UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

POTENCIAL INIBITORIO IN VITRO DE BIFLAVONOIDES DE *Garcinia gardneriana:* UM ESTUDO SOBRE MONOAMINA OXIDASES E CYP19 (AROMATASE)

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Dissertação apresentada por **Maria Angélica Recalde Gil** para obtenção do GRAU DE MESTRE em Ciências Farmacêuticas

Orientador(a): Profa. Dra. Amélia Teresinha Henriques.

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RESUMEN

La planta *Garcinia gardneriana* (Planch. & Triana) Zappi, popularmente conocida en Brasil como "bacupari" ha sido tradicionalmente usada para varios tipos de enfermedades inflamatorias y la evaluación de su composición química, principalmente de las hojas, ha resultado en biflavonoides como compuestos mayoritarios. Estos compuestos fenólicos han demostrado actividad anti-inflamatória validando el uso popular de la planta.

En este trabajo se asilaron a partir de tallos secos de la *Garcinia gardneriana* los biflavonoides: moreloflavona, que consiste en una naringenina unida covalentemente a luteolina, Gb-2a que es un compuesto que consiste en una naringenina unida a un eriodictyol y Gb-2a-7-*O*-glucose. Estos compuestos ya han sido previamente evaluados en diversas actividades como anti inflamatorios y anti antioxidantes pero no se tiene reporte de su actividad como inhibidores enzimáticos. Sin embargo, los monomeros que los conforman han sido evaluados en la inhibición de la aromatasa y con resultados positivos como en la actividad antidepresiva, para la cual comúnmente son usados los inibidores de MAO-A.

En el proceso de aislamiento también fueron encontrados compuestos terpenoides como lupeol y friedelina.

Los biflavonoides aislados y purificados se usaron para evaluar la inhibición enzimática "*in vitro*" en monoaminooxidasas (MAO-A, MAO-B) y aromatasa. Los compuestos presentaron una respuesta positiva calculada con IC_{50} de hasta 5,47 µM y 1,35 µM para la inhibición de las enzimas MAO-A y aromatasa respectivamente, abriendo el camino a una nueva propuesta de relacionar estas dos enzimas para tratamiento de cánceres hormonodependientes y transtornos de ansiedad y depresión.

Palabras clave: Garcinia gardneriana, biflavonoides, aromatasa, monoaminooxidasa, inhibidores.

ABSTRACT

The plant Garcinia gardneriana (Planch. & Triana) Zappi, popularly known in Brazil as "bacupari" has traditionally been used for various types of inflammatory diseases and the evaluation of their chemical composition, mainly of leaves, has resulted in biflavonoids as major compounds. These phenolic compounds have shown anti-inflammatory activity validating the popular use of the plant.

In this work was isolated from dried branches of *Garcinia gardneriana* the biflavonoids: morelloflavone, that is an naringenin covalently linked to luteolin, Gb-2a which is an naringenin linked to eriodictyol and Gb-2a- 7-O-glucose. These compounds have been previously evaluated in various activities such as anti-inflammatory and anti-antioxidants but there is no report of its activity as enzymatic inhibitors. However, the monomers that form it, have been evaluated in the inhibition of aromatase and antidepressant activity with positive outcome, which commonly are used MAO-A inhibitors.

In the isolation process were also founded terpenoid compounds as lupeol and friedelin

The isolated and purified biflavonoids were used to evaluate enzyme inhibition "in vitro" in monoamine oxidases (MAO-A MAO-B) and aromatase. The compounds showed a positive response even of IC50 5,47 µM and 1,35 µM for MAO-A inhibition of and aromatase enzyme respectively; discovering a way for a new proposal to link both enzymes for treatment of hormone-dependent cancers and anxiety and depression disorders.

Keywords: Garcinia gardneriana, biflavonoids, aromatase, monoamine oxidase inhibitors.

CONTENIDO

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1. INTRODUCCION GENERAL

La investigación de metabolitos obtenidos a partir de fuentes naturales como plantas, animales y microorganismos, tiene un nuevo enfoque para el desarrollo de fármacos. El interés en estos productos es debido principalmente a la complejidad química estructural y capacidad de interactuar con centros biológicos. Esto puede ser medido a partir de datos que indican que aproximadamente el 50% de los fármacos aprobados por la FDA entre 1981 a 2010 eran productos naturales o compuestos sintéticos derivados de estos (Newman Y Cragg, 2012). Sin embargo, el levantamiento de los medicamentos aprobados para la comercialización en todo el mundo entre los años 2005 y 2010 mostró que sólo 19 medicamentos eran productos naturales o derivados de estos (Mishra Y Tiwari, 2011).

Garcinia gardneriana (Planch. & Triana) Zappi, es una angioesperma, popularmente conocida como "bacupari", y nativa de la familia clusiaceae que es tradicionalmente usada para tratamiento de infecciones, dolores y varios tipos de inflamación. En su composición química, diversas xantonas, esteroides, triterpenos y biflavonoides ya han sido descritos, a partir de sus hojas, estos últimos demostrando actividad anti-inflamatoria (Correa et al., 1984).

Varios estudios han determinado que ciertos flavonoides y compuestos sintéticos relacionados son potencialmente inhibidores de la MAO, demostrando también otros efectos beneficiosos para prevenir procesos degenerativos. Ya se ha descrito las propiedades IMAO (inhibidores de la monoamina oxidasa) de jaceosidina, eupafolina, luteolina y apigenina (Chimenti et al., 2010), sin embargo, no hay ningún reporte de esta actividad concerniente a biflavonoides.

Algunos flavonoides son conocidos por eliminar los radicales formados bajo una serie de condiciones, incluyendo anoxia, la oxidación de lípidos y la inflamación. Los flavonoides también pueden formar quelatos de iones multivalentes tales como Fe^{3+} y Al^{3+} (Gabor et al., 1988).

Además, los compuestos fenólicos, entre estos los flavonoides, pueden presentar también importantes actividades como inhibidores de diversas enzimas, entre esas la

monoamina oxidasa (MAO). En un estudio se demostró que la expresión de la monoamina oxidasa-A (MAO-A) puede contribuir al crecimiento de cáncer de alto grado y que las drogas antidepresivas para inhibición de MAO-A podrían tener aplicaciones en el tratamiento del cánceres seleccionados, incluyendo de próstata.(Peehl et al., 2008) lo que indica otro camino de estudio para la prevención o tratamiento de cáncer, ya que los cambios en la expresión de MAO-A pueden representar un biomarcador potencial de cánceres, profundizando la inhibición de MAO con otras enzimas relacionadas con crecimiento tumoral como la aromatasa que está asociada con los canceres de mama y próstata.

2. OBJETIVOS

2.1 OBJETIVO GENERAL

Teniendo en cuenta la información previa, el objetivo general de este trabajo fue la obtención de compuestos a partir de los extractos de las ramas secas de Garcinia gardneriana y su evaluación en la actividad inhibitoria *in vitro* frente a enzimas relacionadas a las enfermedades neurodegenerativas (MAO) y enzimas relacionadas con los canceres hormonodependientes (aromatasa)

2.2 OBJETIVOS ESPECIFICOS

- Obtención de los extractos a partir de ramas secas de la especie.
- Evaluación de los extractos por técnicas cromatográficas.
- Obtención de los compuestos aislados y purificados.
- Caracterización de los compuestos.
- Realización de ensayos de inhibición enzimática frente a las isoformas A y B de la monoamina oxidasa.
- Realización de ensayos de inhibición enzimática frente a la aromatasa (CYP19)

3. REVISIÓN

3.1 BIFLAVONOIDES COMO COMPUESTOS ACTIVOS

Los compuestos naturales de plantas medicinales son recursos importantes para el desarrollo de fármacos. Recientemente, una considerable atención se ha centrado en fitoquímicos dietéticos y medicamentos que inhiben, invierten o retardan las enfermedades causadas por procesos inflamatorios y oxidativos. Los esfuerzos están enfocados en aislamiento y determinación de moléculas para nuevos fármacos, entre los que sobresalen el grupo de polifenoles. Las actividades biológicas de polifenoles sobre las plantas, así como los mecanismos de protección contra enfermedades cardiovasculares y neurodegenerativas, el cáncer y la diabetes, podría atribuirse a estos procesos inflamatorios y oxidativos (Fidelis, et al., 2014)

El primer biflavonoide natural, fue aislado en 1929 por Furukawa de *Ginkgo biloba* L. (Lin et al., 1997) como un pigmento amarillo, que más tarde fue nombrado ginkgetina, el cual ha sido evaluado en varias actividades biológicas. Aunque existen diversas actividades evaluadas de los biflavonoides de diversas plantas, aún restan muchas actividades a ser evaluadas por estos metabolitos.

Las partes más usadas para estudios tanto químicos como biológicos en las plantas son las hojas, popularmente usadas para infusiones, por ser la parte de la planta más fácil de recolectar y la de menor destrucción. Sin embargo la presencia de estos metabolitos está distribuida en toda la planta en diferentes proporciones (Fidelis, et al., 2014) y por lo tanto en estudios fitoquímicos debe considerarse las distintas partes de la planta para identificación de nuevos metabolitos.

Por otra parte, los estudios del genero *Garcinia* han revelado su riqueza en biflavonoides y estos, a su vez han demostrado gran variedad de actividades biológicas y enzimáticas. De esta manera, se realizó en este trabajo el aislamiento de polifenoles a partir de tallos secos de *G. gardneriana* , para dar paso a la investigación de actividades *in vitro*, sobre blancos enzimáticos. El resultado de la evaluación biológica y enzimática

de biflavonoides podría servir para nuevas propuestas de potencial fitomedicina y suplementos dietarios para la prevención de diversas enfermedades.

Varios biflavonoides han sido destacados por su potente actividad en el proceso anti inflamatorio, y aunque, una respuesta excesiva a este proceso hace que la morbilidad y la mortalidad aumente en las enfermedades con un componente inflamatorio importante tales como la artritis reumatoide, enfermedad de Crohn y la enfermedades como Alzheimer, esclerosis múltiple, cerebro y la isquemia del corazón, no se ha reportado toxicidad de estos compuestos (Pereañez, et al., 2014)

3.2 BIFLAVONOIDES Y ACTIVIDADES BIOLOGICAS.

Algunos biflavonoides, como la morelloflavona, muestran actividades biológicas importantes, tales como antitumoral, antiviral, de vasodilatación, antimicrobiana, (Fidelis et al., 2014) y posee actividad antioxidante demostrada en varios sistemas experimentales (Gil y Sanz et al., 1997)

Los biflavonoides morelloflavona y Gb-2a aislados de las hojas de *Garcinia gardneriana* han sido intensamente evaluados en actividades anti-inflamatorias que confirman el uso popular de la planta y la convierte en una fuente primaria de metabolitos para medicamentos de uso anti-inflamatorio, ya que también han sido probados “in vivo”. (Castardo et al., 2008). Gb-2a es un biflavonoide común en el género *Garcinia* que consiste en una naringenina unida a un eriodictyol. (Fig.1. 4)

Moreloflavona es un compuesto, que consiste en una naringenina (Fig.1. 1) unida covalentemente a luteolina (Fig.1. 2). Posee además, una amplia gama de actividades biológicas y enzimaticas (Tabla 1). De este biflavonoide se tienen reportes de diversas actividades biológicas, como efectos antiaterogénicos (Decha-Dier et al., 2008), angiogénesis antitumoral (Pang et al., 2009), inhibición potencialmente los hongos mediante el bloqueo de la actividad de ácido graso sintasa (FAS), inhibición de la cepa

Plasmodium falciparum W2 resistente a la cloroquina y otros fármacos (Ngouamegne et al., 2008)

El biflavonoide amentoflavona, un compuesto que consiste en una apigenina (Fig. 1.3) unida a una luteolina, ha demostrado actividad inhibitoria sobre el coronavirus s (CoV), SARS-CoV, causante del síndrome respiratorio agudo (Ryu et al., 2010), incluso con mayor efectividad que sus flavonas correspondientes.

La ginkgetina, un biflavonoide que también posee una luteolina como uno de sus componentes, ha demostrado actividad anti inflamatoria, anti-viral en la influenza, actividad anti fúngica e inibición en células de cáncer de próstata PC-3 (You et al., 2013) aunque el mecanismo subyacente de la ginkgetina como anti tumoral sigue siendo poco claro.

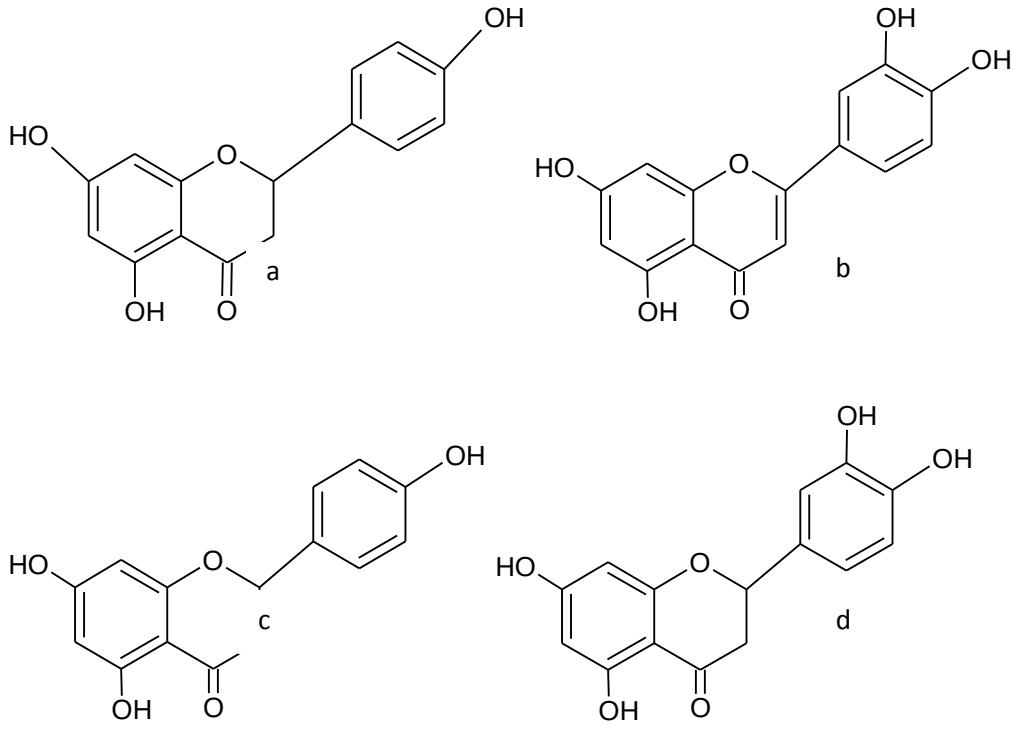


Fig. 1. (a)Naringenina, (b) Luteolina, (c) Apigenina, (d) Eriodictyol

3.3 ACTIVIDAD ENZIMATICA

En una revisión de actividades enzimáticas por biflavonoides puede encontrarse unos con mayor destaque, como la moreloflavona, que además de sus diversas actividades biológicas ya descritas, ha demostrado inhibición de la topoisomerasa de ADN (Fidelis et al., 2014), también inhibe fuertemente la tirosinasa, la principal enzima responsable de melanizacion en la piel (Masuda et al., 2005), además de las mencionadas en la tabla 1.

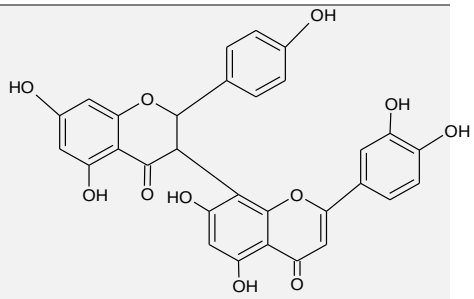
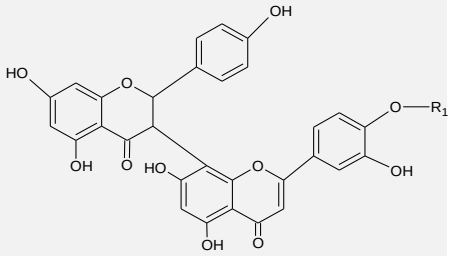
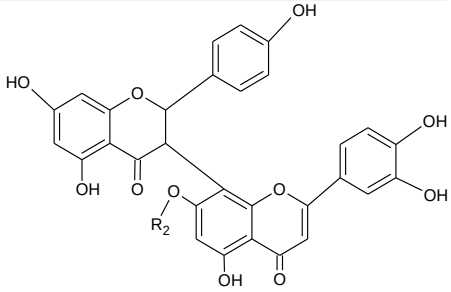
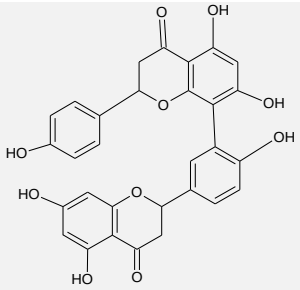
La atención ha sido creciente en derivados de este tipo de compuestos, ya que su actividad biológica ha demostrado resultados, como el caso del derivado semi sintetico Morelloflavona-7,4´,7´´,3´´´,4´´´ penta-O-acetil que mostró una fuerte actividad inhibidora contra la enzima cisteína proteasas de Leishmania y otras dos isoformas (Gontijo et al., 2012) y puede representar un medicamento innovador candidato para el tratamiento de la leishmaniosis.

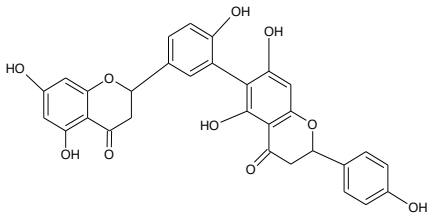
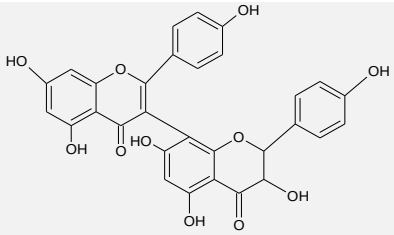
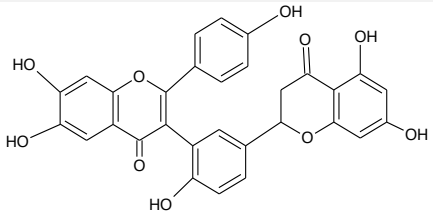
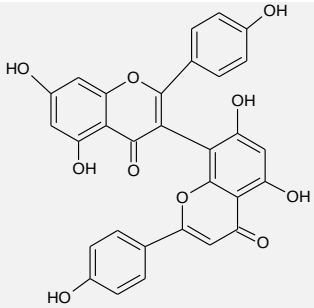
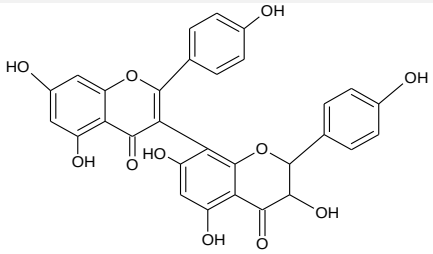
No es frecuente encontrar biflavonoides con reporte de actividad inhibitoria de la aromatasa, pero, las flavonas naringenina, apigenina, y luteolina (Balunas et al., 2011), correspondientes a monómeros de biflavonoides aislados de *Garcinia gardneriana* han sido los más activos en esta actividad, con valores aproximados de IC50 de 2,9 µM, 1,2 µM, y 1,2 µM respectivamente

El biflavonoide GB1, asilado de *Garcinia kola*, es el único biflavonoide expuesto en la literatura, que ha sido evaluado en atividad inhibitoria de la aromatasa, exhibiendo actividades inhibidoras de la la aromatasa, (IC_{50} 11,3µM) y también de la α-glucosidasa (IC_{50} 0,9µM) aunque no resultó tóxico en las líneas celulares tumorales ensayadas. (Antia et al., 2010)

Algunas actividades inhibitorias enzimáticas de biflavonoides aislados de diferentes de plantas se presentan a continuación en la tabla 1.

Tabla 1. Actividades de inhibición enzimática de biflavonoides

BIFLAVONOI DE NATURAL	PLANTA	ACTIVIDAD DE INIBICIÓN	REFERENCIA	ESTRUCTURA
Morelloflavone	*Garcinia madruno*	fosfolipasas A2 desde sinovial humano y la fuente de veneno de abeja	Pereañez, et al., 2014)	
	G. multiflora	VIH-1 (tipo LAV-1)	(Lin et al., 1997)	
	G.dulcis	3-hidroxi-3-metil-glutaril-CoA reductasa (HMG-CoA)	(Tuansulong, et al., 2011)	
	G. brasiliensis	r-CPB2.8, cisteína proteasa recombinante tipo 2.8r-CPB3, cisteína proteasa recombinante tipo 3	(Gontijo et al., 2012)	
Morelloflavone-4'''-O-β-D-Glycosyl	*G. brasiliensis*	r-CPB2.8, cisteína proteasa recombinante tipo 2.8 r-CPB3, cisteína proteasa recombinante tipo 3	(Gontijo et al., 2012)	 R_1. Glucosa
Morelloflavone-7″-O-β-D-Glycoside o Fukugiside	*G. brasiliensis*	r-CPB2.8 Y r-CPB3	(Gontijo et al., 2012)	 R_2. Glucosa
Amentoflavona	*Selaginella tamariscina*	proteína tirosina fosfatasa 1B	(Nguyen et al., 2015)	

Robustaflavona	*S. tamariscina*	proteína fosfatasa 1B	tirosina	(Nguyen et al., 2015)	
Cupressuflavona	*S. tamariscina*	proteína fosfatasa 1B	tirosina	(Nguyen et al., 2015)	
Taiwaniaflavona	*S. tamariscina*	proteína fosfatasa 1B	tirosina	(Nguyen et al., 2015)	
3,8''-Biapigenina	*S.tamariscina*	proteína fosfatasa 1B	tirosina	(Nguyen et al., 2015)	
GB1	*Garcinia kola*	*α*-Glucosidasa,y Aromatasa		(Antia et al., 2010)	

4. MANUSCRITO I

Manuscrito sometido a Arch. Pharm. Chem. Life Sci

Monoamine oxidase inhibitory activity of the biflavonoids obtained from branches of *Garcinia gardneriana* (Clusiaceae)

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ABSTRACT

Garcinia gardneriana is chemically characterized by the presence of biflavonoids. Taking into account that flavonoids are able to inhibit monoamine oxidase (MAO) activity, in the present study, the chemical composition of the branches' extract of the plant is described for the first time and the MAO inhibitory activity of the isolated biflavonoids was evaluated. Based on spectroscopic and spectrometric data, it was possible to identify volkesiflavone, morelloflavone (1), Gb-2a (2) and Gb-2a-7-O-glucose (3) for the ethyl acetate fraction of the ethanol extract of the branches. Compounds 1-3 were evaluated in vitro and demonstrated the capacity to inhibit MAO-A activity with an IC50 ranging from 5.05 to 10.7 µM, and from 20.7 to 66.2 µM for MAO-B. These inhibitions corroborate with previous IC50 obtained for monomeric flavonoids, with a higher selectivity for MAO-A isoform. The obtained results indicate that biflavonoids might be promising structures for the identification of new MAO inhibitory compounds.

KEY WORDS: *Garcinia gardneriana*, biflavonoids, monoaminoxidase inhibitors

INTRODUCTION

Several biological activities had already been reported for different species of *Garcinia* L., such as anti-inflammatory, antioxidant, and antitumoral (Acuña, et al., 2012)(Ayepola et al., 2014). *Garcinia gardneriana* (Planch. & Triana) Zappi (Guttiferae) is a tree widely distributed on the Brazilian Atlantic Forest biome and easily cultivated (Castardo et al., 2008). It is popularly known as "bacupari" in the South of Brazil and it is traditionally used for the treatment of infections and various types of pain and inflammation (Campos et al., 2013). Regarding the chemical composition, different classes of secondary metabolites had already been isolated from diverse organs of the species, including terpenoids, steroids and phenolic compounds. Concerning the leaves, biflavonoids core compounds have been described, including volkensiflavone, GB-2a, morellofavone (or fukugetin) and fukugeside (Luzzi et al., 1997).

Some flavonoids are known to eliminate the radicals formed under a number of circumstances, including anoxia, lipid oxidation and inflammation. Flavonoids may also chelate multivalent ions such as Fe^{3+} and Al^{3+} (Rodrigues et al., 2015). The presence of a significant amount of these ions contributes to the development of neurodegenerative diseases. Therefore, the elimination of free radicals, chelation of metal ions and inhibition of specific enzymes such as monoamine oxidase (MAO) can contribute to the neuroprotective properties of compounds in neurodegenerative situations (Sloley et al., 2000).

MAO-A inhibitors are used in the treatment of depression and anxiety disorders, while MAO-B inhibitors are used for the symptomatic treatment of Parkinson's Disease, such as the N-propargyl drugs (Song et al., 2013). Both isozymes seem to also be involved in the etiology of Alzheimer's Disease (AD). There are some reports of increased MAO-B activity in the hippocampus and cerebral cortex of patients with AD. Changes in MAO-A appear to be more complex, since increased enzymatic activity or increased expression of mRNA are observed in a large number of brain regions, including the neocortex of the frontal lobe, the parietal cortex, occipital cortex, temporal cortex and frontal cortex (Kennedy et al., 2009).

Several studies have determined that certain flavonoids and related synthetic compounds are potentially MAO inhibitors, also demonstrating other beneficial effects to prevent degenerative processes. It has already been described the MAO inhibitory properties of luteolin (MAO-A IC_{50} 4.9 µM, MAO-B IC_{50} 59.7 µM), apigenin (MAO-A IC_{50} 1.7 µM, MAO-B IC_{50} 12.8 µM) and quercetin (MAO-A IC_{50} 2.8 µM, MAO-B IC_{50} 90.0 µM) (Chimenti et al., 2010). The flavonoids quercitrin, isoquercitrin, rutin and quercetin isolated from *Melastoma candidum* D. were also evaluated as MAOIs with IC_{50} values for MAO-B of 6.19, 11.64, 10.89 and 3.89 µM respectively. These flavonoids also demonstrated antioxidant activity (Mei-Hsien Lee et al., 2001).

Taking into account that *Garcinia* biflavonoids had already demonstrated antioxidant properties and that several monomeric flavonoids display MAO inhibitory potential, this study deals with the evaluation of the effect of a *G. gardneriana* biflavonoid enriched fraction and isolated biflavonoids on MAO-A and -B activities. Moreover, the

chemical characterization of the extract and isolated biflavonoids are achieved by HPLC-DAD, UPLC-DAD-MS and NMR analysis.

MATERIALS AND METHODS

Chemicals

Kynuramine dihydrobromide, pargyline hydrochloride, clorgyline hydrochloride, pentadecane and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human MAO-A and MAO-B Supersomes™ were acquired from BD Gentest (Woburn, MA, USA). Diethyl ether was acquired from Tedia Company, Inc. (Fairfield, OH, USA). All remaining chemicals used were of analytical grade and were purchased from Sigma-Aldrich. Buffer was prepared using Milli-Q water.

Plant material

Garcinia gardneriana was collected in Taquara/RS (29º39'02"S 50º46'50"W). The species was identified by Dr. Sérgio Augusto de Loreto Bordignon and a voucher was deposited at the herbarium of the Departmento de Botânica of the Universidade Federal do Rio Grande do Sul under the number ICN 180888

Chemical extraction and isolation

Ethanol extraction 1:10 was performed by maceration of the dried branches (82g) of the tree, three consecutive times for periods of 48 hours. This extract was suspended in water and partitioned with hexane, dichloromethane and ethyl acetate. At the end, each solvent was evaporated by rotary evaporator under vacuum.

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Two biflavonoids were isolated from the ethyl acetate fraction (4,99g). This fraction was subjected to column chromatography using silica gel as stationary phase. As eluent, a gradient of dichloromethane/methanol was used, ranging from 100:0 until 0:100. Fractions obtained of this column (F2 and F3) were again submitted to column chromatography. Compound **1** was isolated with 2.0% of yield by column chromatography from fraction F3 using a triphasic gradient of dichloromethane/acetone/methanol. F2 was submitted to column chromatography using a gradient solvent of dichloromethane/acetone obtaining the fraction 2-82. Finally, the fraction 2-82 (107mg) was recromatographed using an isocratic elution of dichloromethane/ethyl acetate/methanol (70:15:15, v/v/v), affording compound **3** (4.8% yield). These compounds were analyzed by NMR and the spectra compared to those already reported in the literature. Compound **2** was previously isolated, as described by Luzzi et al. (1997).

Sample dilution and preparation

Sample solutions were prepared by dissolving the compounds and fraction in HPLC grade MeOH. For the HPLC analysis the samples and mobile phases were filtered under vacuum using a 0.45 µm Millipore membrane filter. For the UPLC analysis, the samples were filtered using 0.22 µm membrane filter. Sonication was performed for 20 minutes in order to eliminate air bubbles. The ethyl acetate fraction was co-injected with the reference samples of biflavonoids (**1**)-(**3**). All the samples were injected at 1 mg/mL.

HPLC-PDA and UPLC-MS conditions and parameters

The ethyl acetate fraction and isolated biflavonoids were analyzed by HPLC-DAD and UPLC-DAD-MS. Analytical HPLC was performed using e2695 Waters Alliance® analytical module equipped with a 2998 PDA detector (Waters, Milford, MA, USA). For the method development, *o*-phosphoric acid 0.1% in water (phase A) and *o*-phosphoric

acid 0.1% in acetonitrile (phase B) as a mobiles phases were evaluated. A column Kromasil® C_{18}, lenght 15cm, i.d 4,6mm. o.d: 8mm, 5µm particle (100 Å pore size) was selected. The flow rate was 0.70 mL/min and the injection volume of 10µL. After optimization, the samples were analyzed by gradient elution: 78%A- 22%B (0-20min), 74%A- 26%B (20-60 min), 60%A- 40%B at 60 minute, 40%A- 60%B at 80 minute and 0%A- 100%B (82-87 min). The chromatograms were detected at 254 nm. Data analysis was performed with Empower™ Software.

For UPLC-DAD-MS analysis, a MicromassLCT Premier Time of Flight mass spectrometer from Waters (Milford, MA, USA) with an electrospray (ESI) interface coupled with an ACQUITY UPLC® system from Waters was used. As stationary phase, a column ACQUITY UPLC® BEH C_{18} 1.7µm, 2.1x50mm conditioned at 30 °C was used. The analysis was performed in positive ion mode in a range m/z 100–1000, capillary voltage 3000V, cone voltage 30v, extraction Cone 3.3V, source temperature 100°C, and desolvation temperature 250°C. The solvent system was the same as described for HPLC, however with a different gradient: Initial 78%A- 22%B (0-2.15min), 76%A- 24%B (2.15-6.69 min), 60%A- 40%B (7.82 min), 40%A- 60%B (8.95 min), 0%A- 100%B (9.18-9.75 min). The data analysis was performed with Software MassLynx™ 4.1.

MAO inhibitory assay

MAO inhibitory assay was performed using human recombinant MAO-A or MAO-B (Passos et al., 2013). The assays were carried out in black polystyrene 96-well microtiter plates. The plates were preincubated for 20 minutes at 37 °C with 158 µL of potassium phosphate buffer pH 7.4, 2 µL of the sample diluted in DMSO and 20 µL of kynuramine 0.5 mM. Later, it was added 20 µL of the enzyme (MAO-A 0.09 mg/mL or MAO-B 0.15 mg/mL) and, after the incubation (30 min at 37°C, equipment Agimaxx AG-4P), 75 µL of NaOH 1M was used to stop the reaction. As control, it was used DMSO 1% and clorgyline 10^{-6} M (100% of inhibition) and 10^{-8} M (50% of inhibition) for MAO-A, or pargyline 10^{-5} M (100% of inhibition) and 10^{-7} M (50% of inhibition) for MAO-B. Fluorescence readings were measured on a Wallac EnVision high throughput screening microplate reader

(PerkinElmer Life and Analytical Sciences, Turku, Finland), at an excitation wavelength of λ = 315 nm and an emission wavelength of λ = 380 nm. The extract was tested in concentrations ranging from 6 to 200 µg/mL and biflavonoids (**1-3**) were tested in concentrations ranging from 3 to 100 µM.

Statistical analysis

Data analysis was performed with Prism 5.0 (GraphPad Software, Inc., CA, USA) and the IC_{50} was calculated by adjusting the experimental data (% of inhibition versus concentration) to nonlinear regression curves.

RESULTS AND DISCUSSION

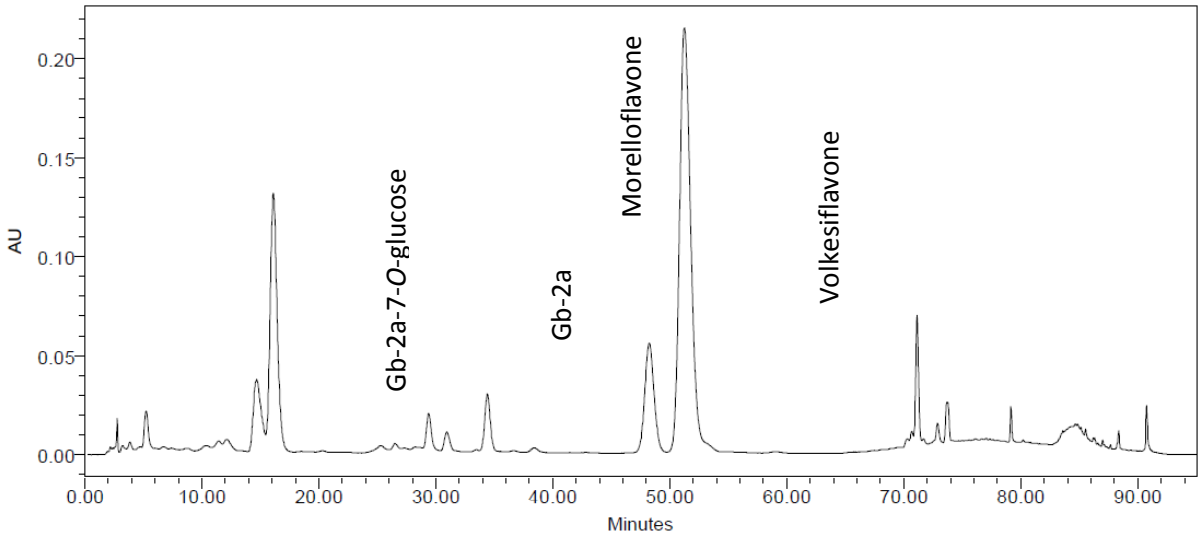
In previous studies of *Garcinia gardneriana,* biflavonoids were isolated from the leaves (Castardo et al., 2008; Luzzi et al., 1997; Verdi et al 2004; Otuki et al., 2011). The leaves have been focus of studies since it is used in folk Brazilian medicine because of its anti-inflammatory activity. However, bark and seeds have also been evaluated due to their anti-inflammatory activity, but no promising results were obtained. Preliminary phytochemical investigation of the bark and seeds extracts compared with that obtained from the leaves indicated a significant difference in the chemical composition, with lower biflavonoids content for the formers (Otuki et al., 2011). Xanthones have also been described for the roots (Monache et al., 1984). In the present study, the branches were used for chemical and pharmacological analysis, taking into account that, as far as we know, no previous studies were performed with this part of the plant.

After ethanol extraction, the calculated yield was 14%. The partition with different solvents afforded the fractions hexane (yield 15%), dichloromethane (yield 29%) and ethyl acetate (yield 43.5%), and the remaining corresponding to the aqueous fraction. Each fraction was analyzed by TLC. For hexane and dichloromethane fractions, similar bands corresponding to terpenoids and steroids were observed, unlike the fraction of ethyl acetate in which it was detected different bands corresponding to phenolic compounds.

Considering that there are some reports demonstrating that flavonoids are able to inhibit MAO activity (Jäger, 2011) the ethyl acetate fraction of *G. garneriana* was evaluated by *in vitro* assay. It was observed that the fraction was able to impair MAO-A and -B activities in the same extend, with an IC_{50} of about 22 µg/mL. Aiming to identify the compounds responsible for this activity, the **fraction** was analyzed. The HPLC analysis of the fraction also indicated the presence of phenolic compounds, corroborating with results observed by TLC. The obtained chromatogram can be observed in figure 1. By the UV profile of the different peaks, it was possible to infer the presence of flavonoids in the fraction. Taking into account that HPLC-DAD analysis is not sufficient to identify the compounds, the same fraction was injected in UPLC-DAD-MS in order to perform a deeper analysis of the chemical profile of the fraction. By the fragmentation of some peaks, it was possible to observe different pseudo-molecular ions $[M+H]^+$ at 557, 559, and 541. With the calculation and comparison of corresponding mass data of these compounds, they were identified as: Morelloflavone (**1**) ($C_{30}H_{20}O_{11}$, MW 556.4732 g/mol), Gb-2a (**2**) ($C_{30}H_{22}O_{11}$, MW 558.4732 g/mol), and volkensiflavone ($C_{30}H_{20}O_{10}$, MW 540.4738 g/mol). Since these biflavonoids have already been isolated from *G. gardneriana* leaves previously (Luzzi et al., 1997), these compounds were co-injected with authentic samples, affording the same retention time as the peaks highlighted in figure 1. Therefore, based on UV spectra, retention time and mass data, it was possible to confirm the structure of the biflavonoids.

In addition to the already described biflavonoids (**1**)-(**2**), another compound was identified in the ethyl acetate fraction. By column chromatography, compound (**3**) was isolated. By HPLC-DAD analysis, it was possible to observe that the UV profile had the same profile as that exhibited by Gb-2a. However, by UPLC-MS analysis, a molecular ion peak at 740 was observed with a base peak at 559, the same observed for Gb-2a. Taking into account the difference of 180, a Gb-2a glucose structure was proposed. By NMR analysis and comparison with spectral data (Compagnone et al., 2008; Li et al., 2014), the structure Gb-2a-7-*O*-glucose (**3**) was confirmed.

Fig. 1. Chromatogram obtained by the HPLC-PDA analysis of the ethyl acetate fraction of *Garcinia gardneriana* at 1mg/mL. Mobile phase was composed of *o*-phosphoric acid 0.1% in water (phase A) and *o*-phosphoric acid 0.1% in acetonitrile (phase B) eluted in a Kromasil® C_{18} column (15cm, i.d 4,6mm, 5µm, 100 Å) with a flow rate of 0.70 mL/min. Detection was monitored at 254 nm.



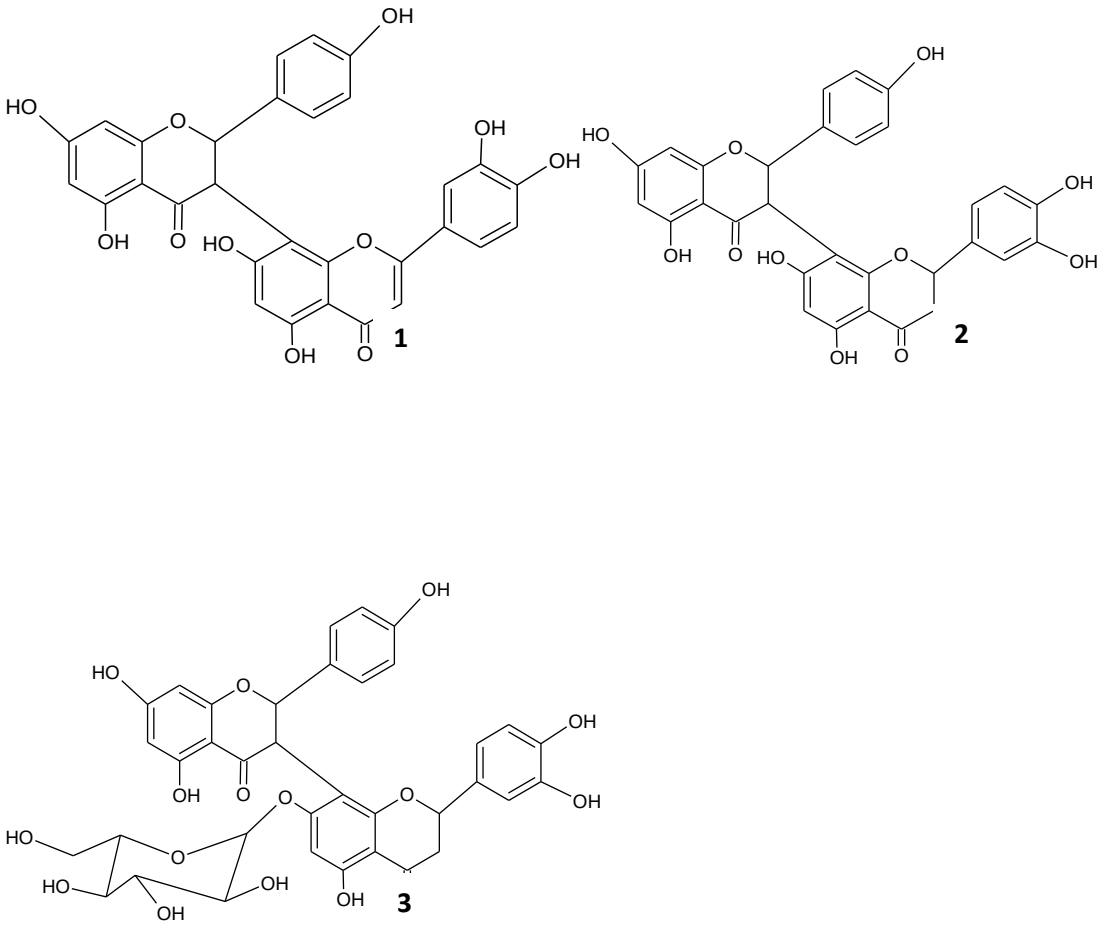


Fig. 2. Isolated biflavonoids structure: Morelloflavone (1), Gb-2a (2), and Gb-2a-7-*O*-glucose (3).

Aiming to evaluate if these biflavonoids have the capacity to inhibit MAO activity, morelloflavone (**1**), Gb-2a (**2**) and Gb-2a-7-*O*-glucose (**3**) were submitted to *in vitro* evaluation. The extent of MAO activity is associated with various behavioral abnormalities. Inhibition of MAO-A predominantly affects neurotransmitters considered to be important in depression and anxiety disorders. On the other hand, MAO-B inhibitors would increase the basal dopamine levels in the nigrostriatal dopaminergic input pathway (Yamada & Yasuhara, 2004).

Although the focus of studies on the involvement of MAO inhibitors in neuroprotection has been on MAO-B inhibitors, there is a growing evidence demonstrating that MAO-A inhibitors may also have neuroprotective properties. So, the presence of MAO inhibitors in plants extracts suggest that such preparations may be investigated for neurodegenerative diseases. Selective MAO-A inhibitors may also exert protective effects such as moclobemide, a reversible MAO-A inhibitor, that has been reported to have anti-Parkinson activity and neuroprotective effects in a model of cerebral ischemia (Song et al., 2013).

As observed in table 1, these compounds were more selective to MAO-A, with IC_{50} ranging from 5.05 to 10.7 µM in comparison to 20.7 to 66.2 µM for MAO-B. This might be due to the smaller entrance cavity (290 A^3) and substrate cavity for MAO-B (~400 A^3) when compared to the monopartite cavity of the MAO-A enzyme (~550 A^3) (Binda et al., 2011). Some flavonoids had already demonstrated the MAO inhibitory effect. In fact, most of them were more selective to MAO-A. Chaurasiya et al. (2014) demonstrated that galangin, apigenin, and quercetin were able to inhibit MAO-A activity with an IC_{50} ranging from 0.13 to 2.44 µM, being 2 to 28 times more selective to this enzyme in comparison to the MAO-B isoform. This selectivity corroborates the study of Chimenti et al. (2010).

The obtained results indicate that biflavonoids might be promising structures for the identification of new MAO inhibitory compounds. In addition, taking into account their ion chelation capacity, a multifunctional activity related to neurodegenerative diseases should be expected, affording promising structures for drug design.

Table 1. Quantitative *in vitro* inhibitory effects of natural biflavonoids in MAO-A and MAO-B

	IC_{50} (μM)		
	MAO-A	**MAO-B**	**Selectivity index**
Morelloflavone	5.05	66.2	13.1
Gb-2a	5.47	56.7	10.4
Gb-2a-7-*O*-glucose	10.7	20.7	1.9
Clorgyline	0.004	NT	-
Pargyline	NT	0.13	-

NT = not tested

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5. MANUSCRITO II

Manuscrito en Preparación

Aromatase (CYP19) inhibition by biflavonoids obtained from branches of *Garcinia gardneriana* (Clusiaceae)

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ABSTRACT

Overexpression of aromatase in breast cancer cells may substantially influence breast cancer progression and maintenance. Some monomeric flavonoids isolated from the genus *Garcinia* have been demonstrating anti-cancer activity and have been evaluated on different activities, including their chemopreventive properties and aromatase inhibition. In this work, the biflavonoids isolated from *Garcinia gardneriana*, morelloflavone (**1**), Gb-2a (**2**) and Gb-2a-7-*O*-glucose (**3**) were submitted to *in vitro* assay to evaluate if these compounds have the capacity to inhibit aromatase. The achieved results showing that the biflavonoid Gb-2a had stronger activity (IC_{50} 1,35μM) than morelloflavone (IC_{50} 3,09μM), with similar structure and had better activity than Gb-2a-7-*O*-glucose (IC_{50} 7,67μM).

KEY WORDS

Garcinia gardneriana, biflavonoids, aromatase (CYP19) inhibition

INTRODUCTION

There are more than 180 drugs available for cancer treatment approved by the Food and Drug Administration (FDA), with great prospects for growth, considering that the agency has approved an average of ten new drugs per year (Kinch, 2014). To date, six new drugs were approved this year in the US (CenterWatch, 2015). Nevertheless, the observed side effects, including anemia and reduction of the immune response, are still a problem. In addition, the chemotherapeutic agents resistance cases have been detected on a recurring basis (Wellington, 2015). Therefore, the constant search for new compounds capable of acting as more potent and more selective chemotherapy is fully justified.

It is known that natural products are an important source of bioactive compounds (secondary metabolites). In fact, as recently highlighted by Giddings and Newman (2014), more than 50% of the available drugs in cancer treatment or are natural products or derivatives of these. The success achieved by this class of compounds is primarily due to the ability of plants and other organisms to synthesize extremely complex structures that would be difficult to be proposed by traditional organic synthesis studies.

Metabolites isolated from the genus *Garcinia* have been demonstrating anti-cancer activity. Garcinol, obtained from *Garcinia indica* (Liu, et al., 2015), was evaluated *in vitro* and *in vivo* showing induction of apopotosis and cell cycle arrest, inhitition of angiogenesis and modulation of gene expression. In addition, xanthones are the most studied compounds in *Garcinia* genus due to their anti-carcinogenic activity. Three new xanthones from *Garcinia pedunculata* , named pedunxanthone D-F, demonstrated cytotoxicity against Hela (human cervical cancer) and NCI-H460 (human lung cancer) cells (Vo, et al., 2015). Prenylated xanthones isolated from *Garcinia nujiangensis*, named Nujiangxanthone C-F, were able to inhibit with high selectivity the HeLa cancer cells and HepG2 (liver hepatocellular carcinoma). (Tang, et al., 2015).

Some biflavonoids from *Garcinia* have been evaluated on different activities, including their chemopreventive properties. Kolaviron isolated from *Garcinia kola*, demonstrated the ability to scavenge free radicals, inhibit stress response proteins and interfere with DNA binding activities of some transcription factors (Farombi & Owoeye, 2011a). Ginkgetin from *Ginkgo biloba* was able to induce apoptosis in prostate cancer cells via activation of caspase-3 and inhibition of survival genes as a potent chemotherapeutic agent for prostate cancer treatment (You et al., 2013). The biflavonoid GB1, isolated as major compound from *Garcinia kola* and frequently founded in other species of *Garcinia*, has demonstrated inhibitory activity against aromatase (CYP19)(Antia et al., 2010)

Aromatase inhibitors (AIs) prevent the conversion of androstendione to 17b-estradiol and are extensively used for the treatment and to reduce the risk of breast cancer

in postmenopausal women. Although AIs are widely used for cancer treatment, they can cause severe adverse effects, like the tamoxifen, a drug used for reduce incidence of breast cancer which increases endometrial cancer and venous thromboembolic events (Sestak, 2014) and is common that young women undergoing AIs feel untimely menopause symptoms. Overexpression of aromatase in breast cancer cells may substantially influence breast cancer progression and maintenance (Way, et al., 2004). Aromatase is the only enzyme in vertebrates to catalyze final step of estrogen biosynthesis, being considered a key target for the development of drug against estrogen-dependent breast cancer (Guo et al., 2014). Identification and development of naturally occurring compounds, such as flavonoids, could be the alternative for prevention and cancer treatment.

Considering that *Garcinia* biflavonoids had already demonstrated antioxidant and anti-inflammatory activities, both desired activities in anticancer compounds, and that GB1, a *Garcinia* biflavonoid, was able to inhibit aromatase activity, in this study we aim at evaluate the effect of a *G. gardneriana* isolated biflavonoids in aromatase activity.

MATERIALS AND METHODS

Chemicals

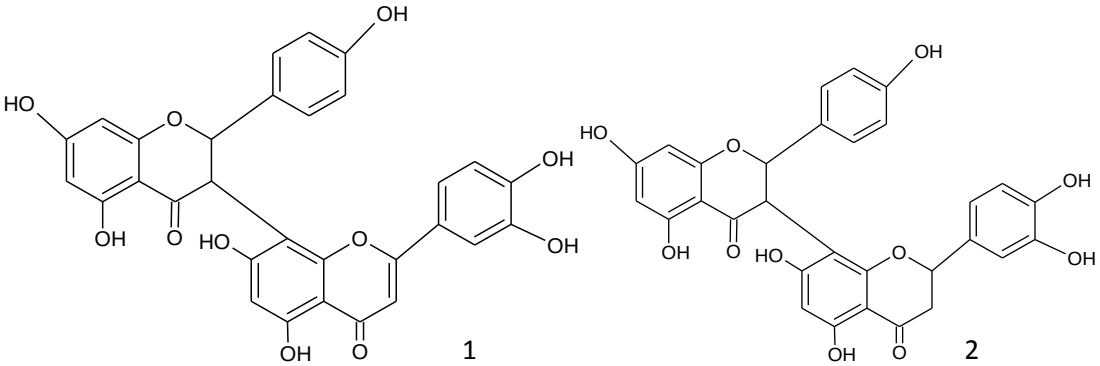
Dibenzylfluorescein, D-Glucose-6-Phosphate, Glucose-6-Phosphate dehydrogenase IX type obtained from baker´s yeast (*S. cerevisiae*), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human aromatase CPY19 were acquired from BD Gentest (Woburn, MA, USA). Bovine albumin (BSA), was obtained from Aldrich Chemical Company Inc. (USA). All remaining chemicals used were of analytical grade and were purchased from Sigma-Aldrich.

Plant material

Garcinia gardneriana was collected in Taquara, Rio Grande do Sul, in November 2013. The species was identified by Dr. Sérgio Augusto de Loreto Bordignon and a voucher was deposited at the herbarium of the Departmento de Botânica of the Universidade Federal do Rio Grande do Sul under the number ICN 180888

Chemical extraction and isolation

Extraction and isolation was performed as already described by “Monoamine oxidase inhibitory activity of the biflavonoids obtained from branches *Garcinia gardneriana* (Clusiaceae)” (chapter 2 of this document). Briefly, the branches were submitted to ethanol extraction and this was partitioned with hexane, dichloromethane and ethyl acetate. Biflavonoids were isolated by consecutive columns chromatography from ethyl acetate fraction using silica gel as stationary phase and a gradient solvents mixture, obtaining two different biflavonoids. Their chemical structure were determined as Morelloflavone (**1**) and Gb-2a-7-*O*-glucose (**3**) by MS and NMR experiments and compared to previous studies (Luzzi et al., 1997). Gb-2a (**2**) (Figure 1), were previously isolated, as described by Luzzi et al. (1997).



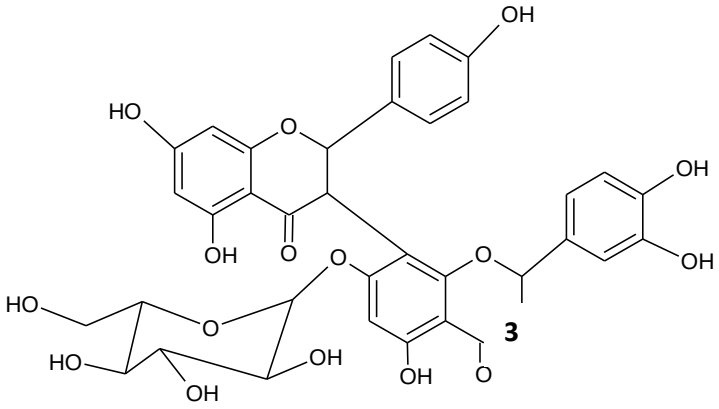


Fig. 1. Biflavonoids structure: (1) Morelloflavone , (2) Gb-2a, (3) Gb-2a-7-*O*-glucose

Aromatase inhibitory assay

Solution Preparation

Each solution was prepared as follows:

Solution A: β- Nicotinamide adenine dinucleotide phosphate β-NADP (final concentration 26.0 mM), Glucose-6-Phosphate dehydrogenase (final concentration 66.67 mM) magnesium chloride (final concentration 60.0 mM) in Milli-Q water; **Solution B**: Glucose-6-Phosphate dehydrogenase 40 µ/mL; **C/SD mixture**: potassium phosphate buffer (pH 7.4), bovine serum albumin (BSA) (final concentration 0,05%), solution A (final concentration in assay: NADP 1,18mM; MgCl2 30mM; G-6-P 3,3mM), Solution B (G-6-PH o, 36U/mL); **E/S mixture**: potassium phosphate buffer (pH 7.4), BSA (final concentration of 0.2%), dibenzylfluorescein (final concentration 0.4 µM), CYP19 (final concentration 0.2nM).

The effect of the biflavonoids (morelloflavone, Gb-2a and Gb-2a-7-*O*-glucose) on the aromatase activity was evaluated by the detection of fluorescein, product of the hydrolysis of dibenzylfluorescein mediated by aromatase. It was added 90 µL of the C/SD

solution in a non-sterile black 96-well plate, responsible for the “in situ” NADPH generation. Ethanol was used as negative control. Ten µL of the samples (3.12 to 100 µM/mL) were added, using as blank 100 µL of the C/SD mixture, followed by agitation for 30 seconds,at 10 cycles/min. The plates were pre-incubated for 10 min at 37 ° C. Later, it was added 100 µL of the enzyme-substrate mixture (E/S), except in the blank, followed by agitation for 30 seconds at 10 cycles/min. After incubation at 37 ° C for 30 min, the reaction was stopped by adding of 75µL of NaOH 5 M. Finally, it was added 100 µL E/S in wells for the blank. The microplate was agitated at 10 cycles/min and room temperature for 5 min, followed by incubation at 37 °C for 2h. Resulting fluorescence was determined by fluorimeter, using the wavelength λ=485 nm for excitation and λ =535 nm for emission.

Before starting the tests with samples, naringenine inhibition curve (74.46 µM final concentration to 0.57 µM) was performed in order to determine possible deviations.

RESULTS AND DISCUSSION

About 300 natural compounds, mainly flavonoids, have been evaluated for their effects on aromatase activity in noncellular, cellular, and *in vivo* studies (Farombi and Owoeye, 2011a). In fact, flavonoids have been reported as the most active class of natural product with AI activity. Some of the most active flavonoids include monomers like naringenin (5,7,4'-flavanone), luteolin (5,7,3',4'- tetrahydroxyflavone) and eriodictyol, that has shown strong activity in microsomal aromatase assays (Balunas M.J. et al., 2011). However, just a few biflavonoids had already been evaluated due to their AI properties.

Previous chemical studies of *Garcinia gardneriana* leaves led to the isolation of several biflavonoids (Luzzi et al., 1997). In this study, morelloflavone (**1),** Gb-2a-7-*O*-glucose (**3**) and Gb-2a (2), obtained from the extract of the branches of the plant, were submitted to *in vitro* assays in order to evaluate their capacity to inhibit aromatase. As observed in table 1, all the biflavonoids displayed very low IC_{50}, ranging from 1.35 to 7.67 µM. Accordingly to the classification adopted by Balunas et al. (2011), both morelloflavone and Gb-2a would be considered strongly active compounds (IC_{50} < 5 µM). In comparison

to GB1, obtained from *Garcinia kola* and previously evaluated due to its AI properties (Antia et al., 2010), it is observed that Gb-2a is about eight times more potent. Morelloflavone is a naringenin attached to a luteolin, and Gb-2a is a naringenin attached to an eriodictyol (flavanone). All these monomers were able to inhibit aromatase activity with high potency (van Meeuwen et al., 2008)

In addition, biflavonoids like ginkgetin has been reported to exert cytotoxic effect in human ovarian adenocarcinoma cell line,and inhibition of survival genes in PC-3 prostate cancer cells(You et al., 2013).

Aromatase inhibitors (AIs), are the standard therapy for block the synthesis of estrogens. This reduces estrogen levels, and slows the growth of cancers like breast and prostate cancer.

Few different natural compounds have been reported against aromatase activity (Guo et al., 2014) and biflavonoids have not been evaluated with this enzyme, except the biflavonoid GB1. However several biflavonoids have been evaluated for allied biologic activities, as isoled biflavonoids from *Vernonia amigdalina*(Farombi and Owoeye, 2011b), and from *Garcinia kola* (Ayepola, et.al., 2014) that have been reported antioxidant activity and chemopreventive properties showing the biflavonoids, advantages over monomeric flavonoids.

The biflavonoid formed by the flavonoids naringin and luteolin (morelloflavone) had an IC_{50} 3.09 µM as result. The biflavonoid formed by naringenin and eriodictyol (Gb-2a) had as result an IC_{50}1,35 µM, lastly, the biflavonoid formed by naringenin and eriodictyol-glycosylated (Gb-2a-7-O-glucose) had as result an IC_{50} 7,67µM, spoting that the results obtained for each biflavonoid are close to the lower limits reported for each monomer that conforms it.

The order of inhibition activity starting with Gb-2a could be due to the presence of eriodictyol in this structure, being that according (Balunas, 2011) whit range of IC_{50} (0,6 a 5,3 µM), it has a stronger activity that luteolin. Therefore it is necessary to evaluate the stage of the enzymatic process is inhibited by this bioflavonoids

For other hand, the isolated biflavonoids, Gb-2a glucoside, morelloflavone and similar biflavonoids have been already related important anti-inflammatory effects (Castardo et al., 2008), considering that the intensity of the inflammatory response is crucial; an insufficient response can lead to immunodeficiency, which gives rise to infections and cancer, these could be a potential thorough target study.

Table 1. Quantitative *in vitro* inhibitory effects of natural biflavonoids in CPY19

Compound	**IC_{50} (μM) aromatase**
Morelloflavone	3,09
Gb-2a	1,35
Gb-2a-7-*O*-glucose	7,67
Naringenine	1,4-4,2

CONCLUSIONS

Natural aromatase inhibitors are important target for the treatment of estrogen-dependent cancers. The inhibition of aromatase by biflavonoids obtained from *Garcinia gardneriana* showed that in addition to its anti-inflammatory activity have inhibitory effect on aromatase like this monomers. A study “in vivo” is advisable because the whole-body inhibition of aromatase causes severe adverse effects. Furthermore, considering the strong activity of the monomers constituting the tested biflavonoids, and the results shown in this work, it will be necessary to evaluate the mechanism of inhibition of these molecules, because the size of the biflavonoids in theory could reduce the effect but apparently in practice don’t occurs.

6. CONSIDERACIONES GENERALES

Los biflavonoides fueron aislados de ramas *G. gardneriana* por columna cromatográfica usando como fase estacionaria gel de sílice. Considerando que estos tienen estructuras químicas muy similares y una alta polaridad debido a la presencia de varios grupos hidroxilo, se entiende que estas características se vean reflejadas en un bajo rendimiento de estos compuestos cuando se utiliza gel de sílice como adsorbente en la columna cromatografía (Girardi, et al., 2005) lo que podría sugerir la búsqueda y aplicación de métodos diferentes para aislar compuestos de esta planta y obtener mayores rendimientos o nuevos productos.

Además, debe considerarse el estudio químico de las diferentes partes de la planta en diferentes épocas de colecta para determinar los compuestos mayoritarios en cada tiempo y parte de la planta.

En el proceso de aislamiento de compuestos de este trabajo, los compuestos mayoritarios resultaron ser biflavonoides con presencia de monómeros recurrentes en la literatura como naringenina, luteolina y eriodictyol. Estos monómeros han sido previamente evaluados para diversos tipos de actividades, entre esas, inhibición enzimática de la aromatasa por diversos métodos dando resultados fuertemente positivos (Balunas et.al., 2011).

La recopilación de resultados obtenidos para naringenina están un rango de IC_{50} (2,9 a 17,0 µM), para luteolina IC_{50} (1,2 a 25,0 µM) y para eriodictyol IC_{50} (0,6 a 5,3 µM), lo que coincide con los resultados obtenidos para cada biflavonoide y podría sugerir relación estructural.

El monómero eriodictyol-glicosilado no reporta resultados enzimáticos en la literatura consultada.

Este ensayo enzimático podría ser una herramienta útil para la detección de nuevos productos activos, en especial de los derivados de especies vegetales comunes, evitando o disminuyendo los efectos secundarios de los tratamientos convencionales

Los resultados de IC50 para MAO-A son considerados relativamente similares para moreloflavona y Gb-2a, reportando una óptima actividad inhibitoria para esta enzima, lo que no pasa con el biflavonoide Gb-2a-7-*O*-glucosa, lo que confirma la necesidad de estudiar el mecanismo de funcionamiento de estos metabolitos y descifrar si el resultado está influenciado por el sustituyente glucosa o por la conformación de la enzima, ya que los resultados obtenidos para la inhibición de la aromatasa también indican una menor respuesta con el biflavonoide glicosilado.

Inhibidores de la aromatasa son objetos de estudio importantes para visualizar el descubrimiento de compuestos anti-tumorales, en especial aquellos denominados hormonodependientes,, que necesitan de hormonas para su crecimiento como el caso de cáncer de próstata e mama y que, así mismo, su tratamiento se realiza mediante el detenimiento de los procesos hormonales que causan el crecimiento de los tejidos correspondientes, causando efectos secundarios indeseables en diferentes procesos naturales del paciente.

La respuesta anti inflamatoria de estos biflavonoides también se ha relacionado con transtornos emocionales como ansiedad y depresión, los cuales son tratados con inhibidores de la MAO-A, donde la intensidad de la respuesta inflamatoria de los compuestos es crucial, pues, una respuesta insuficiente puede dar lugar a inmunodeficiencia resultando en infecciones y cáncer.

El efecto antidepresivo en el uso tradicional de algunas plantas como *Mentha aquatica L* (Olsen, et al., 2008) ha sido atribuidos a la naringenina y quercetina y la luteolina, los cuales se han sugerido para ser compuestos potenciales antidepresivos porque ejercen un efecto supresor sobre la reacción de MAO-A (Bandaruk, et al., 2014). Estos hechos pueden ser corroborados con los resultados presentados en este trabajo, los cuales demuestran que los biflavonoides moreloflavona, Gb-2a y Gb-2a-7-*O*-glucosa tienen actividad sobre las enzima aromatasa y MAO-A, además de su fuerte respuesta anti inflamatoria. Esto confirma la necesidad de buscar el mecanismo de funcionamiento de inhibición de estos compuestos sobre las enzimas, pues al parecer, el tamaño de las moléculas no es el principal determinante para su desempeño con estos blancos de estudio basándose en los resultados obtenidos por los monómeros.

Estudios demuestran que la expresión de MAO-A está relacionada con el desarrollo de cánceres como el de próstata, lo que podría sugerir que los inhibidores de MAO-A podrían usarse para el tratamiento de cáncer y la depresión presentada en el tratamiento de cáncer de mama que es realizada con la inhibición de la aromatasa.

Con todos los resultados observados, se concluye que se debe realizar una profundización en la relación de MAO-A y aromatasa para encontrar nuevas propuestas de fármacos para tratamiento de cáncer de mama evitando los trastornos depresivos, además del estudio "*in vivo*" de los resultados presentados anteriormente.

7. CONCLUSIONES

Frente a los resultados obtenidos en este trabajo se puede concluir que:

- Fué posible realizar un análisis químico de los extractos de los tallos secos de *Garcinia gardneriana* colectadas en Rio Grande do Sul, evaluando sus perfiles cromatográficos.

- Los compuestos mayoritarios fueron asilados por cromatografia de columna..

- Los compuestos fueron caracterizados como biflavonoides, principalmente por sus espectros de masa y la comparación de sus estructuras con referencias de muestras y datos de la literatura.

- Los compuestos aislados en mayor cantidad fueron evaluados tanto en su actividad de inhibición de las enzimas MAO A y B como en aromatasa.

- En la evaluación de la actividad inhibitoria a la enzima Monoaminaoxidasa, los compuestos aislados demostraron actividad mas pronunciada frente a la isoforma A.

- Los compuestos demostraron actividad inhibitoria frente a la enzima aromtasa.

- Como conclusión final se puede inferir que la especie *G gardneriana* es una importante fuente de compuestos bioactivos e puede considerarse una fuente para la continuación de estudios relacionados a actividades conjuntas en estas enzimas y otras patologías diversas relacionadas como enfermedades neurodegenerativas y cáncer hormono-dependientes;

8. REFERENCIAS

Acuña, U. M., Dastmalchi, K., Basile, M. J., & Kennelly, E. J. (2012). Quantitative high-performance liquid chromatography photo-diode array (HPLC-PDA) analysis of benzophenones and biflavonoids in eight Garcinia species. *Journal of Food Composition and Analysis*, *25*(2), 215–220. http://doi.org/10.1016/j.jfca.2011.10.006

Antia B.S., Pansanit A., Ekpa O.D., Ekpe U.J., Mahidol C., Kitta koop P. α-Glucosidase Inhibitory, Aromatase Inhibitory, and Antiplasmodial Activities of a Biflavonoid GB1 from Garcinia kolaStem Bark. Pharmacology Planta Med 2010; 76(3): 276-277 DOI: 10.1055/s-0029-1186081

Ayepola, O. R., Cerf, M. E., Brooks, N. L., & Oguntibeju, O. O. (2014). Kolaviron, a biflavonoid complex of Garcinia kola seeds modulates apoptosis by suppressing oxidative stress and inflammation in diabetes-induced nephrotoxic rats. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*, *21*(14), 1785–93. http://doi.org/10.1016/j.phymed.2014.09.006

Balunas M.J. Su B. , Brueggemeier R.W., and K. A. D. (2011). NIH Public Access. Medicinal Chemistry, 8(6), 646–682.

Bandaruk, Y., Mukai, R., & Terao, J. (2014). Cellular uptake of quercetin and luteolin and their effects on monoamine oxidase-A in human neuroblastoma SH-SY5Y cells. *Toxicology Reports*, *1*, 639–649.

Campos, P. M., Horinouchi, C. D. D. S., Prudente, A. D. S., Cechinel-Filho, V., Cabrini, D. D. A., & Otuki, M. F. (2013). Effect of a Garcinia gardneriana (Planchon and Triana) Zappi hydroalcoholic extract on melanogenesis in B16F10 melanoma cells. *Journal of Ethnopharmacology*, *148*(1), 199–204. http://doi.org/10.1016/j.jep.2013.03.079

Castardo, J. C., Prudente, A. S., Ferreira, J., Guimarães, C. L., Monache, F. D., Filho, V. C., … Cabrini, D. a. (2008). Anti-inflammatory effects of hydroalcoholic extract and two biflavonoids from Garcinia gardneriana leaves in mouse paw oedema. *Journal of Ethnopharmacology*, *118*(3), 405–11. http://doi.org/10.1016/j.jep.2008.05.002

Chaurasiya, N., Ibrahim, M., Muhammad, I., Walker, L., & Tekwani, B. (2014). Monoamine Oxidase Inhibitory Constituents of Propolis: Kinetics and Mechanism of Inhibition of Recombinant Human MAO-A and MAO-B. *Molecules*, *19*(11), 18936–18952. http://doi.org/10.3390/molecules191118936

Chimenti F., Fioravanti R. , Bolasco A. , Chimenti P., Secci D., Rossi F. , Yáñez M. , Orallo F., Ortuso F. , Alcaro S., Cirilli R. , Ferretti R. , Sanna M. L. 2010. A new series of flavones, thioflavones, and flavanones as selective monoamine oxidase-B inhibitors Bioorganic & Medicinal Chemistry v 18 , 1273–1279

Correa, M.P.,. Dicionario das plantas uteis do Brasil e das exóticas cultivadas. Ministério da Agricultura, IBDF, Rio de Janeiro, pp. 318–337. 1984

Farombi, E. O., & Owoeye, O. (2011a). Antioxidative and chemopreventive properties of Vernonia amygdalina and Garcinia biflavonoid. *International Journal of Environmental Research and Public Health*, *8*(6), 2533–55. http://doi.org/10.3390/ijerph8062533

Fidelis, Q. C., Ribeiro, T. a. N., Araújo, M. F., & de Carvalho, M. G. (2014). Ouratea genus: chemical and pharmacological aspects. *Revista Brasileira de Farmacognosia*, *24*(1), 1–19. http://doi.org/10.1590/0102-695X20142413361

Gabor, M. V., Middleton, E., Harborne, J. B., Beretz. A Szent-Gyorgyi and the bioflavonoids: new results and perspectives of pharmacological research into benzo-pyrone derivatives. Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties. Alan R. Liss Inc., New York, pp 1-15. 1988.

Girardi, L. G. J., Morsch, M., Oliveira, A. E., Cechinel-Filho, V., & Rodrigues, C. A. (2005). Separation of bioactive biflavonoids from Rheedia gardneriana using chitosan modified with benzaldehyde. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences*, *60*(5-6), 408–410

Gontijo, V. S., Judice, W. a S., Codonho, B., Pereira, I. O., Assis, D. M., Januário, J. P., … Santos, M. H. Dos. (2012). Leishmanicidal, antiproteolytic and antioxidant evaluation of natural biflavonoids isolated from Garcinia brasiliensis and their semisynthetic derivatives. *European Journal of Medicinal Chemistry*, *58*, 613–623. http://doi.org/10.1016/j.ejmech.2012.06.021

Guo, J., Yuan, Y., Lu, D., Du, B., Xiong, L., Shi, J., … Wang, F. (2014). Two natural products, trans-phytol and (22E)-ergosta-6,9,22-triene-3β,5α,8α-triol, inhibit the biosynthesis of estrogen in human ovarian granulosa cells by aromatase (CYP19). *Toxicology and Applied Pharmacology*, *279*(1), 23–32. http://doi.org/10.1016/j.taap.2014.05.008

Kennedy SH, Holt A, Baker GB. 2009. Monoamine oxidase inhibitors. In: Sadock BJ, Sadock VA,Ruiz P, editors. Kaplan and Sadock's comprehensive textbook of psychiatry. 9th ed.Philadelphia: Lippincott Williams & Wilkins;. p. 3154–63

Li Yunfang , Yu Chen, Chuying Xiao, Dan Chena, Yuxiu Xiao, Zhinan Mei. (2014) Rapid screening and identification of a-amylase inhibitors from Garcinia xanthochymus using enzyme-immobilized magnetic nanoparticles coupled with HPLC and MS. Journal of Chromatography B, 960 166‑173

Lin, Y., Anderson, H., Flavin, M. T., Pai, Y. S., Mata-greenwood, E., Pengsuparp, T., … Chen, F. (1997). In Vitro Anti-HIV Activity of Biflavonoids Isolated from Rhus succedanea and Garcinia multiflora, *3864*(97), 884–888.

Liu, C., Ho, P. C., Cheng, F., Sethi, G., & Zhi, L. (2015). Garcinol : Current status of its anti-oxidative , anti-inflammatory and anti-cancer effects, *362*, 8–14.

Luzzi, R., Guimarães, C. L., Verdi, L. G., Simionatto, E. L., Delle Monache, F., Yunes, R. a, … Cechinel-Filho, V. (1997). Isolation of biflavonoids with analgesic activity from Rheedia gardneriana leaves. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*, *4*(2), 141–4. http://doi.org/10.1016/S0944-7113(97)80060-8

Mei-Hsien Lee, Rong-Dih Lin, Lee-Yen Shen, Ling-Ling Yang, Kun-Ying Yen and Wen-Chi Hou. 2001. Monoamine Oxidase B and Free Radical Scavenging Activities of Natural Flavonoids in Melastoma candidum D. Don. *J. Agric. Food Chem.*, 2001, 49 (11), pp 5551–5555

Mishra B.B, Tiwari V.K. Natural products: an evolving role in future drug discovery. Eur J Med Chem. 2011 Oct;46(10):4769-807. doi: 10.1016/j.ejmech.2011.07.057

Monache, G. D., Monache, D., Waterman, P. G., & Crichton, E. G. (1984). MINOR XANTHONES FROM RHEEDZA GARDiVERZANA *, *23*(8), 1757–1759.

Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products*, *75*(3), 311–335. http://doi.org/10.1021/np200906s

Nguyen, P.-H., Ji, D.-J., Han, Y.-R., Choi, J.-S., Rhyu, D.-Y., Min, B.-S., & Woo, M.-H. (2015). Selaginellin and biflavonoids as protein tyrosine phosphatase 1B inhibitors from Selaginella tamariscina and their glucose uptake stimulatory effects. *Bioorganic & Medicinal Chemistry*, *23*(13), 3730–3737. http://doi.org/10.1016/j.bmc.2015.04.00

Olsen, H. T., Stafford, G. I., van Staden, J., Christensen, S. B., & Jäger, A. K. (2008). Isolation of the MAO-inhibitor naringenin from Mentha aquatica L. *Journal of Ethnopharmacology*, *117*(3), 500–502. http://doi.org/10.1016/j.jep.2008.02.015

Otuki, M. F., Bernardi, C. a, Prudente, A. S., Laskoski, K., Gomig, F., Horinouchi, C. D. S., … Cabrini, D. a. (2011). Garcinia gardneriana (Planchon & Triana) Zappi. (Clusiaceae) as a topical anti-inflammatory alternative for cutaneous inflammation. *Basic & Clinical Pharmacology & Toxicology*, *109*(1), 56–62. http://doi.org/10.1111/j.1742-7843.2011.00689.x

Passos, C. S., Simões-Pires, C. a, Nurisso, A., Soldi, T. C., Kato, L., de Oliveira, C. M. a, … Henriques, A. T. (2013). Indole alkaloids of Psychotria as multifunctional cholinesterases and monoamine oxidases inhibitors. *Phytochemistry*, *86*, 8–20. http://doi.org/10.1016/j.phytochem.2012.11.015

Peehl, D. M., Coram, M., Khine, H., Reese, S., Nolley, R., & Zhao, H. (2008). The significance of monoamine oxidase-A expression in high grade prostate cancer. *The Journal of Urology*, *180*(5), 2206–11. http://doi.org/10.1016/j.juro.2008.07.019

Pereañez, J. A., Patiño, A. C., Núñez, V., & Osorio, E. (2014). The biflavonoid morelloflavone inhibits the enzymatic and biological activities of a snake venom phospholipase A2. *Chemico-Biological Interactions*, *220*, 94–101. http://doi.org/10.1016/j.cbi.2014.06.015

Press, D. (2014). Preventative therapies for healthy women at high risk of breast cancer, 423–430.

Rybaczyk, L. a, Bashaw, M. J., Pathak, D. R., & Huang, K. (2008). An indicator of cancer: downregulation of monoamine oxidase-A in multiple organs and species. *BMC Genomics*, *9*, 134. http://doi.org/10.1186/1471-2164-9-134

Ryu, Y. B., Jeong, H. J., Kim, J. H., Kim, Y. M., Park, J. Y., Kim, D., … Lee, W. S. (2010). Biflavonoids from Torreya nucifera displaying SARS-CoV 3CLpro inhibition. *Bioorganic and Medicinal Chemistry*, *18*(22), 7940–7947. http://doi.org/10.1016/j.bmc.2010.09.035

Sloley, L. J. Urichuk, P. Morley*, J. Durkin*, J. J. Shan, P. K. T. Pang Coutts, And R. T.2000. Identification of Kaempferol as a Monoamine Oxidase Inhibitor and Potential Neuroprotectant in Extracts of Ginkgo Biloba Leaves. J. Pharm. Pharmacol, 52: 451±459

Song, M., Matveychuk, D., Mackenzie, E. M., Duchcherer, M., Mousseau, D. D., & Baker, G. B. (2013). Progress in Neuro-Psychopharmacology & Biological Psychiatry An update on amine oxidase inhibitors : Multifaceted drugs, *44*, 118–124.

Tang, Z., Xia, Z., Qiao, S., Jiang, C., & Shen, G. (2015). Fitoterapia Four new cytotoxic xanthones from Garcinia nujiangensis, *102*, 109–114.

Trisuwan, K., Rukachaisirikul, V., & Phongpaichit, S. (2013). Phytochemistry Letters Tetraoxygenated xanthones and biflavanoids from the twigs of Garcinia merguensis, *6*, 511–513

Tuansulong, K. A., Hutadilok-Towatana, N., Mahabusarakam, W., Pinkaew, D., & Fujise, K. (2011). Morelloflavone from Garcinia dulcis as a novel biflavonoid inhibitor of HMG-CoA reductase. *Phytotherapy Research*, *25*(3), 424–428. http://doi.org/10.1002/ptr.3286

Van Meeuwen, J. a., Nijmeijer, S., Mutarapat, T., Ruchirawat, S., de Jong, P. C., Piersma, a. H., & van den Berg, M. (2008). Aromatase inhibition by synthetic lactones and flavonoids in human placental microsomes and breast fibroblasts - A comparative study. *Toxicology and Applied Pharmacology*, *228*(3), 269–276. http://doi.org/10.1016/j.taap.2007.12.007

Verdi Luiz G., Pizzolatti, Moacir G., Montanher Ana Beatriz P., Brighente Inês M.C. , Júnior Artur Smânia, Smânia Elza de F.A. Simionatto Edesio L., Delle Monache Franco (2004) Antibacterial and brine shrimp lethality tests of biflavonoids and derivatives of Rheedia gardneriana. , Fitoterapia 75 (2004) 360–363 0367-326X/04/$ - see front matter _ 2004 Elsevier B.V. All rights reserved.

Vo, H. T., Ngo, N. T. N., Bui, T. Q., Pham, H. D., & Nguyen, L. D. (2015). Phytochemistry Letters Geranylated tetraoxygenated xanthones from the pericarp of Garcinia pedunculata, *13*, 119–122.

Way, T.-D., Lee, H.-H., Kao, M.-C., & Lin, J.-K. (2004). Black tea polyphenol theaflavins inhibit aromatase activity and attenuate tamoxifen resistance in HER2/neu-transfected human breast cancer cells through tyrosine kinase suppression. *European Journal of Cancer (Oxford, England : 1990)*, *40*(14), 2165–74. http://doi.org/10.1016/j.ejca.2004.06.018

Yamada, M., & Yasuhara, H. (2004). Clinical pharmacology of MAO inhibitors: safety and future. *Neurotoxicology*, *25*(1-2), 215–21. http://doi.org/10.1016/S0161-813X(03)00097-4.

You, O. H., Kim, S.-H., Kim, B., Sohn, E. J., Lee, H.-J., Shim, B.-S., … Kim, S.-H. (2013). Ginkgetin induces apoptosis via activation of caspase and inhibition of survival genes in PC-3 prostate cancer cells. *Bioorganic & Medicinal Chemistry Letters*, *23*(9), 2692–5. http://doi.org/10.1016/j.bmcl.2013.02.080

Yunfang Li a,b, Yu Chenc, Chuying Xiaob, Dan Chena, Yuxiu Xiaoa, Zhinan Mei. (2014) Rapid screening and identification of a-amylase inhibitors from Garcinia xanthochymus using enzyme-immobilized magnetic nanoparticles coupled with HPLC and MS. Journal of Chromatography B, 960 166–173

9. ANEXOS

FIG. 1. [1] H NMR Spectrum of Gb-2a-7-o-Glucose (MeOD solvent, 300MHz)

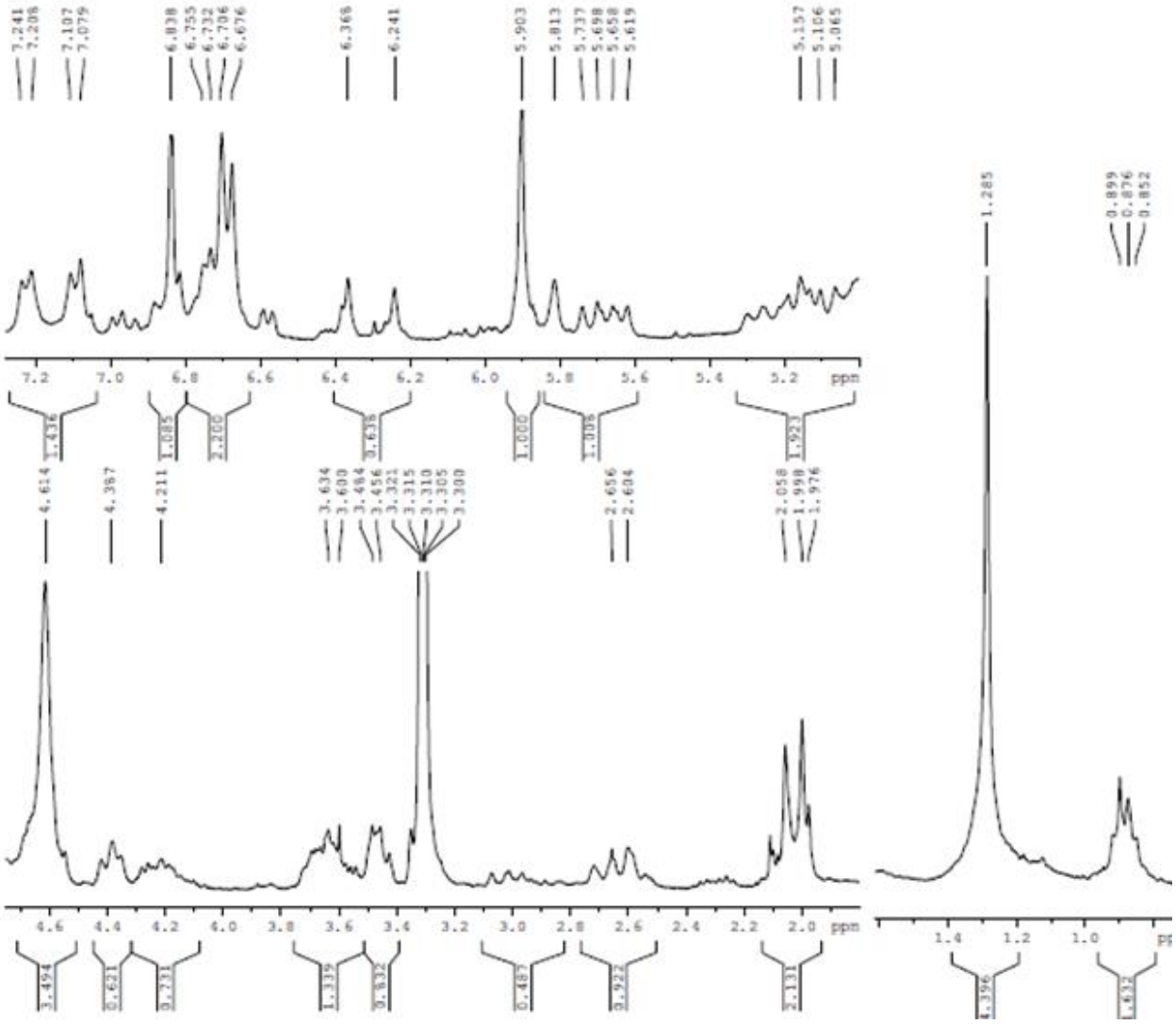
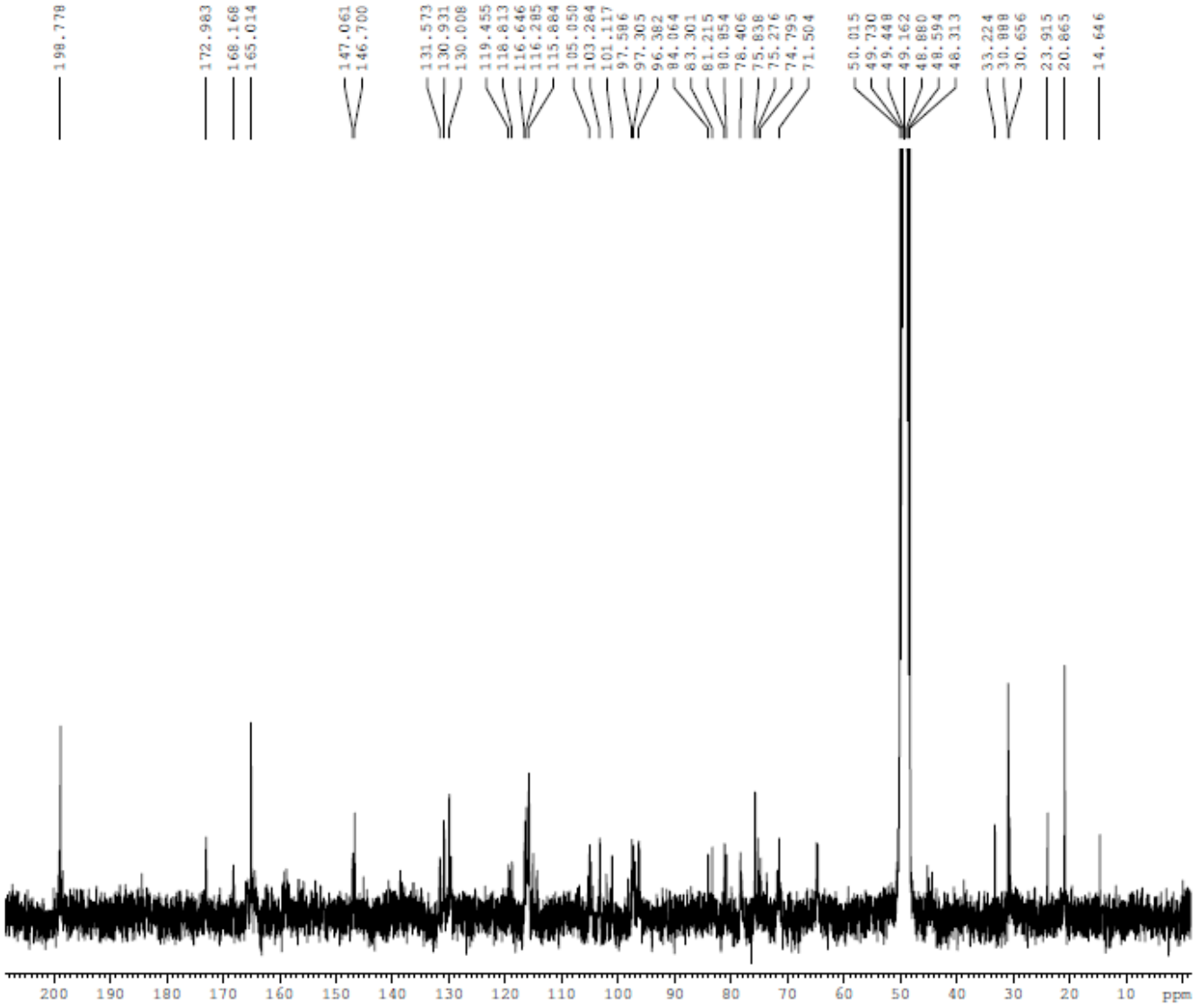


FIG. 1. ^{13}C NMR Spectrum of Gb-2a-7-o-Glucose (MeOD solvent, 300MHz)



Gig. 3. MSMS Morelloflavone

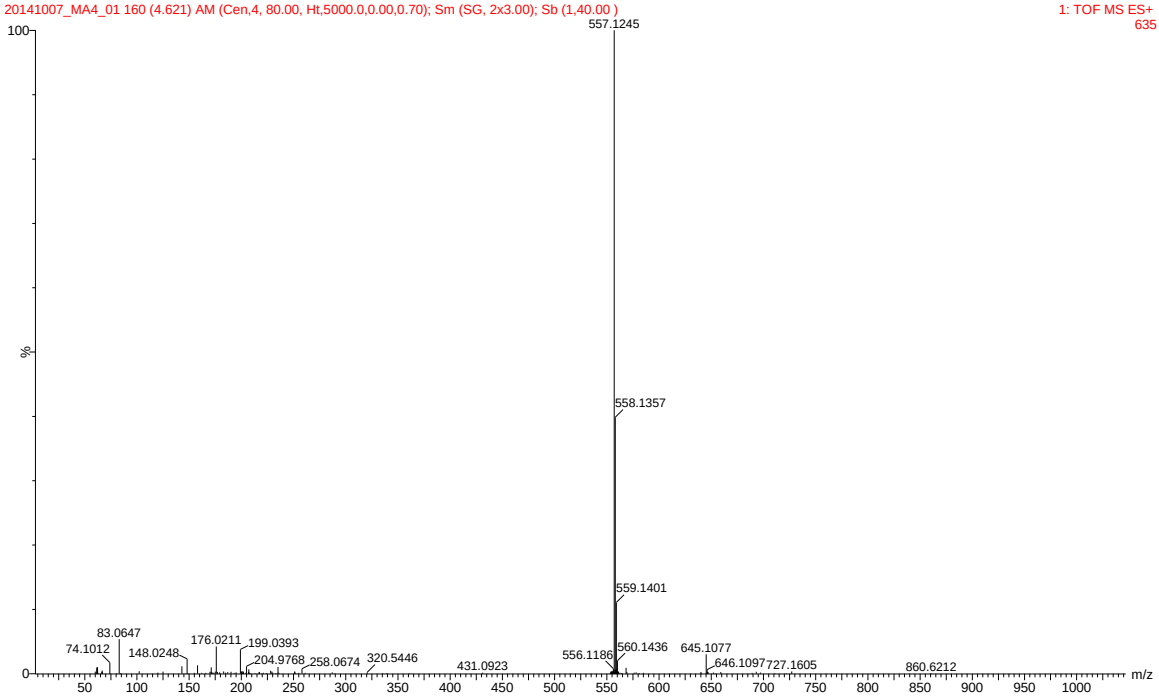


Fig. 4. MSMS Gb-2a

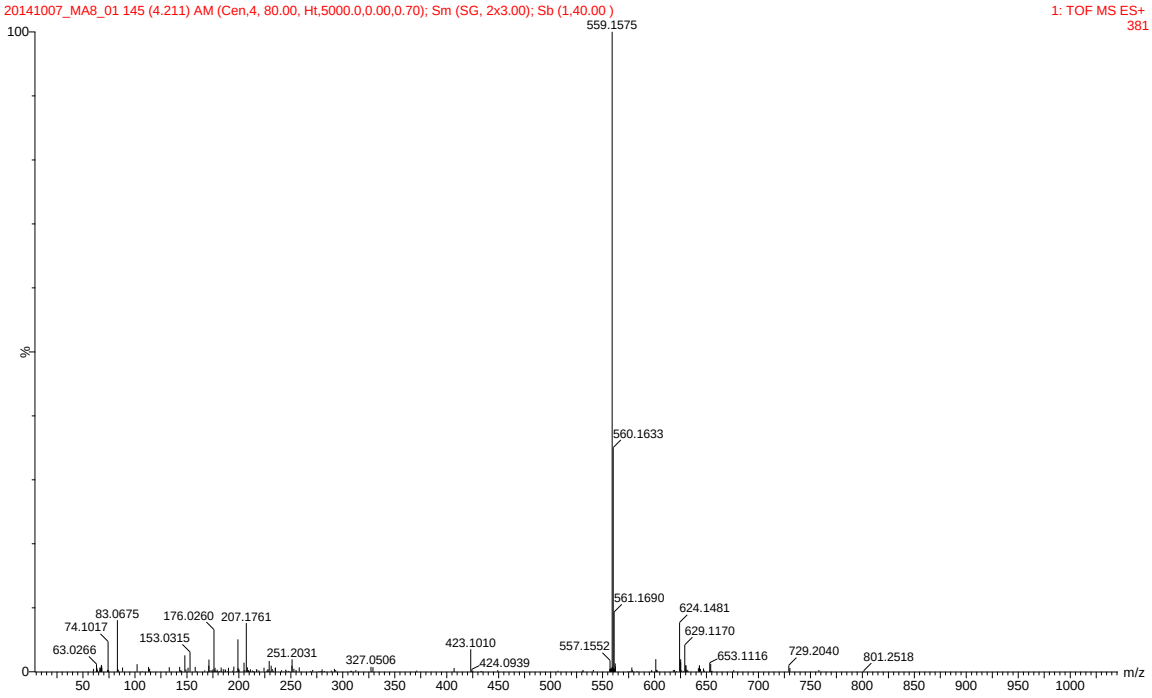


Fig 5. MSMS Gb-2a-7-O-Glucose