas áreas de bioquímica e fisiologia de microrganismos, bioquímica e fisiologia vegetal, biologia celular, fisiologia animal e humana e bioquímica de alimentos, com ênfase no desenvolvimento de suplementos com propriedades nutricionais e funcionais para grupos específicos, como diabéticos.

DEDICO

Aos meus pais, Paulo e Lúcia,

Irmãos, Matheus e Fernanda,

Ao meu marido Willian,

E aos meus orientadores Silvio e Cecília pela confiança,

paciência, compreensão e apoio nesta fase.

A vocês meu eterno amor e gratidão.

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APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de dois artigos científicos. O primeiro deles foi submetido e aceito na revista *Anais da Academia Brasileira de Ciências,* em julho de 2016, que no momento possuía QUALIS B2 para Ciência de Alimentos. O segundo artigo, foi aceito para a publicação na revista *Journal of Food Science and Technology*, com extrato qualis B1 (2017) na área de Ciência de Alimentos, com fator de impacto 1, 241 (ano de 2015).

ARTIGO 1

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Title: New seminal variety of *Stevia rebaudiana*: Obtaining fractions with high antioxidant potential of leaves.

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Cecília Edna Mareze da Costa; Department of Physiological Sciences; State University of Maringa
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Title: Fortification of the whey protein isolate antioxidant and antidiabetic activity with fraction rich in phenolic compounds obtained from *Stevia rebaudiana* (bert.). Bertoni leaves.

RESUMO GERAL

RESUMO GERAL: FORTICAÇÃO DA ATIVIDADE ANTIOXIDANTE E ANTIDIABÉTICA DE *WHEY PROTEIN* ISOLADO COM FRAÇÃO RICA EM COMPOSTOS FENÓLICOS OBTIDOS DE FOLHAS DE STEVIA.

INTRODUÇÃO: *Stevia rebaudiana* (Bert.). Bertoni é mundialmente conhecida e explorada por apresentar naturalmente em suas folhas, glicosídeos de esteviol de alto poder edulcorante. Entre estes compostos, os mais proeminentes são o esteviosídeo e o rebaudiosídeo A, ambos estudados nas últimas décadas também por suas propriedades antidiabéticas. Entretanto, além dos glicosídeos de esteviol, as folhas de estévia foram descritas como fonte de compostos fenólicos e flavonoides. Muitos esforços têm sido feitos para isolar e caracterizar frações ricas em compostos fenólicos de folhas de estévia, de uma nova variedade seminal e de elite, Stevia UEM-13, com o objetivo de avaliar seu potencial de uso como fonte multifuncional de compostos fenólicos. Este estudo relaciona a obtenção de uma fração de Stevia com alto conteúdo de compostos fenólicos, que teve sua composição centesimal determinada e também, seus principais fenólicos e flavonoides presentes foram identificados, por meio da análise LCMS. A fração com potencial para alimentos fortificantes foi adicionada em um isolado proteico obtido a partir de soro de leite (WPI), e o suplemento desenvolvido foi testado quanto aos seus efeitos antioxidantes e antidiabéticos.

OBJETIVO: O objetivo deste estudo foi determinar a composição e o potencial antioxidante de folhas de uma nova seminal variedade de *Stevia rebaudiana* (Stevia UEM-13), determinar os teores de glicosídeos de esteviol, compostos fenólicos, flavonoides e de capacidade antioxidantes de diferentes extratos e frações obtidos a partir das folhas de Stevia UEM-13; e avaliar o potencial da fração com alta capacidade antioxidante, obtida pelo fracionamento em acetato de etila, como fortificadora ou enriquecedora da atividade antioxidante e antidiabética de isolado proteico do soro do leite (*Whey protein)* produzido por processos de separação por membranas.

MATERIAIS E MÉTODOS: As plantas de *Stevia rebaudiana* da variedade seminal UEM 13 cultivadas em NEPRON (UEM) foram coletadas no de estágio máximo crescimento vegetativo. Estes arbustos foram previamente secos em estufa a 60°C e as folhas foram subsequentemente separadas dos caules e ramos, colocadas em sacos de polietileno e

armazenadas a -18°C antes do desenvolvimento dos diferentes extratos. Os reagentes químicos foram adquiridos a *Sigma-Aldrich*. Diferentes extratos e frações foram obtidos a partir dessas folhas e foram analisados quanto aos teores de glicosídeos totais (CLAE), compostos fenólicos (Folin), flavonoides totais e capacidade antioxidante (DPPH). O Extrato aquoso das folhas de Stevia UEM 13 foi obtido por meio de uma extração aquosa de uma amostra de 2,0 g de folhas, adicionadas de 250 ml de água destiladas e aquecidas a 100 ºC. Amostras das folhas dessa variedade foram ainda usadas para extrações com os solventes etanol absoluto e metanol, os quais foram utilizados para extração em aparelho *Sohxlet* e maceração (somente etanol absoluto). O extrato metanólico, por apresentar maior rendimento de extração dos compostos de interesse foi fracionado com hexano, clorofórmio, acetato de etila e isobutanol e as frações obtidas foram analisadas. A fração de acetato de etila (ASF), por apresentar-se livre de glicosídeos e com alta capacidade antioxidante foi analisada em LCMS/MS, os compostos fenólicos e flavonoides foram identificados, e além disso sua composição proximal foi determinada. ASF foi usada como fração fortificadora de um isolado proteico do soro do leite (ASF foi adicionada ao WPI na concentração de 0,2%). A obtenção de WPI foi feita de acordo com Milani et al. (2016) com modificações. O soro foi concentrado em sistema de ultrafiltração (UF), diafiltração (DF) e (NF). Uma amostra deste concentrado foi seca em secador por pulverização. Os processos UF e DF foram feitos em um sistema com membranas filtrantes de polietersulfona (corte de 10 kD), marca *Koch*, em configuração espiral, com área de 50 cm^2. Foram realizados 12 ciclos de diafiltração. A nanofiltração foi realizada em sistema de osmose reversa, composta por duas membranas de poliamida com massa molecular de corte 180D (*Koch*) e 500D (*Millipore*), ambas em configuração espiral, com área de 50 cm^2 cada. A secagem do WPI foi realizada em atomizador secador spray. Um teste de citotoxicidade também foi realizado (teste MTT). Para testar a atividade antidiabética foram estabelecidos 4 grupos experimentais que receberam os seguintes suplementos diariamente: 1) WPI - isolado de proteína de soro de leite (100 mg/kg); 2) ASF - fração de estévia (0,2 mg/kg); 3) WPI + ASF - proteína isolada de soro de leite fortificada com 0,2% da fração de estévia (100 mg/ kg); 4) DC- diabético controle; recebeu apenas água e ração. Um quinto grupo controle (C), não diabético e não suplementado também foi estabelecido. A suplementação oral foi realizada diariamente, às 08:00 horas, por um período de 30 dias, por gavagem esofágica. Semanalmente foram avaliados glicemia de jejum e no estado alimentado, e peso corporal. Após os tratamentos foram avaliadas as taxas plasmáticas e séricas de glicose, colesterol total, colesterol HDL, triglicérides, frutosamina,

AST e ALT. A capacidade antioxidante total também foi avaliada no plasma de todos os grupos de animais após o tratamento.

RESULTADOS E DISCUSSÃO: Entre os principais resultados das diferentes extrações realizadas a partir das folhas de Stevia UEM-13, podemos destacar que o extrato metanólico apresentou maior rendimento de extração dos compostos de interesse quando comparado ao extrato etanólico e por isso foi fracionado com diferentes solventes. A fração isobotanólica mostrou maior teor de glicosídeos (65,3%). A fração obtida em acetato de etila apresentou o maior conteúdo de compostos fenólicos e flavonoides (524.20 mg equivalente de ácido gálico/g; 380.62 μg equivalente de quercetina/g, respectivamente), e também alta capacidade antioxidante e apresentou-se com baixo teor de glicosídeos. Os principais compostos identificados por LCMS/MS foram o ácido cafeico, quercetina-3-o-glicosídeo, cianidina-3-glicosídeo, kaempferol, quercetina, apigenina, ácido rozmarínico, ácido cloriogênico e ácido dicafeoilquínico. A fração de estevia em acetato de etila (ASF) foi utilizada como fonte multifuncional de compostos fenólicos na fortificação do isolado de proteínas de soro de leite (WPI) obtido por processos de separação de membranas. WPI fortificado com 0,2% de ASF teve sua atividade antioxidante aumentada em 80%. O WPI fortificado com ASF apresentou efeitos antidiabéticos mais pronunciados em relação ao WPI não fortificado, principalmente no controle glicêmico de animais diabéticos induzidos por estreptozotocina. Importantes efeitos antioxidantes *in vitro e in vivo* foram mostrados pela fração de Stevia ASF, o que contribuiu de forma expressiva para fortificar o WPI. Este estudo pioneiro mostra que a ASF pode ser usada para enriquecer as propriedades antioxidantes e antidiabéticas do WPI.

CONCLUSÕES: A avaliação da extração e do fracionamento desta planta utilizando diferentes solventes e metodologias, resultou em extratos de diferentes capacidades antioxidantes, que podem ser utilizados para enriquecer a literatura e contribuir para sua aplicação em alimentos, produtos farmacêuticos e cosméticos. Uma dieta rica em antioxidantes pode contribuir significativamente para prevenir doenças degenerativas, doenças cardiovasculares e metabólicas, como o diabetes mellitus. Assim, os alimentos naturais e industrializados enriquecidos com flavonoides e compostos fenólicos contribuem para a redução dessas doenças. O presente estudo mostrou que os extratos de etanol, metanol e acetato de etilo de folhas de *Stevia rebaudiana* (Stevia UEM-13) possuem um grande potencial antioxidante e podem ser usados como possíveis aditivos para melhorar a funcionalidade de alimentos e bebidas.

Por meio dos processos de separação das membranas, foi possível obter a partir do soro do leite, um isolado (WPI) com uma concentração importante de proteínas. A fração Stevia, ASF, obtida com 3,82% de rendimento, exibiu compostos fenólicos como principais constituintes. Os principais ácidos fenólicos e flavonoides identificados na ASF estão de acordo com a literatura. A ASF na concentração de 0,2% promoveu aumento de 80% na atividade antioxidante WPI. Os testes fisiológicos mostraram que os três suplementos (ASF, WPI e WPI + ASF) foram benéficos para melhorar o controle metabólico de ratos diabéticos. O WPI fortificado com ASF foi o suplemento alimentar com maior funcionalidade, mostrando que a ASF tem potencial para ser utilizada como fortificador da atividade antioxidante e antidiabética de alimentos como o WPI, e possivelmente enriquecendo outros alimentos ou suplementos.

Palavras-chaves: Isolado protéico de soro; fração antioxidante de *Stevia rebaudiana*; compostos fenólicos; flavonoides; diabetes mellitus.

GENERAL ABSTRACT

FORTIFICATION OF THE WHEY PROTEIN ISOLATE ANTIOXIDANT AND ANTIDIABETIC ACTIVITY WITH FRACTION RICH IN PHENOLIC COMPOUNDS OBTAINED FROM STEVIA.

INTRODUCTION: *Stevia rebaudiana* (Bert.). Bertoni is worldwide known and explored by presenting high sweetener power steviol glycosides naturally in its leaves. Among these compounds, the most prominent ones are the stevioside and the rebaudioside A, both have been studied in the last decades also by their antidiabetic properties. However, besides the steviol glycosides, the stevia leaves have been described as phenolic and flavonoids compounds source, with antioxidant and antidiabetic source. Many efforts have been made directed to isolating and characterizing fractions rich in phenolic compounds from stevia leaves of a new seminal and elite variety, Stevia UEM-13, with the purpose of evaluating their potential of use as multi-functional source of phenolic compounds. This study relates the obtainment of a stevia fraction with high phenolic compounds content, which had its centesimal composition determined and also, its main present phenolic and flavonoids were identified, through LCMS analysis. The fraction with potential for fortifying foods was added in a protein isolate obtained from whey (WPI), and the developed supplement was tested for its antioxidant potential and antidiabetic effects.

OBJECTIVE: The objective of this study was to determine the composition and antioxidant potential of leaves of a new seminal variety of *Stevia rebaudiana* (Stevia UEM-13), to determine the levels of steviol glycosides, phenolic compounds, flavonoids and antioxidant capacity of different extracts and fractions obtained from the leaves of Stevia UEM-13 and to evaluate the potential of the fraction with high antioxidant capacity, obtained by fractionation with ethyl acetate, as a fortifier of the antioxidant and antidiabetic activity of whey protein isolate produced by membrane separation processes.

MATERIALS AND METHODS: The plants of *Stevia rebaudiana* of the seminal variety UEM 13 grown at NEPRON (UEM) were collected at maximum vegetative growth stage. These shrubs were previously dried in an oven at 60°C and the leaves were subsequently separated from the stems and branches, placed in polyethylene bags, and stored at -18°C before developing different extracts. The chemical reagents were purchased from Sigma-

Aldrich. Different extracts and fractions were obtained from these leaves and were analyzed for total glycosides (HPLC), phenolic compounds (Folin) and total flavonoids and antioxidant capacity (DPPH). Aqueous extract of Stevia leaves UEM-13 was obtained by means of an aqueous extraction of a sample of 2.0 g of leaves, added with 250 ml of distilled water and heated to 100 ºC. Samples from the same sheet were also used for extractions with the solvents absolute ethanol and methanol, which were used for extraction in Sohxlet apparatus and maceration (absolute ethanol only). The methanolic extract, due to the higher extraction yield of the compounds of interest, was fractionated with hexane, chloroform, ethyl acetate and isobutanol and the fractions obtained were analyzed. The ethyl acetate fraction (ASF), because it was free of glycosides and with high antioxidant capacity, was analyzed in LCMS/MS and the phenolic and flavonoid compounds were identified, and in addition its proximal composition was determined. ASF was used as a fortifying fraction of a whey protein isolate (ASF was added at 0.2% concentration to the WPI). The WPI obtaining was made in accordance to Milani et al. (2016) with modifications. The whey was concentrated in ultrafiltration system (UF), diafiltration (DF) and (NF). A sample of this concentrated was dried in spray dryer. The UF and DF processes were made in a system with polyethersulfone filtering membranes (10 kD cut off), Koch brand, in spiral configuration, with 50 cm^2 area. 12 diafiltration cycles were performed. The nanofiltration was performed in reverse osmosis system, composed by two polyamide membranes with 180D cut off molecular mass (Koch) and 500D (Millipore), both in spiral configuration, with 50 cm^2 area each one. The WPI drying was made in a spray dryer atomizer. The MTT test was made in adipose cells to evaluate the ASF fraction cytotoxicity. To test the antidiabetic activity, 4 experimental groups were established, receiving the following supplements daily: 1) WPI - whey protein isolate (100 mg/kg); 2) ASF - stevia fraction (0.2 mg/kg); 3) WPI+ASF - protein isolate of whey fortified with 0.2% of the stevia fraction (100mg/kg); 4) DC- diabetic control; Received only water and feed. A fifth group (C) non-diabetic and non-supplemented was also established. The oral supplementation was performed daily, at 08:00 AM, for a 30 days period, by gavage (the control diabetic animals group received pure water). Fasting and fed blood glucose and body weight were evaluated weekly. After the treatments were evaluated plasma and serum levels of glucose, total cholesterol, HDL cholesterol, triglycerides, fructosamine, AST and ALT. The total antioxidant capacity was also evaluated in the plasma of all groups of animals after treatment.

RESULTS AND DISCUSSION: Among the main results of the different extractions made from the Stevia UEM-13 leaves, we can highlight that the methanolic extract presented higher extraction yield of the compounds of interest when compared to the ethanolic extract and therefore it was fractionated with different solvents. The isobotanolic fraction showed a higher content of glycosides (65.3%). The fraction obtained in ethyl acetate presented the highest content of phenolic compounds and flavonoids (524.20 mg galic acid equivalent/g; 380.62 µg quercetin equivalent/g respectively), and also of antioxidant capacity and presented with low glycosides content. the main identified by LC-MS were caffeic acid, quercetin-3-o-glycoside, cyanidin-3-glucoside, kaempferol, quercetin, apigenin, rozmarinic acid, clhologenic acid e dicaffeoylquinic acid. The stevia fraction in ethyl acetate (ASF) was used as a multi-functional source of phenolic compounds in the fortification of the whey protein isolate (WPI) obtained by membranes separation processes. WPI fortified with 0.2% of ASF had its antioxidant activity increased in 80%. WPI fortified with ASF presented more pronounced antidiabetic effects in relation to the WPI unfortified, mainly in the glycemic control of diabetic animals induced by streptozotocin. In vitro and in vivo antioxidant effects found important in stevia fraction, what contributed in an expressive way for fortifying the WPI. This pioneer study shows that the ASF can be used for enriching the WPI antioxidant and antidiabetic properties.

CONCLUSIONS: The evaluation of extraction and fractionation of this plant using different solvents and methodologies resulted in extracts of different antioxidant capacity, which can be used to enrich the literature and contribute to their application in foods, pharmaceuticals and cosmetics. A diet rich in antioxidants may contribute significantly to prevent degenerative diseases, cardiovascular and metabolic diseases, such as diabetes mellitus. Thus, natural and industrialized foods enriched with flavonoids and phenolic compounds contribute to the reduction of these diseases. The present study showed that ethanol, methanol and ethyl acetate extracts of *Stevia rebaudiana* leaves (Stevia UEM-13) have a great potential antioxidant and so can be used as possible additives to enhance the functionality of food and beverages. Through membranes separation processes it was possible to obtain from whey the whey proteins isolate (WPI), which had its centesimal composition determined; The stevia fraction, obtained from Stevia UEM 13 variety leaves, with 3.82% yield, exhibited phenolic compounds as main constituents. The main identified phenolic acids and flavonoids in the ASF comply with the data literature. The ASF in the 0.2% concentration promoted increase of 80% in the WPI antioxidant activity. The physiological tests showed that the three

supplements (ASF, WPI and WPI+ASF) were beneficial for improving the metabolic control of diabetic mice. The WPI fortified with ASF was the food supplement with expressive functionality, showing that the ASF has potential to be used as a fortifier or an antioxidant and antidiabetic activity enriching of foods or supplements.

Key-words: whey protein isolate; antioxidant fraction of *Stevia rebaudiana*; phenolic compounds; flavonoids; diabetes mellitus;

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Title

New seminal variety of *Stevia rebaudiana*: Obtaining fractions with high antioxidant potential of leaves.

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Key-words: Bioactive compounds; glycosides; leaf extracts; phenolic compounds; new cultivar of Stevia.

Running Title

Variety of *Stevia*, fractions with high antioxidant action

Academy Section

Agrarian Sciences

The aim of this study was to determine the composition and antioxidant potential of leaves of a new variety of *Stevia rebaudiana* (Stevia UEM-13). Stevia leaves of UEM-13 contain rebaudioside A as the main glycoside, while most wild Stevia plants contain stevioside. Furthermore can be multiplied by seed, which reduces the cost of plant culture techniques as other clonal varieties are multiplied by buds, requiring sophisticated and expensive seedling production systems. Ethanol and methanol were used in the extraction to determine the bioactive compounds. The methanolic extract was fractionated sequentially with hexane, chloroform, ethyl acetate and isobutanol, and the highest concentration of phenolic compounds and flavonoids was obtained in the ethyl acetate fraction (524.20 mg galic acid equivalent/g; 380.62 µg quercetin equivalent/g). The glycoside content varied greatly among the fractions (0.5% - 65.3%). Higher antioxidant potential was found in the methanol extract and the ethyl acetate fraction with 93.5% and 97.32%, respectively. In addition to being an excellent source for obtaining of extracts rich in glycoside, this new variety can also be used as raw material for the production of extracts or fractions with a significant amount of antioxidant activity and potential to be used as additives in food.

Introduction

Plants are an important source of structurally different active natural products and biological properties. These active ingredients can play adjuvant and important roles in traditional medicine in many countries (Tadhani and Subhash 2007). The beneficial effects are produced by different active ingredients and other compounds of low molecular weight such as vitamins, carotenoids, flavonoids, anthocyanins, tannins and other phenolic compounds (Wölwer-Rieck 2012, Tadhani and Subhash 2007), which have different mechanisms of action. It is known that phenolic compounds and flavonoids are important for the normal development and protection of plants (Wölwer-Rieck 2012), but their high antioxidant activity as food additives for humans must still be further investigated.

Stevia rebaudiana Bertoni, a plant from the Asteraceae family, is native to Paraguay and it is cultivated in many countries, including Brazil, Argentina, Japan, China, the United States and European countries (Dacome et al. 2005). It is important in the food industry because the leaves contain sweet-tasting diterpene glycosides, among which stevioside and rebaudioside A (RebA) are included (Carakostas et al. 2008). These glycosides are 450 times sweeter than sucrose (Williams and Burdock 2009), which has led to commercial interest, mainly for food and beverages. The Stevia plants have more than 100 compounds (Wölwer-Rieck 2012) and extracts from the leaves have been traditionally used in the treatment of diseases such as diabetes (Tadhani and Subhash 2007, Shukla et al. 2009). Stevia extracts can exert beneficial effects on human health, including anti-hypertension (Chan et al. 2000) and anti-hyperglycemic effects (Abudula et al. 2008), antiviral activities against the human rotavirus, it is not cariogenic and has beneficial properties for glucose metabolism and renal function (Shukla et al. 2009). The beneficial effects of these compounds on human health have been previously studied. Research aimed at obtaining Stevia extracts containing significant levels of sweeteners and these compounds (Gawel-Beben et al. 2015, Kawshik et

al. 2010). The results showed that there is still no consensus on the ideal method of obtaining both compounds (sweeteners and bioactive compounds) in significant concentrations.

The composition of Stevia leaf extracts and the concentration of these bioactive compounds vary according to the solvents used and the different types of extraction methods. Significant results were found in the ethanol and methanol extracts and ethyl acetate solvents (Wölwer-Rieck 2012, Shukla et al. 2009). However, further studies should be conducted on different varieties of plants and improved extraction methods need to be developed for better results. In addition, more studies should be conducted in order to obtain the best method and the best fractions of the bioactive compounds from stevia are present in higher concentrations. The Center for Studies in Natural Products (NEPRON), State University of Maringá (UEM) started in 1990 studies in order to obtain sweeteners or stevia extracts with high levels of RebA, sweetener better sensory profile that stevioside. The first projects ("Biotechnological Applications of *Stevia rebaudiana* Products (Bert.) Bertoni") resulted in the development of a selection of stevia plants with high content of this glycoside. The research resulted in obtaining two varieties (M1 Alvarez and Stevia UEM-320, the latter forward three times as RebA compared with a variety of wild-type plant, and therefore the majority glycoside in the plant selected (Alvarez and Couto 1994).

The cultivation of these clonal varieties, made by cutting method, presented some disadvantages over the years, as difficulties and rooting problems, increased susceptibility to environmental variations and attack predators, and need maintenance ideal conditions for plant growth. Currently the researchers NEPRON are developing projects in order to obtain varieties of stevia third generation that can be reproduced and grown for seed minimizing costs, increasing the viability of farming, reproducibility and marketing, and decreasing the mortality of plants. Thus, this study was developed from a new seminal variety of Stevia rebaudiana called Stevia UEM-13.

The aim of this study was to present the glycoside composition, bioactive compounds and antioxidant potential of leaves from a new variety of *Stevia rebaudiana* (Stevia UEM-13). Was evaluated the concentration of these bioactives in different extracts and fractions produced by different extraction methods, and also identified in which these fractions have greater functional potential for use in food, pharmaceutical and cosmetic products

2. Experimental Section

2.1. Experimental material

The plants of *Stevia rebaudiana* of the seminal variety UEM 13 grown at NEPRON (UEM) were collected at maximum vegetative growth stage. These shrubs were previously dried in an oven at 60°C and the leaves were subsequently separated from the stems and branches, placed in polyethylene bags, and stored at -18°C before developing different extracts. The chemical reagents were purchased from Sigma-Aldrich.

2.2. Aqueous extract from the Stevia leaves UEM-13

A 2.0 g sample of previously ground Stevia leaves UEM-13 were transferred to a 250 ml beaker; 100 mL of deionized distilled water was added and boiled for five minutes. After this procedure, the material was decanted and filtered under vacuum. The procedure was repeated twice with 100 ml and 50 ml of water, respectively. After the last filtration, the extract was transferred to a 250 ml volumetric balloon and filled up with distilled water. We used this extract to analyze glycosides, phenolic compounds and flavonoids.

2.2.1. Ethanol extract of leaves by maceration (EELM)

The extraction system was established using 500 g of previously ground *Stevia rebaudiana* leaves of the seminal variety UEM-13, placed in a 2.0 L glass beaker and 1.5 L of P.A absolute ethanol (99.5%) was added. The system stood for 24 hours in the dark at room

temperature, to obtain the first fraction. The same procedure was repeated to obtain 7 fractions (extraction until exhaustion). The extracts were combined and dried in a rotary evaporator (Buchi brand) at 50°C under vacuum. The powder obtained was used to evaluate the glycosides, phenolic compounds, total flavonoids and antioxidant activity.

2.2.2. Ethanol extract of the leaves by Soxhlet (EELS)

The previously dried and ground *Stevia rebaudiana* leaves (100 g) of the seminal variety UEM-13 were placed in 500 ml of absolute ethanol (99.5%) and added to the system using the Soxhlet apparatus. The extract was filtered and dried in a rotary evaporator (Buchi trademark) at 50°C under vacuum and the dry powder was analyzed.

2.2.3. Methanol extract of the leaves by Soxhlet (MELS)

The previously dried and ground *Stevia rebaudiana* leaves (100 g) of the seminal variety UEM-13 were placed in 500 ml of P.A methanol and added to the system using the Soxhlet apparatus (extraction until exhaustion). The extract was filtered and dried in a rotary evaporator (Buchi brand) at 50°C under vacuum and the powder was analyzed.

2.2.4. Extract fractionated with different solvents

The fractionation was performed as follows, as shown in figure 1:

2.2.4.1 Hexane fraction (HF)

A 100 g sample of dried *Stevia rebaudiana* leaves of the seminal variety UEM-13 were extracted with 400 ml of methanol were packaged in apparatus Soxhlet for 4 hours. The extraction was repeated to obtain a colorless methanolic extract (extraction until exhaustion). The extracts were combined and dried in a rotary evaporator (Büchi brand) at 50°C.

A sample of 35.8 g of dried methanolic extract in a rotary evaporator (brand Buchi) was dissolved with distilled and deionized water (400 ml). The aqueous solution was extracted with 1000 ml of hexane and the mixture was separated in a separation funnel. The process was repeated. The aqueous and hexane fractions were obtained. The hexane fractions were pooled and dried in a rotary evaporator (Buchi trademark) at 50°C under vacuum and the fluid (HF) was analyzed.

2.2.4.2 Chloroform fraction (CF)

The resulting aqueous fraction from the hexane extraction was extracted with 1000 ml of chloroform in duplicate. The mixture was separated in a separation funnel and the aqueous and chloroform fractions were obtained. The chloroform fraction was dried in a rotary evaporator (Buchi brand) at 50°C under vacuum. The resulting powder (CF) was analyzed.

2.2.4.3 Ethyl acetate fraction (EF)

The resulting aqueous fraction from the chloroform extraction was extracted with 1000 ml of ethyl acetate in duplicate. The mixture was separated in a separation funnel and the ethyl acetate (organic) fraction obtained was dried in a rotary evaporator (Buchi brand) at 50°C under vacuum. The powder (EF) was analyzed.

2.2.4.4 Isobutanol fraction (IF)

The resulting aqueous fraction from the ethyl acetate extract was extracted with 1000 ml of isobutanol in duplicate and the fractions were separated in a separation funnel. The fraction with isobutanol dried using rotary evaporator (brand Buchi) at 50°C under vacuum. The resulting powder (IF) was analyzed.

2.2.4.5 Aqueous fraction (AF)

The quantity of the fraction referred to as IF (isobutanol) was reduced in a rotary evaporator and then dried on a bench-top spray dryer (Spray Dryer, Buchi) and the resulting powder (aqueous fraction - AF) was analyzed.

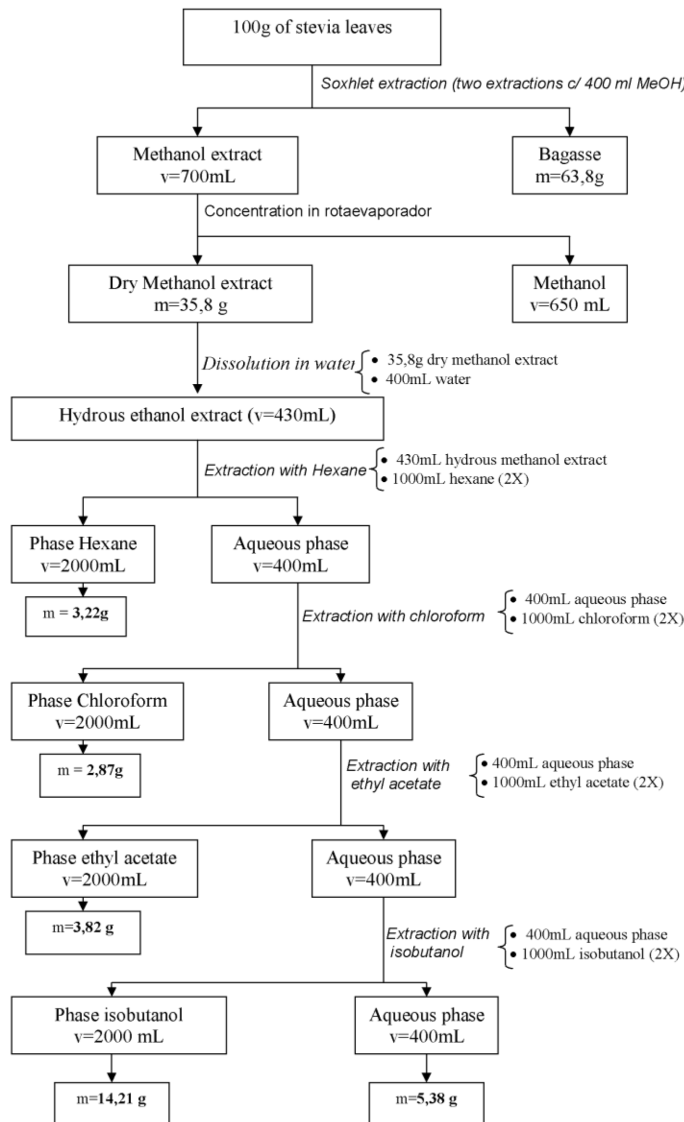

Fig 1. Flow chart for obtaining stevia extract

2.3. Analysis

2.3.1. Glycosides

The glycosides compounds of leaves, extracts and fractions were assayed according to the method described by Dacome et al. 2005.

2.3.2. Total phenolic compounds

The phenolic compounds of leaves, extracts and fractions were assayed according to the method described by Singleton et al. 1999 (with modifications). A solution of 1 mg/ml of aqueous extract of the leaves was prepared to analyze the leaves. For the extract analysis, a solution of absolute ethanol (99.5%) was prepared at a concentration of 0.5 mg/ml of ethanolic leaf extract by maceration, ethanolic leaf extract through Soxhlet and methanolic leaf extract through Soxhlet. The hexane, chloroform and isobutanol fractions were prepared at a concentration of 1 mg/mL of absolute ethanol. The ethyl acetate fraction was solubilized in ethanol at a concentration of 0.1 mg/ml. The concentration of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per extract/g using a standard curve prepared with water for the analysis of leaves (aqueous extract) (0-30 µg/ml) and in ethanol for analysis of extract fractions (0-80 µg/ml).

2.3.3. Total flavonoids

The quantification of total flavonoids was determined by Jia et al. 1999 (with modifications). All extracts and fractions were prepared at a concentration of 1 mg/ml of ethanol, except the ethyl acetate fraction (0,5 mg/ml). The absorbance reading of samples was 510 nm. Data were expressed as rutin and quercetin equivalents.

2.3.4. Antioxidant activity

The elimination of free radical activity from the extracts and *Stevia rebaudiana* fractions of the seminal variety UEM-13 was measured by the ability to eliminate DPPH (Blios 1958,

with modifications). The results were expressed as inhibition percentage of free radicals by the sample according to the following formula: % inhibition = A_{0}- A_t/A_t x 100. The symbol A_0 stands for control absorbance and A_t for absorbance of each extract or fraction. All analyses were performed in triplicate and results expressed as mean values. Gallic acid was used as the standard.

2.4 Statistical Analysis

Each analysis was performed in triplicate. The results of all the analyses were expressed as mean ± standard error of the mean and submitted to ANOVA followed by Tukey's test ($p < 0.05$). We used the Statistical Analysis System (SAS, Institute Inc., Cary, North Carolina, United States, 2006) statistical software version 9.1.

3. Results and discussion

3.1 Total glycosides

Table 1 shows the glycoside content (stevioside and rebaudioside A and C), total phenolics and flavonoids pooled from the leaves used in this study. Importantly, the Stevia leaves UEM-13 contain a higher content of rebaudioside A than of stevioside, and it is a variety of elite that can be reproduced by seeds, also increasing its advantage over crop varieties by cuttings. This is pioneering work with this cultivar. Stevia UEM-13 therefore presented a ratio RebA/Stev much higher (2.27) than the wild varieties that have inferiors values to 1 (Kovylyaeva et al. 2007, Gardana et al. 2010, Gupta et al. 2013, Madan et al. 2010). The new cultivar has significant levels of bioactive compounds when compared with other wild varieties (Gawen-Beben et al. 2015, Pasquel et al. 2000, Wölwer-Rieck 2012).

Table 1. Total glycoside content (stevioside and rebaudioside A and C) from Stevia UEM-13.

	Phenolics compouns	Total flavonoids	Glycosides	Stev	RebA	Reb C	RebA/Stev
Leaves	2,36 %	17,63 %	14.8 %	4.0 %	9.1 %	1.7 %	2.27

Stev= Stevioside; RebA= Rebaudioside A; RebC= Rebaudioside C;

The Stevia leaves UEM-13 used in this study presented 14.8% of total glycosides. This seminal variety showed high rebaudiuoside A content, indicating to be an elite variety. Table 2 shows the extraction yield of glycosides observed in the extraction methods, total glycoside content (stevioside and rebaudioside A, C and D), phenolic compounds (gallic acid equivalents) and total flavonoids (quercetin equivalent). The ethanol extraction method by maceration presented low yield and low percentage of glycosides, when compared with the extraction method in Soxhlet apparatus. Both extracts showed significant amounts of phenolic compounds and flavonoids and a significant percentage of antioxidant activity; these results indicate that treatment with alcoholic solvents may contribute to the extraction of other substances present in the stevia leaves, extracting substantial amounts of glycosides. Periche et al. 2015 study effectives extraction procedures for maximization of the yield of steviol glycosides and total phenolic compounds as well as antioxidant activity in stevia extracts. The results showed significant correlations and experimental conditions greatly influence this achievement. The choice of solvent and extraction method, temperature, time and ratio changes the performance of the extraction. This work indicates that the fractioning with different solvents can change the proportion of sweeteners and antioxidants, showing in most of them a negative correlation.

Pasquel et al. 2000 show that pretreatment of stevia leaves with solvents such as ethanol and CO_2 prior to the conventional extraction process can improve the quality of the sweetener obtained, contributing to the reduction of the bitter aftertaste. Hexane, chloroform and ethyl acetate fractions do not contain significant concentrations of glycosides, unlike

isobutanol that extracted 60.3% of sweeteners, making this the best solvent to extract higher yields of sweeteners in the industrial processes.

Table 2. Total compunds present in the *Stevia rebaudiana* extracts and fractions

Type of extract	Extraction yield (g/100g)	Total glycosides (g/100g)	Phenolics compounds (g/100g)	Total flavonoids (g/g)
EELM	6.13±0,01	26.0±0,01	7.27±0,01	0.28±0,02
EELS	29.50±0,01	31.8±0,01	10.26±0,02	0.32±0,01
MELS	38.82±0,02	30.5±0,05	10.70±0,05	0.35±0,05
HF	8.99±0,01	0.1±0,01	4.47±0,01	0.29±0,01
CF	8.01±0,01	1.1±0,04	3.96±0,03	0.26±0,02
EF	10.67±0,02	0.05±0,01	52.42±0,03	0.38±0,01
IF	39.69±0,01	70.8±0,02	7.29±0,01	0.24±0,01
AF	15.02±0,01	1.14±0,01	2.33±0,01	0.07±0,01

EELM: Ethanolic Extract of Leaves (maceration); EELS: Ethanolic Extract of Leaves (Soxhlet); MELS: Methanolic Extract of Leaves (Soxhlet); HF: Hexane Fraction; CF: Chloroform Fraction; EF: Ethyl Acetate Fraction; IF: Isobutanol Fraction, AF: Aqueous Fraction.

3.2 Total phenolic compounds

The content of phenolic compounds present in the Stevia leaves UEM-13 (aqueous extract of leaves) was 23.61 mg/g GAE (Gallic Acid Equivalent). Figure 2 shows the concentration of phenolic compounds of the *Stevia rebaudiana* leaf extracts and fractions of the variety UEM-13. These results demonstrate the high antioxidant potential of the extracts from the Stevia leaves, particularly the methanol and ethyl acetate extracts (107.08 mg/g, 524.2 mg/g, respectively). Shukla et al. 2009 found 61.5 mg/g GAE from the ethanol extract

of Stevia leaves through Soxhlet. Gawel-Beben et al. 2015 found similar values of phenolic compounds in ethanol extract of dried leaves from *Stevia rabaudiana*. The values found in the present study indicate that these compounds should be further evaluated for their antioxidant potential in food and drinks, and further demonstrates that the ethyl acetate fraction has the greatest potential for will provide additional benefits in terms of oxidative.

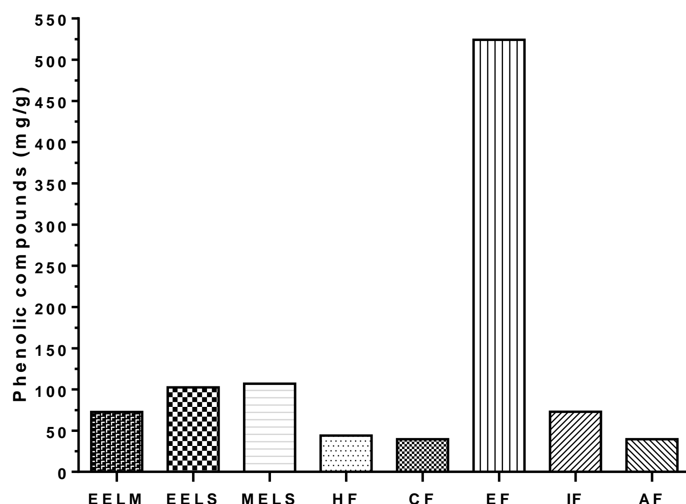

Fig. 2. Total phenolic extracts from Stevia leaves UEM-13 expressed as mg of gallic acid equivalents per extract/g. The letters represent the following: EELM: Ethanolic Extract of Leaves (maceration); EELS: Ethanolic Extract of Leaves (Soxhlet); MELS: Methanolic Extract of Leaves (Soxhlet); HF: Hexane Fraction; CF: Chloroform Fraction; EF: Ethyl acetate Fraction; IF: Isobutanol Fraction, AF = Aqueous Fraction.

3.3 Total flavonoids

Different concentrations of these compounds can be found in leaves, callus, and different *Stevia rebaudiana* extracts, depending on the conditions, type of solvent and extraction method used (Madan et al. 2010). In the present study the concentration of flavonoids present in the Stevia leaves UEM-13 (aqueous extract) was 176.33 µg/mg. After the extraction process, significant concentrations of flavonoids were found in the methanol and ethyl acetate extract, demonstrating the antioxidant potential of the extract (Figures 3A and 3B)

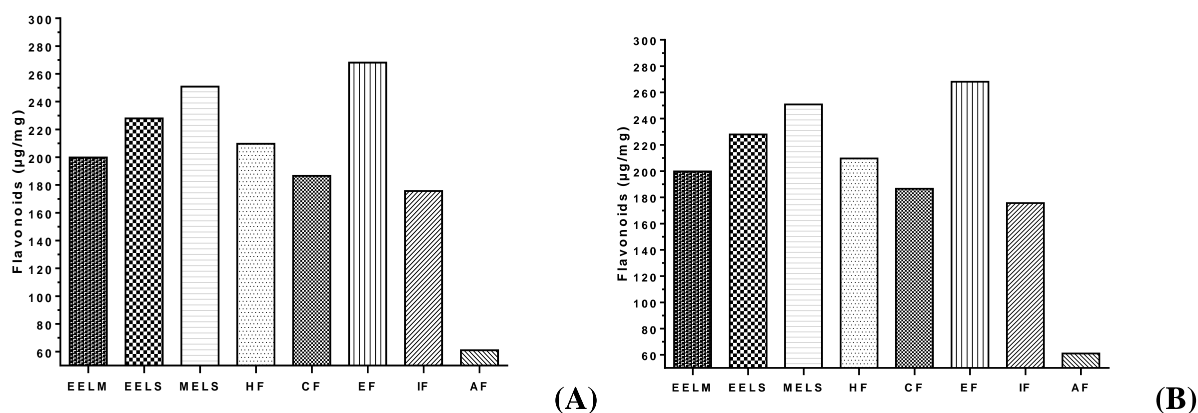

Fig. 3. Total flavonoid extracts from the Stevia leaves UEM-13 expressed as µg of quercetin equivalent per extract/mg (A) and µg of rutin equivalents per extract/mg; (B) µg of quercetin equivalents per extract/mg. The letters represent the following: EELM: Ethanolic Extract of Leaves (maceration); EELS: Ethanolic Extract of Leaves (Soxhlet); MELS: Methanolic Extract of Leaves (Soxhlet); HF: Hexane Fraction; CF: Chloroform Fraction; EF: Ethyl acetate Fraction; IF: Isobutanol Fraction, AF = Aqueous Fraction.

3.4 Antioxidant activity

Studies show that Stevia leaf extracts may exhibit high antioxidant potential (Periche et al. 2015). The antioxidant activity of the aqueous extract from the leaves of Stevia UEM-13 was 42.26% (1 mg/ml). The Figure 4 shows the percentages of inhibition of DPPH radicals of the extracts evaluated in this study. It can be seen that, in general, all extracts showed important antioxidant potential, particularly the methanol extract of leaves obtained by extraction and through Soxhlet. The methanol extract and ethyl acetate fraction showed inhibition of DPPH radical of the 93.5% and 97.32% at a concentration of 1 mg/ml. These extracts had higher levels of phenolic compounds and flavonoids which indicates high antioxidant activity. The IC_{50} for each extract (EEMF, EESF, MESF, HF, CF, EF, IF, AF) were 626.5 µg/ml, 576.70 µg/ml, 534.75 µg/ml, 1175 µg/ml, 963.02 µg/ml, 513.76 µg/ml, 792. 64 µg/ml, 889.20 µg/ml, respectively. The fraction with the hight potential is the ethyl acetate

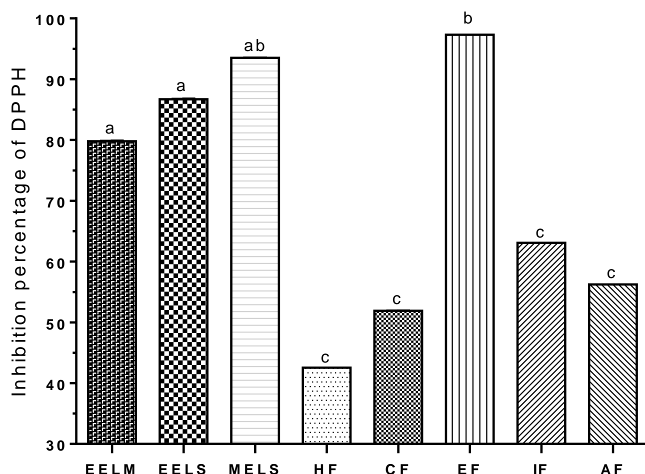

Fig. 4. Percentage of inhibition of DPPH radicals from leaf extracts of Stevia UEM-13 (1 mg/ml). Mean values with different superscript letters indicate significant differences ($P > 0.05$). The letters represent the following: EELM: Ethanolic Extract of Leaves (maceration); EELS: Ethanolic Extract of Leaves (Soxhlet); MELS: Methanolic Extract of Leaves (Soxhlet); HF: Hexane Fraction; CF: Chloroform Fraction; EF: Ethyl acetate Fraction; IF: Isobutanol Fraction, AF = Aqueous Fraction.

4. Conclusion

This study shows for the first time the glycosides content, phenolic compounds and antioxidant activity of seminal variety from *Stevia rebaudiana*, Stevia UEM-13, confirming that a variety with high RebA content and can be reproduced by seeds.

The evaluation of extraction and fractionation of this plant using different solvents and methodologies resulted in extracts of different antioxidant capacity, which can be used to enrich the literature and contribute to their application in foods, pharmaceuticals and cosmetics. A diet rich in antioxidants may contribute significantly to prevent degenerative diseases, cardiovascular and metabolic diseases. Thus, natural and industrialized foods enriched with flavonoids and phenolic compounds contribute to the reduction of these diseases. The present study showed that ethanol, methanol and ethyl acetate extracts of *Stevia*

rebaudiana leaves (Stevia UEM-13) have a great potential antioxidant and so can be used as possible additives to enhance the functionality of food and beverages.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

Resumo

O objetivo deste estudo foi determinar a composição e o potencial antioxidante de folhas de uma nova variedade de *Stevia rebaudiana* (Stevia UEM-13). Folhas de estévia UEM-13 contêm rebaudiosídeo A como o glicosídeo principal, enquanto que a maioria das plantas de estévia selvagens possui esteviosídeo. Além disso, esta variedade pode ser multiplicada por sementes, o que reduz o custo de técnicas de cultivo em relação a outras variedades clonais que são multiplicados por estaquia, que exigem sistemas de produção de plantio sofisticados e dispendiosos. O etanol e o metanol foram usados na extração para determinar os compostos bioactivos. O extrato metanólico foi fraccionado sequencialmente com hexano, clorofórmio, acetato de etila e isobutanol, e a concentração mais elevada de compostos fenólicos e flavonóides foi obtida na fração de acetato de etila (524.20 mg de ácido gálico equivalente/g; 380.62 ug quercetina equivalente/g). O conteúdo glicosídeo variou bastante entre as frações (0.5% - 65.3%). O Maior potencial antioxidante foi encontrado no extrato metanólico e na fração de acetato de etila com 93.5% e 97.32%, respectivamente. Este estudo mostra que além de ser uma excelente fonte para a obtenção de extratos ricos em glicosideo, esta nova variedade pode também ser usada como matéria-prima para a produção de extratos ou frações com uma quantidade significativa de atividade antioxidante e potencial para serem utilizados como aditivos em alimentos.

Key-words: Compostos Bioativos; extratos de folhas; glicosídeos; compostos fenólicos; nova cultivar de estévia,

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Highlights

- Stevia fraction (ASF) obtained with ethyl acetate exhibited high antioxidant capacity.
- Phenolic compounds and flavonoids of ASF were identified by LC-MS/MS.
- ASF promoted an 80% increase in the antioxidant activity of whey protein isolate (WPI).
- The WPI fortified with ASF improved metabolic control in diabetic mice.
- ASF has potential to enhance the antioxidant and antidiabetic capacities of WPI.

Abstract

A stevia fraction (ASF) free of steviol glycosides was extracted from *Stevia rebaudiana* leaves (Stevia UEM-13). ASF essentially constitutes phenolic compounds (52.42%), which were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) as caffeic acid, quercetin-3-*o*-glycoside, cyanidin-3-glucoside, kaempferol, quercetin, apigenin, rozmarinic acid, chlorogenic acid and dicaffeoylquinic acid. ASF was used as a multi-functional source of phenolic compounds to fortify the whey protein isolate (WPI) obtained by membrane separation. WPI fortified with 0.2% ASF showed an 80% increase in its antioxidant activity and more pronounced antidiabetic effects than the unfortified WPI, mainly in the glycemic control of diabetic animals induced by streptozotocin. The *in vitro* and *in vivo* antioxidant effects of ASF may enhance the effects of WPI. Indeed, this pioneering study revealed that ASF can be used to enrich the antioxidant and antidiabetic properties of WPI.

Keywords: whey protein isolate; antioxidant fraction of *Stevia rebaudiana*; phenolic compounds; flavonoids; diabetes mellitus.

1. Introduction

Stevia rebaudiana (Bert.) Bertoni is known worldwide, and its leaves are characterized by the presence of steviol glycosides, which have immense sweetening capacity (Dacome et al. 2005). Among these steviol glycosides, the most prominent compounds are stevioside and rebaudioside A, both of which have been studied in recent decades for their antidiabetic properties (Gregersen et al. 2004; Milani et al. 2016). In addition to steviol glycosides, stevia leaves are a source of phenolic and flavonoid compounds with antioxidant and antidiabetic properties (Ghanta et al. 2007; Shukla et al. 2009; Wölwer-Rieck 2012; Shivanna et al. 2013; Gawel-Beben et al. 2015). Substantial effort has been directed toward isolating and characterizing phenolic compound-rich fractions from stevia leaves and evaluating their potential use as multi-functional sources of phenolic compounds (Ghanta et al. 2007; Shukla et al. 2009; Shivanna et al. 2013; Gawel-Beben et al. 2015). This study describes the isolation of a stevia fraction (ASF) with a high phenolic compound content. The centesimal composition of the fraction was determined, and its main phenolics and flavonoids were identified through liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The fraction determined to have potential for fortifying foods was added to a whey protein isolate (WPI), and the developed supplement was tested for its antioxidant potential and antidiabetic effects.

WPI supplements constitute a rich source of proteins, such as β-lactoglobulin and α-lactoalbumin, peptone protease, immunoglobulins, bovine serum albumin, lactoferrin, and lactoperoxidase, and peptides, such as glycomacropeptide (GMP), which is an excellent source of branched-chain amino acids (BCAAs). When regularly added to the diet, these concentrated or isolated WPI proteins function as adjuvants for the treatment of diseases such as diabetes mellitus (DM). These compounds may promote insulin secretion and assist in metabolic control, antioxidant capacity and satiety (Sgarbieri 2004; Jakubowicz & Froy 2013; Mortensen et al. 2012; Ebaid 2014). The bioactive compounds in ASF can increase the

antioxidant or antidiabetic activities of these proteins, fortify their products and produce even more pronounced effects, especially relating to the metabolic complications of DM.

DM is an endocrine, metabolic disorder that affects thousands of people worldwide and is characterized by deficiency or absence of the production of insulin and its mode of action (Gaudel et al. 2013). This hormone is secreted by β-pancreatic cells and primarily acts in the liver tissue, muscle and adipose tissue to stimulate glycogen, lipid and protein synthesis and inhibit glycogenolysis, lipolysis and proteolysis. In addition to these metabolic effects, insulin plays a fundamental role in capturing glucose from adipose and muscular tissues and releasing it from the liver. Thus, diabetic patients present increased blood glucose levels and decreased antioxidant capacity, which may result in long-term hyperglycemia, further disorders, and even the failure of some organs (Koga et al. 2004; Ton et al. 2014).

DM control and management are complex and involve the oral hypoglycemic use of insulin and other strategies, such as lifestyle changes, mainly including the consumption of a balanced diet, to manage the disease. Hypoglycemic and natural antioxidant sources can be important coadjuvants in the treatment of diabetes (Milani et al. 2016; Gaudel et al. 2013).

Therefore, the objectives of this pioneering study were to detect and chemically characterize a phenolic compound-rich fraction of stevia leaf extract and to evaluate the potential use of this fraction to improve the antioxidant and antidiabetic activities of WPI and produce an effective supplement for DM treatment.

2. Material and Methods

2.1. Materials

ASF was obtained from *S. rebaudiana* leaves (seminal variety: Stevia UEM-13) cultivated at the Universidade Estadual de Maringá (UEM, Maringá, Paraná, Brazil). WPI was obtained from skimmed and pasteurized whey provided by the Flora Milk dairy (Flórida, Paraná,

Brazil). The membranes were made of polyethersulfone and polyamide from Koch and Millipore. The reagents used for the extraction, chemical and chromatographic analyses, adipocyte isolation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and streptozotocin were purchased from *Sigma*. The rodents' feed was obtained from *Nuvilab* (Colombo, Paraná, Brazil), and the specific kits for the plasma and serum dosages were obtained from Gold Analisa (Belo Horizonte, Minas Gerais, Brazil).

2.2. Obtaining the WPI from the milk whey

The WPI was obtained according to Milani et al. (2016) with modifications. The whey was concentrated by ultrafiltration (UF), diafiltration (DF) and nanofiltration (NF). Each concentrated sample was then dried by a *spray dryer*. The UF and DF processes were performed in a system with polyethersulfone filtering membranes (10-kD *cut-off*, 50-cm^2 area; *Koch*) in a spiral configuration, and 12 DF cycles were performed. NF was conducted using a reverse osmosis system composed of two polyamide membranes with cut-off molecular masses of 180 Da (Koch) and 500 Da (*Millipore*), both in a spiral configuration with a 50-cm^2 area. The WPI was dried in a *spray dryer* atomizer (*Büchi*, B-191) using an input temperature of 170ºC, an output temperature of 105ºC, and a flow of 8 mL/min.

2.3. Obtaining the ASF with antioxidant properties

Dry leaves (100 g) that had been previously ground were added to 500 mL of methanol and extracted using a Soxhlet apparatus for 4 hours. The extraction was repeated until a colorless methanolic extract was obtained, which then was filtered and evaporated in a rotary evaporator (Buchi) at 50ºC under vacuum. The resulting powder (35.8 g of dry methanolic extract) was hydrated with 400 mL of deionized water and subjected to fractionation with different solvents (hexane, chloroform, ethyl acetate and isobutanol). ASF obtained by

fractioning with ethyl acetate contained phenolic compounds and exhibited antioxidant activity; therefore, it was tested for biological activity and used for WPI fortification.

2.4. Protein supplement from whey with added ASF (WPI+ASF)

ASF (0.2%) was added to the WPI, and the proportion was determined according to the results obtained by Shivanna et al. (2013), who observed antidiabetic effects in experimental animals fed with feed containing 4% *S. rebaudiana* leaves.

2.5. Physicochemical analysis, bioactive compounds and in vitro antioxidant activity.

2.5.1. Whey and WPI

The total protein and lipids, lactose, mineral residues, humidity, pH and soluble solid contents were determined. The total protein and lipid analyses were performed following AOAC (1995) methodology. The lactose concentrations were obtained by high-efficiency LC connected to a refraction index detector (NH_2 5-μm column with dimensions of 150 x 4.6 mm; acetonitrile and water mobile phase [80:20 v/v]). The standard and the samples were prepared at a concentration of 0.5 mg/mL. The fixed mineral residue (ash), pH and soluble solid content (°Brix) were quantified according to the Instituto Adolfo Lutz methodology (2005). The WPI antioxidant potential was determined by measuring its 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability according to the Blios (1958) methodology. The standard used was gallic acid, and the data were expressed as inhibition percentages. All analyses were conducted in triplicate and were subjected to statistical treatment.

2.5.2. ASF

The proteins, lipids, fixed mineral residues and humidity concentrations were determined following the methodology mentioned in the previous section. The total glycoside concentration was measured using the method described by Dacome et al. (2005). The total phenolic compounds were determined according to Singleton, Orthofer & Lamuela-Raventos

(1999) and the Folin-Ciocalteu method; the data were expressed in gallic acid equivalents, which was used as a standard. The total flavonoid quantity was measured according to Jia et al. (1999), and the results were expressed in standard quercetin equivalents. The antioxidant potential was determined according to the methodology described in the previous section. All analyses were conducted in triplicate and were subjected to statistical treatment.

2.5.2.1. Identification of phenolic compounds

LC-MS and LC-MS/MS analyses were performed using an LC (Waters 1525µ) and spectrometer (*Quattro micro API* model) (Beverly, Massachusetts, USA) with a triple quadrupole (QqQ) mass analyzer, electrospray ionization, and a C18 column (250x4.6 mm; *Thermo Scientific*). The analyses were performed at 270 nm. The mobile phase consisted of water containing 0.1% of formic acid (solvent A) and acetonitrile containing 0.1% of formic acid (solvent B); the gradient was as follows: 0 min, 50% solvent A and 50% solvent B; 1 min, 30% solvent A and 70% solvent B; 2 min, 15% solvent A and 85% solvent B; 10 min, 5% solvent A and 95% solvent B; 12 min, 50% solvent A and 50% solvent B; and 15 min, 50% solvent A and 50% solvent B. The mass spectra were collected with ESI in positive or negative ion mode. The parameters were as follows: capillary voltage, 2.5 kV; cone voltage, 25 V; source temperature, 150°C; desolvation temperature, 250°C; gas caudal cone, 50 Lh-1; and electrovoltage, 30-40 eV. The compounds were identified based on the equipment databases and the following databases and literature: **The Metabolomics Innovation Centre (TMIC),** *METLIN: A Metabolite Mass Spectral Database* and *Plataform for RIKEN metabolomics* and literature data (Shivanna et al. 2013; Gawel-Beben et al. 2015).

2.5.3. Rodents feed

The total protein, total lipids, acidity and gross fiber in the feed provided to the experimental animals were determined in compliance with AOAC (1995) methodology. The ash and humidity were quantified according to the methodology described in section 2.5.1.

2.6. MTT assay of the cytotoxicity of ASF

The MTT assay to evaluate the cytotoxicity of ASF was conducted in adipose cells. The adipocytes were isolated according to Rodbell (1964), with some adjustments. Approximately 1 g of retroperitoneal adipose tissue from male Wistar mice was fragmented and added to 4 mL of digestive buffer (Dulbecco's modified Eagle's medium [DMEM]/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 25 mM; bovine serum albumin [BSA] fraction V, 4%; collagenase II, 1.25 mg/mL; pH 7.4) in a water bath at 37ºC with orbital shaking (90 rpm) for 90 minutes. Then, the digested tissue was filtered, placed in a conical flask and washed three times with 25 mL of GBT buffer (EARLE/HEPES [20 mM] containing BSA [1%] and sodium pyruvate [1 mM] without glucose; pH 7.4 at 37ºC). The infranatant was aspirated, resulting in a cellular suspension with approximately 3.0×10^6 cells/mL. In each well of a 96-well plate, 200-µL aliquots of GBT buffer (n=6) containing different ASF concentrations (1, 0.1, 0.01 and 0.001 mg/mL) were added. Then, 60 µL of the cellular suspension (1.8×10^5 cells) was added to each well and incubated for 60 minutes under 5% CO_2 atmosphere at 37ºC. Subsequently, the medium was replaced with 100 µL of MTT solution (0.5 mg/mL) and incubated (37ºC, 5% CO_2 atmosphere) for 120 minutes. Then, the MTT solution was replaced with 100 µL of dimethyl sulfoxide (DMSO) and shaken for 5 minutes, and the reading was performed at 540 nm. The data were expressed as viability percentages relative to the control.

2.7. Antidiabetic property evaluation

2.7.1. Experimental animals

The experimental protocol was approved by the UEM Ethics Conduct in Using Animals in Experimentation Committee (Protocol nº 8796250415). Male Wistar mice (60 days old) were obtained from the UEM Central Biotherium. The animals were stored in collective cages (46x24x20 cm; five animals per cage) or in individual metabolic cages and kept in the sectoral biotherium of the UEM Physiologic Science Department under the following conditions: 23ºC, 12-hour light/dark photoperiod, and water and feed (*Nuvilab*®, Colombo, PR) *ad libitum*.

2.7.2. Diabetes induction and experimental groups

After overnight fasting for 12 hours, the animals were sedated with sodium thiopental (40 mg/kg of p.c, i.p) to induce diabetes (streptozotocin, 40 mg/kg, v.i). On the third day after diabetes induction, the animals that showed fasting glycemia equal to or higher than 200 mg/dL and glycemia in the fed condition equal to or higher than 300 mg/dL were selected. The glycemic values and body weight were used to create four groups of diabetic animals that presented the same degree of diabetes severity before starting the feed supplementation. Five experimental groups were established with n=10 animals per group: one group of non-diabetic animals (ND=Non-Diabetic) and four groups of diabetic animals (DC=Diabetic Control, DW=Diabetic supplemented with WPI, and DSW=Diabetic supplemented with WPI+ASF).

2.7.3. Feed supplementation

Three types of feed supplements were used: 1) WPI (100 mg/kg), 2) ASF (0.2 mg/kg), and 3) WPI+ASF (0.2%) (100 mg/kg). The oral supplementation was performed daily at 08:00 AM for a 30-day period by gavage (the control diabetic animals group received pure water). The dose and supplementation period were established based on previous studies (Shivanna et al. 2013; Ma & Mu, 2016).

2.7.4. Physiological parameters evaluated

Body weight and glycemia: Every week the body weight, fasting glycemia and fed condition glycemia were recorded. The glycemia was determined in blood samples obtained from caudal puncture and analyzed using a glucometer from the *MediSence Optium*.

Glucose tolerance test (GTT): At the end of the treatment, after fasting overnight for 12 hours, the animals were submitted to oral GTT. After blood sampling (caudal puncture) for glycemia at time zero, the animals received an overload of glucose (1.5 g/kg, gavage). New blood samples were collected 15, 30, 60, 90 and 120 minutes after the glucose overload, and the glycemia was determined using a glucometer from *MediSence Optium*.

Twelve-hour glycemic curve: At the end of the treatment, blood sampling (caudal puncture) was performed at 6:00 PM after the animals had been subjected to daytime fasting (12 hours). Then, the animals received feed *ad libitum*, and new blood samples were collected at 20:00, 22:00 and 06:00 on the next day. The glycemia was determined using a glucometer from *MediSence Optium*.

Water and food intake and excreted urine volume: At the end of the treatment, the animals were put in individual metabolic cages, and their water intake, food intake and excreted urine volume were recorded over a 24-hour period.

Animal euthanasia: After fasting overnight for 12 hours, the animals were euthanized by anesthetic overload (sodium thiopental, 120 mg/kg, i.p.). After median laparotomy, blood samples were collected through the inferior vena cava, and then, the retroperitoneal and periepididymal fat deposits, gastrocnemius and soleus muscles, testicles, seminal vesicles, kidneys, liver and spleen were removed and weighed.

Biochemical dosage: Blood glucose concentrations, total cholesterol, high-density lipoprotein (HDL), triglycerides, aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

were determined using colorimetric methods (Gold Analisa®, Belo Horizonte, MG) and spectrophotometry (Bioplus2000®, São Paulo, SP). The 2,2-azinobis-3-ethyl-benzotiazolin-6-sulfonic acid (ABTS) radical was used to analyze the blood total antioxidant capacity (CAT) (Erel 2004).

2.8. Statistical Analysis

The results were presented as the average ± standard error of the mean (S.E.M) and were submitted to variance analysis using the Tukey test ($p<0.05$). The statistical program SAS (Statistical Analysis System 2006, version 9.1) and *GraphPad Prism* version 5.0® were used.

3. Results and Discussion

This study found unprecedented results that highlight the antidiabetic potential of a food supplement formulated from WPI fortified with ASF.

3.1. Physicochemical characterization of the obtained WPI

The whey used to produce the WPI had a soluble solid content of 4.4 ºBRIX and pH of 4.8. To obtain *whey* with a protein content of approximately 90%, 8 L of serum was ultrafiltered at 40ºC and then subjected to 12 cycles of DF and NF. The centesimal compositions of the whey and WPI are shown in **Table 1**. The procedure used was determined to efficiently concentrate the whey proteins to almost 90%, with only 7% of lactose. According to these results, the product complied with high-quality standards (Baldasso, Barros & Tessaro 2011; Patel 2015; Milani et al. 2016). WPI, whey protein concentrate (WPC) and whey protein hydrosylate (WPH) were previously shown to exhibit antioxidant properties (Patel et al., 2015). The WPI obtained in this study showed an antioxidant capacity of 41.7% (concentration: 1 mg/mL), similar to the value reported by Peng, Youling & Xiong (2009).

[Table 1]

3.2. Proximal composition of the obtained ASF

The physicochemical characteristics of the ASF are presented in **Table 2**. More than 50% of the proximal composition consisted of phenolic compounds, and almost 35% was proteins, with only 0.05% sweetener glycosides. These results suggest that the procedure used was efficient for obtaining the powder fraction with high antioxidant capacity (97.3%) without sweetener function. Wölwer-Rieck (2012), López et al. (2016) and Gawel-Beben et al. (2015) also reported the total phenolic and flavonoid compounds in stevia extracts and indicated that these compounds confer, together with other compounds, important antioxidant activities. **Table 2** also shows the main bioactive compounds (caffeic acid, quercetin-3-*o*-glycoside, cyanidin-3-glucoside, kaempferol, quercetin, apigenin, rozmarinic acid, chologenic acid and dicaffeoylquinic acid) identified by LC-MS (supplementary material). These compounds were also identified by Shivanna et al. (2013) and Gawel-Beben et al. (2015) in aqueous and alcoholic stevia extracts. ASF contained 52.42% phenolic compounds, and when it was added to WPI at a concentration of 0.2% (WPI+ASF), the antioxidant activity increased to 80%. This is the first study in which a fraction extracted from *S. rebaudiana* leaves was used for WPI fortification. The significant increase in antioxidant activity that resulted from the combination of WPI with ASF may make this material suitable as an antioxidant protein supplement with important applications in clinical nutrition.

[**Table 2**]

3.3. ASF cytotoxicity

The presence of antioxidants in plants and food is related to their beneficial effects for the prevention and treatment of several diseases and metabolic disorders (Krishnaiah et al., 2011). Previous studies have demonstrated that stevia extracts contain significant antioxidant concentrations, especially phenolics and flavonoids (Wölwer-Rieck 2012). However, high levels of antioxidants may increase the toxicity of these extracts, and thus, these extracts must be studied further. The toxicity of various steviol glycoside-rich stevia extracts has been

tested in different cellular models (Gawel-Beben et al., 2015). Nevertheless, studies evaluating the toxicity of extracts and fractions rich in other components, such as phenolics and flavonoids, in different cells remain lacking.

No significant differences (data not shown) in the viability percentage of the adipocytes incubated in the absence or presence of different concentrations of ASF (0.001, 0.01, 0.1 and 1 mg/mL) were found using the MTT assay. Concentrations up to a thousand times higher than those added to the WPI were used. Thus, ASF has potential applications in the fortification of food supplements.

3.4. Diabetic condition characterization

The lowest body weight gain **(Table 3)**, fasting hyperglycemia **(Figure 1A)** and hyperglycemia in the fed condition **(Figure 1B)**, excess excreted urine and water intake, and reduction in adipose tissue deposits **(Table 3)** were observed in the diabetic animal groups compared with those in animals from the non-diabetic group. These results confirmed the induction of diabetes by streptozotocin. This diabetogenic drug causes DNA alkylation and the death of ß-pancreatic cells and, consequently, reduced insulin secretion (Junod et al. 2009; Lenzen 2008).

[**Table 3]**

[Figure 1]

3.5. Feed composition

The functional effects of WPI, ASF and WPI+ASF supplementation were evaluated in diabetic mice fed with commercial mice feed containing 24% protein, 5.4% lipids, 6.5% minerals, 17.3% fiber, 10.4% moinsture and 1.9% acidity.

3.6. Evaluating the antidiabetic properties of food supplements

Body weight, adiposity and hydration: The evolution of the body weight during the supplementation period did not differ among the diabetic animal groups. However, animals that received ASF supplementation (the isolate alone [DS group] or added to the whey [DSW group]) showed significantly higher body weight gains (i.e., the difference between the final and initial weights) ($p<0.05$) than the control diabetic animals **(Table 3).** The retroperitoneal and periepididymal fat deposits were significantly larger in the three groups of diabetic animals that received supplementation than in the control ($p<0.05$). No significant differences were found in the food intake; however, the excreted urine volume and water intake were significantly smaller in the DSW group ($p<0.05$) **(Table 3).** No significant differences were found in the weights of the liver, kidney, spleen, testicles, seminal vesicles and gastrocnemius and soleus muscles (data not shown). These results showed that the three supplements were efficient in reducing the catabolic effects of streptozotocin-induced diabetes caused by the lack of insulin action and that DSW exhibited the best antidiabetic effect.

Glycemic homeostasis: **Figure 1A** shows the weekly fasting glycemia values (blood sampling at 6:00 PM after 10 hours of daytime fasting) during the supplementation period. The DSW group's AUC (área under the curve) values were significantly lower than those the other groups of diabetic mice ($p<0.05$); additionally, lower glycemia values ($p<0.05$) were recorded beginning at the 14th day of supplementation. The DW and DS groups also showed reduced fed condition glycemia but only from the 21st day of supplementation onwards. The glycemia in the fed condition (blood sampling at 08:00 AM with food *ad libitum* overnight) is shown in **Figure 1B**. No significant differences were recorded in the ASC values among the groups of diabetic animals, but beginning on the 14th day of supplementation, the DSW group showed

values that were significantly lower ($p<0.05$) than those of the DC group. Moreover, on the 21st day of the experimental period, all groups of diabetic mice that received food supplementation showed lower fasting glycemia values than the DC group ($p<0.05$). **Figure 2** presents the glycemia values during the oral GTT. The three groups of diabetic mice that received supplementation showed better glucose tolerance and significantly lower ASC values than the diabetic control group ($p<0.05$). The DW and DWS groups exhibited values that were significantly lower than the DC group ($p<0.05$) at all of the evaluated time points during the GTT. However, their values did not differ from those of the ND group, indicating that both were effective in restoring glycemic control, even after a glucose overload. The DS group's levels were significantly lower than those of the DC group at the 15-, 30- and 60-minute time points of the GTT, indicating that ASF exerts an important effect on glycemic homeostasis. The 12-hour (overnight) glycemic curves revealed that the diabetic animals that received supplementation had lower glycemic values at the four experimental time points (18:00, 20:00, 22:00 and 06:00); however, these differences were not significant relative to those of the DC group. The best glycemic control was observed in the diabetic animals that received supplementation and were in the fasting glycemia group. These results were recorded on the euthanasia day **(Table 4)** when the animals were subjected to a 12-hour (overnight) fast. The values recorded for the supplemented diabetic animal groups were significantly lower than those of the DC group ($p<0.05$), and therefore, the three administered food supplements effectively improved the glycemic control in diabetic mice. Notably, the DWS group presented lower glycemia than the DW and DS groups ($p<0.05$), suggesting the benefits of fortifying the WPI with ASF and indicating that the fortified supplement (WPI+ASF) had better functional quality and could potentially be used as an adjuvant in DM treatment and to prevent associated diseases. Previous studies have shown that whey protein can be an important functional food for diabetics (Milani et al. 2016; Patel 2015; Ebaid 2014;

Gaudel et al. 2013; Jakubowicz & Froy, 2013; Ton et al. 2014; Tong et al. 2011). Indeed, several studies have confirmed the hypoglycemic and antidiabetic properties of *S. rebaudiana* products (i.e., extracts and isolate glycosides) (Tadhani, Patel & Subhash 2007; Shivanna et al. 2013; Wölwer-Rieck 2012). The reduction in the glycemia levels determined in fasting and fed conditions in the DS group corroborate the results of these previous studies, demonstrating that stevia polyphenols effectively produce these effects. However, ASF that contains no glycosides cannot be considered to promote hypoglycemic activities (Shivanna et al. 2013; Wölwer-Rieck 2012).

Fructosamine: Another parameter that was related to the best glycemic control in the diabetic animals that received supplementation relative to the control group was the fructosamine plasma concentration. This value represents, in a generic way, the glycated proteins that become stable ketamines, which constitute the parcel most strongly linked to albumin. Thus, the blood fructosamine levels reflect the glycemic control in the past weeks because a direct relationship exists between hyperglycemia and the degree of blood protein glycation. The functional disorders resulting from protein glycation are diverse and are the main determinants of vascular diseases associated with DM (Koga et al. 2004). Significant reductions in the plasma fructosamine levels in the DW, DS and DWS groups were determined in relation to that in the DC group ($p<0.05$) (Table 4). Frid et al. (2005) observed reductions in the glycated hemoglobin rates in animals supplemented with whey. Ferri et al. (2006) obtained the same results in animals treated with *S. rebaudiana* products.

Lipidemia: The diabetic animals from the DW, DS and DWS groups presented the best lipid profiles and higher HDL values ($p<0.05$). The total cholesterol concentration was not changed by supplementation, but the triglyceride values in the DW and DWS groups were significantly reduced ($p<0.05$) (Table 4). Mortensen et al. (2012) reported decreased

triglycerides and total cholesterol levels in diabetic patients who received supplementation with different fractions of whey protein. Milani *et al.* (2016) found reductions in the triglyceride and total cholesterol concentrations in diabetic animals supplemented with whey protein sweetened with stevia.

Hepatic enzymes: The serum concentrations of AST and ALT decreased ($p<0.05$) in all diabetic groups that received supplementation (Table 4). Haraguchi et al. (2009) also observed beneficial effects on these parameters when whey supplements were used.

Antioxidant capacity: The *in vitro* antioxidant capacity was evaluated in relation to the DPPH radical scavenging abilities associated with WPI, ASF and WPI+ASF supplementation. The results revealed that WPI presented an antioxidant capacity of approximately 40%, whereas that of ASF was 97% (both were evaluated at the same concentration: 1 mg/mL). WPI+ASF increased the inhibition observed for WPI alone by 80% (at a concentration of 1 mg/mL). Thus, the concentration of the polyphenol-rich fraction tested here increased the WPI antioxidant potential, resulting in superior multi-functional effects in the group of animals that received supplementation (DWS). Ghanta et al. (2007) and Mondaca et al. (2012) also found results indicating that *S. rebaudiana* may be useful as a potential source of natural antioxidants. The *in vivo* CAT was also analyzed in plasma samples from all of the animal groups after the supplementation period, and the results are shown in **Table 4**. The findings corroborated the results of the *in vitro* test, indicating that ASF produced superior antioxidant effects in the animals from the DS group compared to those from the DW group. Furthermore, the plasma values of the DSW group were better than those of the DW group, proving that ASF has antioxidant potential in addition to antidiabetic properties. This study also suggested that the DS group may have exhibited the best metabolic conditions because of

the bioactives present in stevia leaves, which may exhibit higher antioxidant capacities than serum proteins. However, these properties muse be confirmed in future investigations.

4. Conclusions

We obtained WPI from whey through membrane separation and determined its centesimal composition. The main constituents of ASF obtained from Stevia UEM-13 leaves in a yield of 3.82% were phenolic compounds. The primary identified phenolic acids and flavonoids in ASF were consistent with reports in the literature. When 0.2% ASF was added to WPI, its antioxidant activity increased by 80%. Physiological tests revealed that the three supplements (ASF, WPI and WPI+ASF) contributed to improving the metabolic control of diabetic mice. Additionally, WPI+ASF was identified as food supplement with significant functionality, demonstrating that ASF has potential applications as a fortifier with antioxidant and antidiabetic activities that can be used to enrich foods or supplements.

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Table 1 - Proximate composition (g/100) the whey (W) and whey protein isolate (WPI)

Analyse	W	WPI
Total protein	1,61 ± 0,05	89,04 ± 0,02
Total lipids	0,18 ± 0,03	0,07 ± 0,01
Lactose	4,12 ± 0,02	7,01 ± 0,01
Humidity	93,2 ± 0,01	2,03 ± 0,01

Ash	0,90 ± 0,01	1,25 ± 0,01

Data were expressed by means ± S.E.M.

Table 2. Proximate composition (g/100) antioxidante stevia fraction (ASF) and identification by LCMS/MS of phenolics and flavonoids

Analyse	ASF
Total protein	34,5 ± 0,02*
Total lipids	1,00 ± 0,03
Glicosides	0,05 ± 0,01

Humidity	3,04 ± 0,01	
Ashes	2,24 ± 0,01	
Flavonoids	0,038 ± 0,01	
Phenolics	52,42 ± 0,03	
Compounds	RT	M/Z
Caffeic acid	4.78	163
Quercetin-3-*o*-glicoside	4.88	434
Cyanidin-3-glucoside	4.51	449
Kaempferol	5.54	287
Quercetin	5.93	303
Apigenin	6.27	271
Rosmarinic acid	6.58	361
Chologenic acid	4.90	353
Dicaffeoylquinic acid	5.33	515

RT= Retention Time; M/Z = Mass Spectrum. * Data were expressed by means ± S.E.M.

Table 3. Body weight gain (ΔP), weights of retroperitoneal fat (RF) and periepididymal (PF), water intake (WI), food intake (FI) and urine volume (UV) of non-diabetic and diabetic rats.

	ND	**DC**	**DS**	**DW**	**DSW**
ΔP (g)	91,2±10,6*	31,8±5,00**	63,5±13,2	50,5±9,70	59,8±9,70

RF (g/100g)	1,0±0,09*	0,42±0,03***	0,64±0,20	0,80±0,09#	0,54±0,04
PF (g/100g)	0,93±0,07*	0,16±0,02***	0,68±0,16	0,43±0,17	0,50±0,17
WI (mL/dia)	52,0±2,10*	149,1±5,40&	161,2±9,00	118,4±8,70	101,1±3,40
FI (g/dia)	39,44±7,40*	29,06±12,9	30,00±12,5	33,06±3,30	30,50±10,4
UV (mL/dia)	10,75±1,40*	70,75±3,60#	67,00±8,90	65,63±7,72	49,13±5,50

ND = Non-Diabetics; DC = Diabetic Control; DS = Diabetic Supplemented with ASF; DW = Diabetic Supplemented with WPI; DSW = Diabetic Supplemented with WPI + ASF. Data were expressed by means ± S.E.M. * differs from other groups (p <0.05); ** differs from DS and DSW (p<0.05); *** differs from DS, DW e DSW (p<0.05); & differs from DW e DSW; # differs from DWS (p<0.05).

Table 4. Plasma and serum parameters of non-diabetic and diabetic rats.

	ND	DC	DS	DW	DSW

GLI (mg/dL)	96,8 ± 2,5*	395,6±43,0**	240,7±24,0	213,3±11	192,9±9,80#
FTA (mmol/L)	1,00±0,06	1,8±0,06**	1,3±0,10	1,12±0,15	1,1±0,07
COL (mg/dL)	75,4±2,70	65,7±3,40	71,1±4,20	73,8±2,90	73,6±3,40
HDL (mg/dL)	39,8±1,50	35,3±0,90**	42,4±2,20	45,6±1,30	45,5±1,70
TGS (mg/dL)	43,7±1,50	70,1±8,70**	65,8±7,70***	36,3±2,50	34,7±4,50
ALT (U/L)	34,5±0,90*	67,4±3,20**	49,4±1,10	49,4±1,00	45,2±1,00
AST (U/L)	16,9±0,50*	75,5±3,20**	39,0±3,80	33,1±5,60	37,2±2,90
CAT (µM EQT)	202,4±11,3**	227,7±4,10**	394,1±21,0***	324,7±16,80	365,2±4,80

GLU = Glucose; FTA = Fructosamine; COL = Total Cholesterol; HDL = HDL Cholesterol; TGS = Triglycerides; ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase; CAT = Total Antioxidant Capacity; ND = Non-Diabetics; DC = Diabetic Control; DS = Diabetic Supplemented with ASF; DW = Diabetic Supplemented with WPI; DSW = Diabetic Supplemented with WPI + ASF. Data were expressed by means ± S.E.M. * differs from other groups (p<0.05); ** differs from DS, DW e DSW (p<0.05); *** differs from DW e DSW (p<0.05); # differs from other groups (p<0.05).

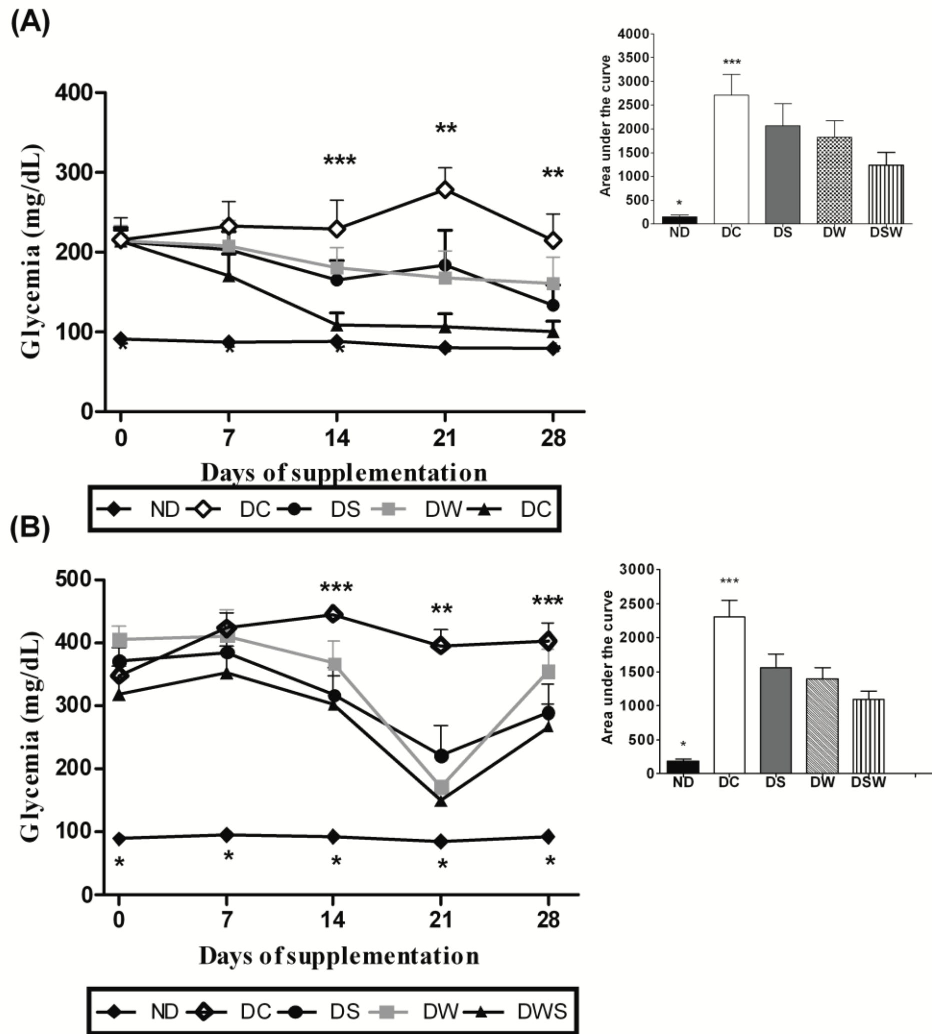

Fig. 1 Fasting glycemia (**a**) and in the fed state (**b**) of non-diabetic mice and diabetic mice. *ND* non-diabetics, *DC* diabetic control, *DS* diabetic supplemented with ASF, *DW* diabetic supplemented with WPI, *DSW* diabetic supplemented with WPI + ASF. Data were expressed by mean ± SEM. Detail of area under the curve (AUC). *differs from the other groups ($p < 0.05$); **differs from the other groups ($p < 0.05$); ***differs from DWS ($p < 0.05$).

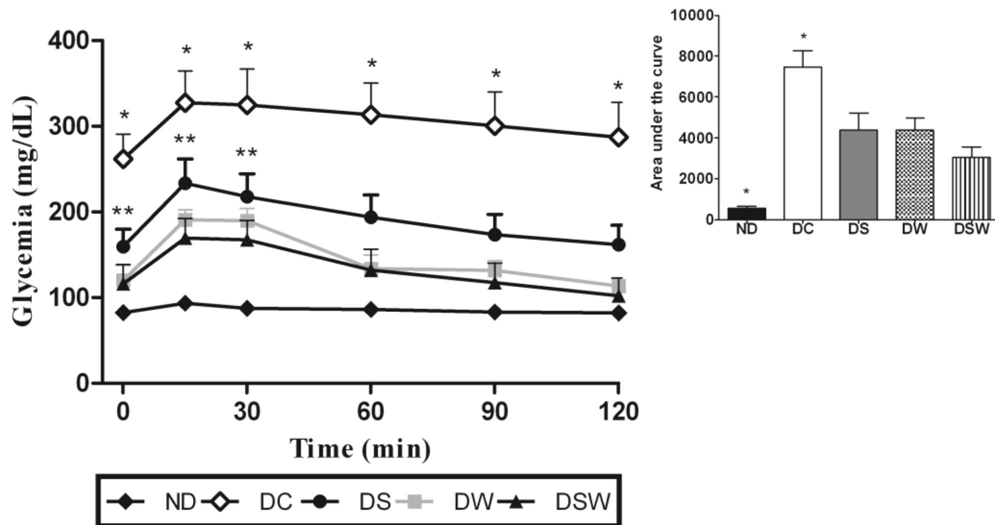

Fig. 2 Glycemia (mg/dL) during oral glucose tolerance test of non-diabetic mice and diabetic mice. *ND* non-diabetics, *DC* diabetic control, *DS* diabetic supplemented with ASF, *DW* diabetic supplemented with WPI, *DSW* diabetic supplemented with WPI + ASF. Data were expressed by mean ± SEM. Detail of area under the curve (AUC). *differs from the other groups ($p < 0.05$); **DS differs from the other groups ($p < 0.05$).

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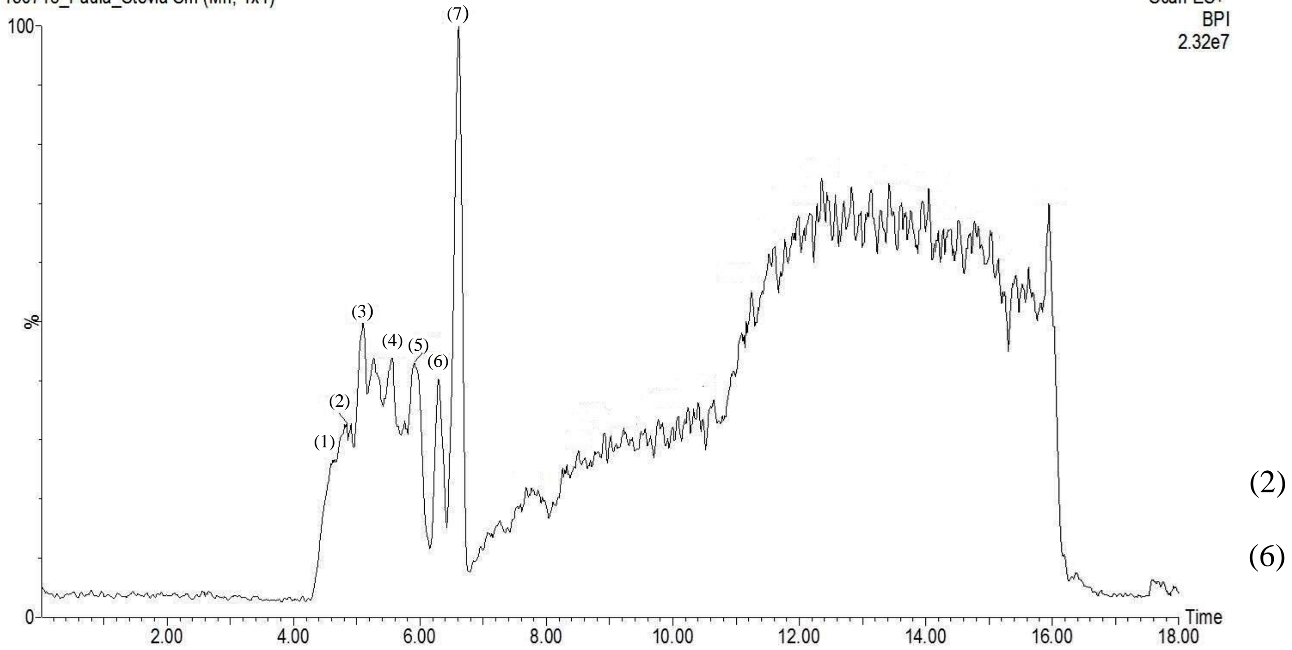


Analise Acetato de Etila Stevia

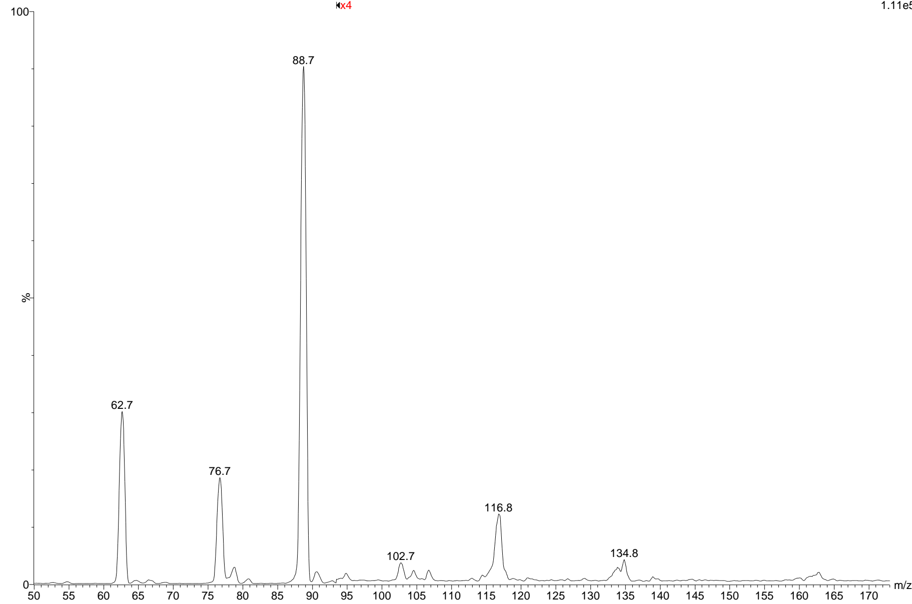


Fig. 4: Caffeic acid

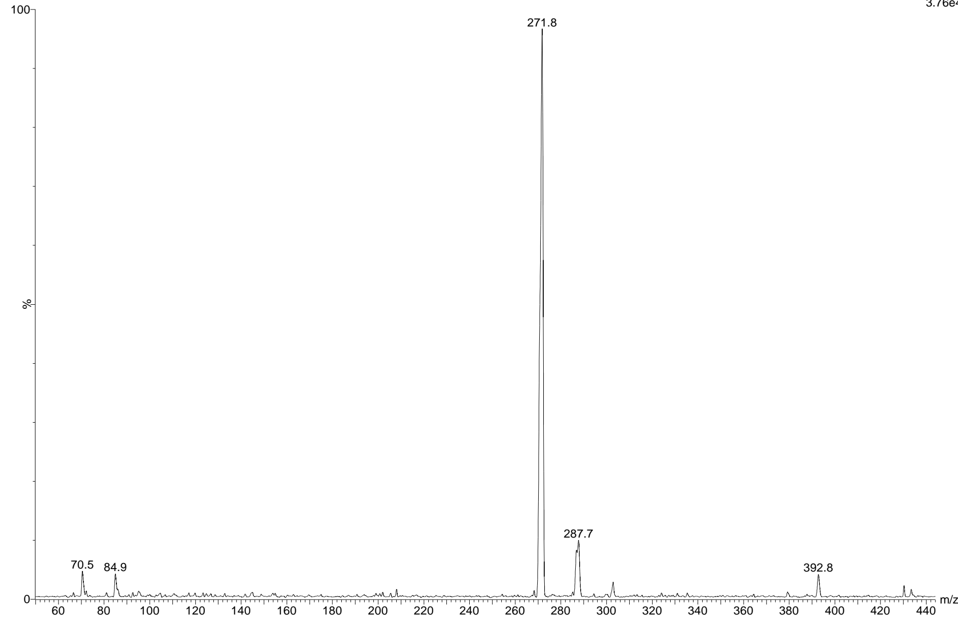


Fig. 5: Quercetin-4-O-glucoside.

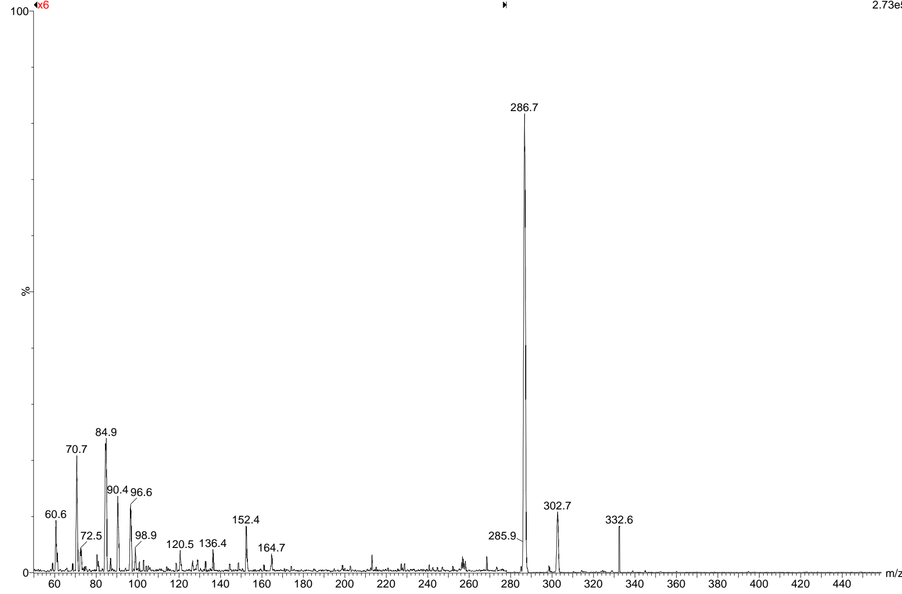


Fig. 6: Cyanidin-3-glucoside.

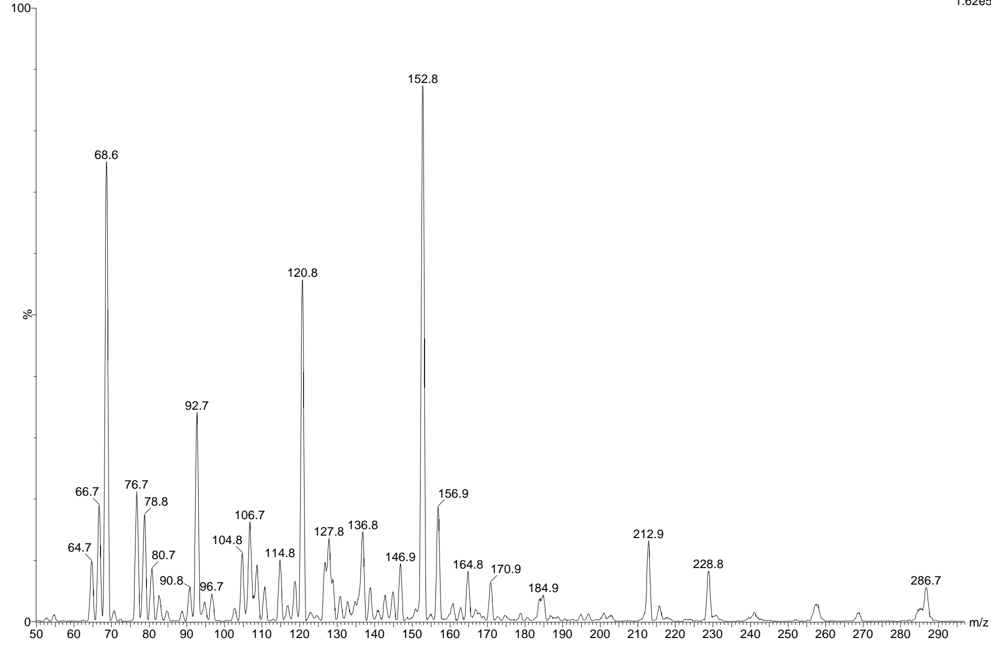


Fig. 7: Kaempferol.

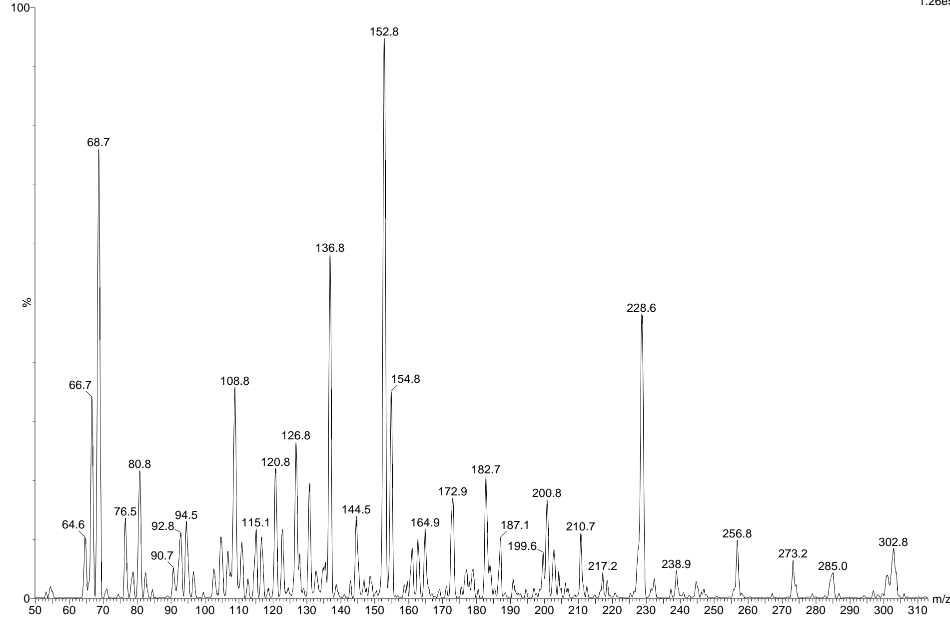


Fig. 8: Quercetin

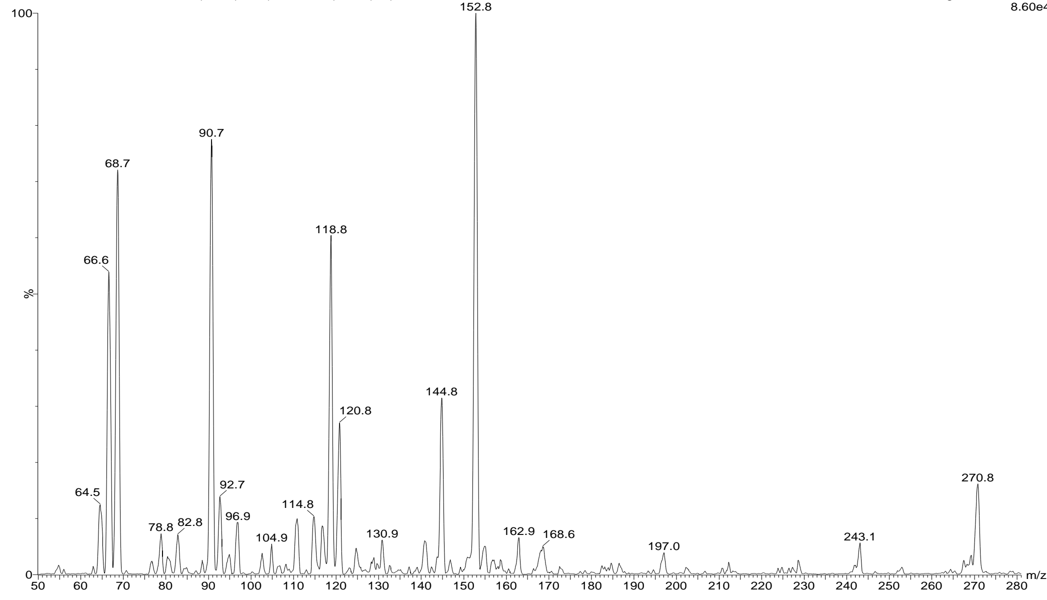


Fig. 9: Apigenin

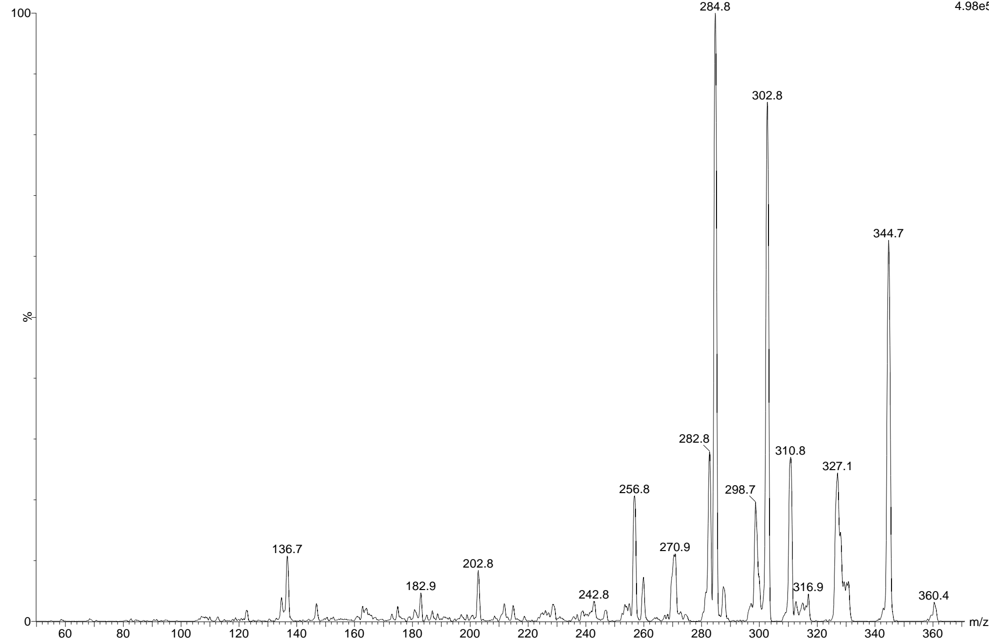
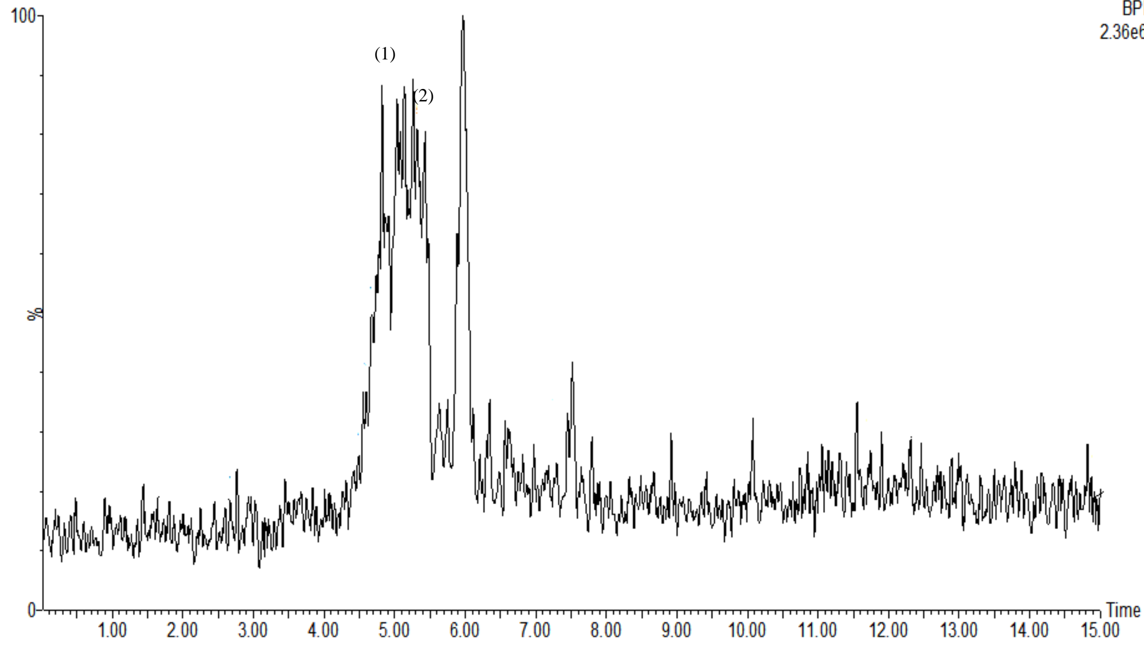


Fig. 10: Rosmarinic acid.



acid. (2)

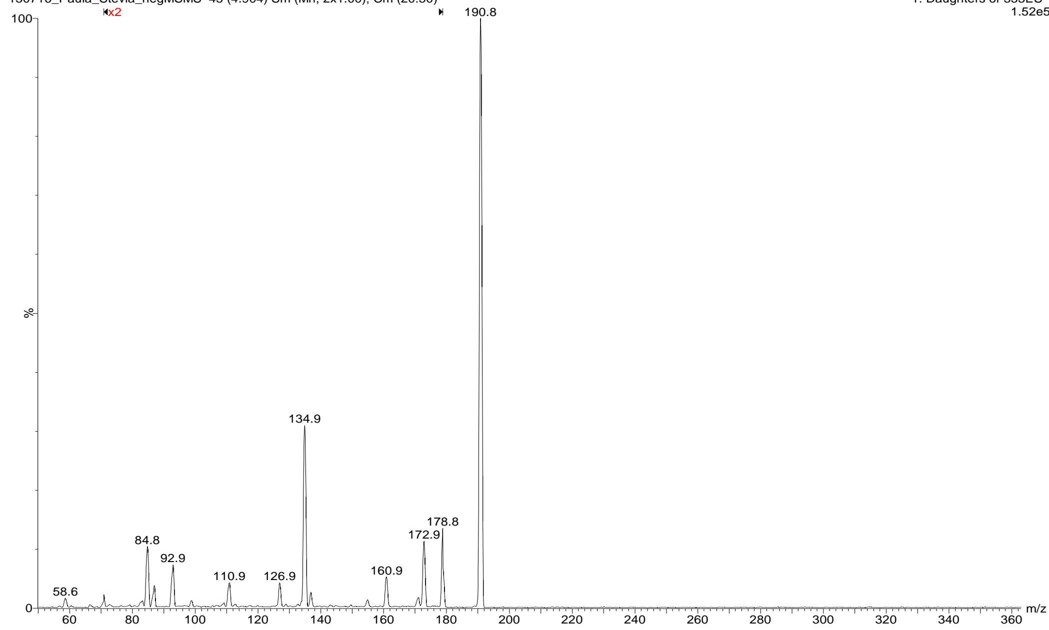


Fig. 12: Chlologenic acid.

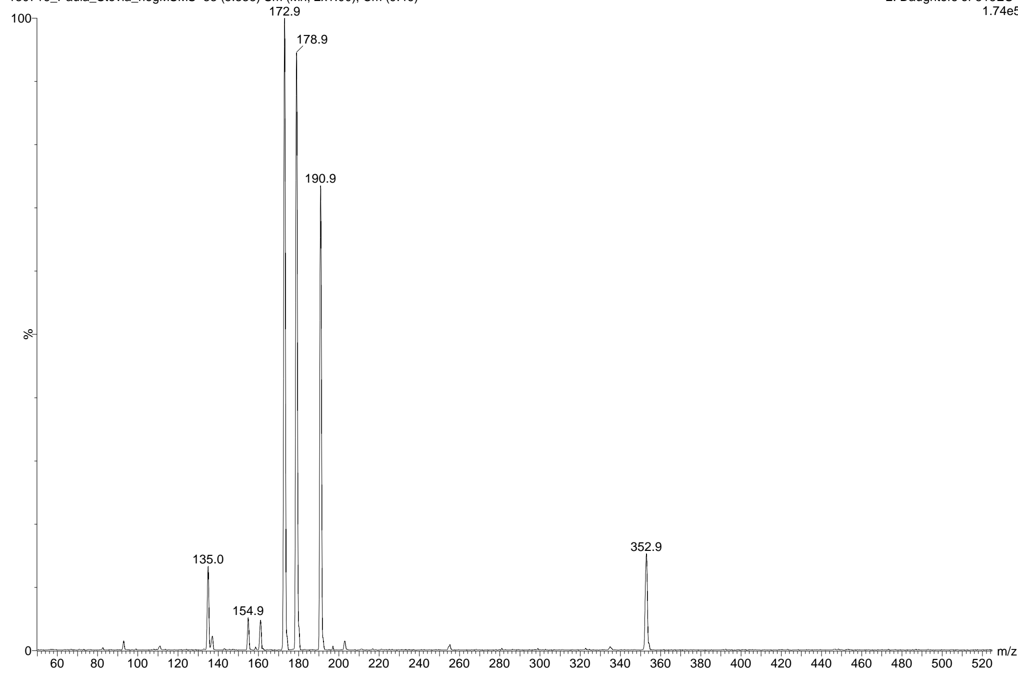


Fig. 13: Dicaffeoylquinic acid.