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FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Uso alternativo do tabaco: extração de 3-*O*-metilquercetina, associação dos flavonóides 3-*O*-metilquercetina e quercetina com ciclodextrina, estudo de modelagem molecular e de permeação cutânea *in vitro* com vistas na sua utilização no tratamento de herpes simplex.

LIEGE CASSIA SCHWINGEL

Orientadora: Profª. Dr. Valquiria Linck Bassani

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Tese apresentada por **Liege Cassia Schwingel** para obtenção do GRAU DE DOUTOR em Ciências Farmacêuticas.

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Profª Dr. Elenara Maria Teixeira Lemos Senna
Universidade Federal de Santa Catarina

Prof Dr. Hubert Karl Stassen
Universidade Federal do Rio Grande do Sul

Profª Dr. Gilsane Linno Von Poser
Universidade Federal do Rio Grande do Sul

Schwingel, Liege Cassia
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Margarida Maria Cordeiro Fonseca Ferreira, CRB 10/480
Heloisa do Canto Canabarro, CRB 10/1036

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"*A mente que se abre a uma nova idéia jamais volta ao seu tamanho original*."

Albert Einstein

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RESUMO

O incentivo para descontinuar o uso do tabaco despertou o interesse em investigar alternativas para o uso da planta, pois a cultura do tabaco é bem estabelecida e apresenta papel econômico relevante na região sul do Brasil. No presente trabalho, o flavonóide 3-*O*-metilquercetina foi isolado a partir das folhas de *Nicotiana tabacum* L. (Solanaceae) por cromatografia em camada delgada preparativa e por cromatografia em coluna, sendo posteriormente caracterizado. A baixa solubilidade em água das agliconas quercetina e 3-*O*-metilquercetina já é bem conhecida, o que tem motivado vários estudos sobre a complexação dessas moléculas com as ciclodextrinas. Com vistas a aprofundar os estudos preliminares de modelagem molecular, realizados para compreender o fenômeno de complexação da 3-*O*-metilquercetina com ciclodextrina, no presente trabalho foi realizado um estudo de modelagem molecular utilizando mecânica quântica, em que os mínimos de energia foram obtidos pelo método semi-empírico Recife Model 1. Os resultados revelaram que, em água, a inserção do anel B na cavidade da *β*-ciclodextrina pela borda das hidroxilas secundárias é a conformação mais provável, enquanto que no vácuo, esta é obtida pela inserção do anel A pela borda das hidroxilas primárias. Ainda constam o desenvolvimento de formulações de hidrogéis de quitosana e hipromelose para aplicação tópica contendo os flavonóides quercetina ou 3-*O*-metilquercetina, e suas associações com β-ciclodextrina, visando ao estudo da atividade antiviral *in vivo* frente ao Vírus Herpes Simplex Tipo 1 em modelo de camundongo nu imunodeprimido. Os hidrogéis foram caracterizados e sua permeação nas diferentes camadas da pele foi avaliada. A avaliação do perfil de liberação e penetração/retenção dos flavonóides 3-*O*-metilquercetina e quercetina nas camadas da pele a partir de hidrogéis de hipromelose ou quitosana demonstrou que, na ausência de ciclodextrina, o primeiro é mais adequado para a liberação e penetração do flavonóide metilado, enquanto que o segundo mostrou favorecer a liberação e penetração do flavonóide de maior polaridade, a quercetina. A *β*-ciclodextrina influenciou a liberação dos flavonóides da base e a penetração destes nas camadas da pele, especialmente na epiderme mais profunda, local alvo para o tratamento do herpes simplex tipo 1.

Palavras-chave: *Nicotiana tabacum;* 3-*O*-metilquercetina; quercetina; β-ciclodextrina; permeação cutânea; Vírus Herpes Simplex Tipo 1

ABSTRACT

The incentive to discontinue tobacco use awakened interest in investigating alternatives to the use of the plant, because the tobacco growing is well established and plays an important role in the economics of southern Brazil. In this study, the flavonoid 3-*O*-methylquercetin was isolated from leaves of *Nicotiana tabacum* L. (Solanaceae) by either preparative thin-layer chromatography or column chromatography, and characterized. The low water solubility of the aglycones quercetin and 3-*O*-methylquercetin is well known, which has motivated many studies on these molecules complexation with cyclodextrins. In order to extend the preliminary molecular modeling studies carried out to understand the phenomenon of 3-*O*-methylquercetin complexation with cyclodextrin, a molecular modeling study was performed using quantum mechanics, where the energy minima were obtained by semi-empirical Recife Model 1 method. The results reveal that in water, the insertion of the B ring into the *β*-cyclodextrin cavity through the secondary hydroxyl rim is the most probable conformation, while in vacuum, the most probable conformation is obtained by inserting the A ring into the cavity through the primary hydroxyl rim. The development of semi-solid formulations for skin topical application containing quercetin and/or 3-*O*-methylquercetin and their associations with *β*-cyclodextrin is also included, in order to study the *in vivo* antiviral activity against the Herpes Simplex Virus Type 1 in immunocompromised (nude) mice model. The hydrogels were characterized and its permeation in different skin layers was evaluated. The evaluation of release and penetration/ retention of the flavonoid 3-*O*-methylquercetin and quercetin in the skin layers from hydropropyl methylcellulose or chitosan hydrogels showed that in the absence of cyclodextrin, the former is more appropriate for the release and penetration of methylated flavonoids, while the latter favored the release and penetration of the more polar flavonoid, quercetin. The *β*-cyclodextrin influenced the release and penetration of flavonoids on the skin layers, especially the epidermis, which is target for the treatment of herpes simplex virus type 1.

Keywords: *Nicotiana tabacum*; 3-*O*-methylquercetin; quercetin; *β*-cyclodextrin, skin permeation; Herpes Simplex Virus Type 1.

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I. INTRODUÇÃO

A cultura do tabaco exerce importante papel no desenvolvimento da região sul do Brasil, que concentra quase a totalidade da produção nacional, seja pela arrecadação de impostos ou devido à importante demanda por mão-de-obra de natureza familiar. Considerando a expectativa de que o consumo da planta decline em todo o mundo nos próximos anos e que o Brasil, maior exportador mundial, encontre dificuldades para escoar a produção, o impacto que a redução global do consumo poderá trazer no longo prazo sobre a demanda de produção abre espaço para a busca de alternativas economicamente viáveis. Apesar de existirem alternativas ao tabaco, a falta de recursos para investimentos, ausência de canais de comercialização para grande parte das culturas agrícolas tradicionais, juntamente com outras restrições tais como limitações na infra-estrutura de transporte e armazenamento, são fatores que contribuem para dificultar a transição da cultura do tabaco para cultivos alternativos. Neste contexto, a utilização do tabaco pela indústria farmacêutica torna-se uma alternativa interessante a ser considerada, e o isolamento do flavonóide 3-*O*-metilquercetina a partir das folhas da planta é inserido nesta realidade de forma inovadora e inédita.

A infecção pelo Vírus Herpes Simplex Tipo 1 é muito comum em humanos, sendo uma das principais causas de morbidade, especialmente em pacientes imunodeprimidos. A busca de novas alternativas para a terapia antiviral tem sido motivada pelo elevado custo das terapias disponíveis e/ou os potenciais efeitos colaterais e, em especial, pelo desenvolvimento de resistência viral a esses medicamentos.

A administração tópica de antiviral consiste numa alternativa exequível para a redução dos efeitos colaterais que seriam potencialmente obtidos pelas demais vias. A efetividade de agentes tópicos é determinada pela sua capacidade de permear através do epitélio, estando disponível nas terminações nervosas sensoriais, onde ocorre a replicação viral. Em geral, a terapia tópica é menos efetiva do que outras formas de intervenção, parcialmente devido a problemas relacionados à falta de acessibilidade ao sítio de ação, o que remete à necessidade de estudos tecnológicos detalhados das formulações.

I.1. Vírus Herpes Simplex Tipo 1

Os medicamentos utilizados na terapia antiviral, no caso de infecções causadas pelos vírus herpes simplex tipo 1 e tipo 2, contém em sua composição substâncias tais como idoxuridina, trifluoridina, ibacitabina, vidarabina, citarabina, além do aciclovir e seus derivados fanciclovir, pencivlovir, ganciclovir, valaciclovir e foscarnet (DE CLERCQ, 1993; BRADY e BERNSTEIN, 2004; LIMA, 2004; TIERNEY JR *et al*., 2005; FATAHZADEH e SCHWARTZ, 2007). O elevado custo das terapias e/ou os potenciais efeitos colaterais e, em especial, o desenvolvimento de resistência viral aos medicamentos contendo estas substâncias ativas tem motivado a busca de novas alternativas para a terapia antiviral (McCORMACK, 1996). A resistência ao aciclovir e análogos de nucleosídeo pode ocorrer pela mutação na timidina kinase do HSV-1 ou DNA-polimerase. Portanto, novos antivirais com mecanismos de ação diferentes são necessários (KHAN *et al*., 2005).

Neste contexto, diversos estudos relativos à atividade anti-herpética de extratos de plantas medicinais e de substâncias isoladas a partir dos mesmos têm sido descritos na literatura (KHAN *et al*., 2005). SIMÕES e colaboradores (1999) relataram atividade antiviral de extratos aquosos e hidroalcoólicos das inflorescências de *Achyrocline satureioides* (marcela). Mais recentemente, BETTEGA e colaboradores (2004) demonstraram atividade inibitória *in vitro* do produto seco por aspersão frente à replicação do vírus herpético tipo 1 (HSV-1), sendo que a atividade foi atribuída principalmente à presença de constituintes flavonoídicos, em especial, à quercetina e 3-*O*-metilquercetina.

AMOROS e colaboradores (1992) investigaram a atividade *in vitro* da própolis e de seus principais flavonóides, e constataram ação antiviral da quercetina. Em 2001, GONÇALVES e colaboradores publicaram um estudo sobre a atividade antiviral *in vitro* de extratos de *Vitex polygama* (Verbenaceae) ricos em flavonóides (especialmente quercetina, luteolina e 3-*O*-metilquercetina) contra o HSV-1 resistente ao aciclovir.

A seleção do modelo de camundongo nu imunodeprimido para a avaliação de infecções cutâneas pelo HSV-1 é justificada pela comparabilidade das

circunstâncias ocorridas durante a infecção herpética de pacientes imunodeprimidos. Além disso, muitas cepas do HSV resistentes ao aciclovir produzem manifestações significativas em camundongos atímicos, entretanto a sua virulência reduzida resulta na manifestação apenas moderada da doença em camundongos imunocompetentes.

I.2. *Nicotiana tabacum*

O tabaco (*Nicotiana tabacum* L.) pertence à família Solanaceae (mesma da batata e do tomate). É considerado um híbrido provável entre *N. sylvestris* e *N. otophora* ou *N. tomentosiformis* (GOODSPEED, 1954), constituindo um anfidiplóide, com 2n = 4x = 48. Tem como origem provável a América do Sul, mais precisamente o Noroeste da Argentina e a região dos Andes. É cultivada entre as latitudes de 60ºN e 38ºS (AKEHURST, 1981).

O cultivo do tabaco é perene (altura média de 1 a 2 metros) e constitui-se em uma das principais atividades agrícolas da região Sul do Brasil, devido ao sistema de produção que possibilita o cultivo em pequenas áreas, com boa remuneração para os pequenos agricultores, o que lhe confere relevante papel sócio-econômico, além de se constituir em matéria-prima para um importante complexo industrial.

Espécies do gênero *Nicotiana* são referidas como tóxicas, ornamentais, e algumas são fonte de substâncias inseticidas, como a anabasina, a nicotina e a nornicotina (VIEIRA *et al*., 2003). Já *Nicotiana tabacum* L. é amplamente conhecida por sua importância econômica, como fonte de matéria-prima para a indústria do tabaco, por suas propriedades estimulantes e por ser muito utilizada em pesquisas científicas nas áreas de farmácia, fisiologia, virologia e engenharia genética (GOODSPEED, 1954; HAWKES, 1999; HUNZIKER, 2001).

Estudos mostraram que os tricomas existentes no tabaco excretam flavonóides em resposta ao estresse ambiental e ao ataque de herbívoros, e que 3-*O*-metilquercetina e outros derivados metilados da quercetina são os principais flavonóides encontrados em exsudatos de *Nicotiana* (YANG *et al*., 1960; WOLLENWEBER e DÖRR, 1995).

I.2.1. Quercetina

O flavonóide quercetina é um composto polifenólico difundido no reino vegetal e possui uma gama de atividades relatadas, dentre as quais se destacam as atividades antioxidante e antiviral. Estudos realizados por KAUL e colaboradores (1985) demonstraram que a quercetina reduziu de forma concentração-dependente a infectividade de diversos vírus testados, incluindo HSV-1, atuando por meio de uma interação irreversível com os vírus. VRIJSEN e colaboradores (1988) demonstraram a atividade virucida da quercetina contra vírus envelopados, como é o caso do vírus herpético tipo 1. O mecanismo referente à atividade antiviral da quercetina parece estar relacionado com a sua capacidade de ligação à proteína viral e à interferência na síntese de ácido nucléico viral (FORMICA e REGELSON, 1995; GONÇALVES *et al*., 2001).

I.2.2. 3-*O*-Metilquercetina

A 3-*O*-metilquercetina é um derivado metilado da quercetina, e também tem apresentado importante atividade sobre diversos vírus (VAN HOFF *et al.*, 1984; KAUL *et al.*, 1985; CASTRILLO *et al*., 1986; SEMPLE *et al.*, 1999; SIMÕES *et al*., 1999). Atua no bloqueio da replicação do poliovírus pela interferência na cópia intermediária da fita-simples do RNA viral (CASTRILLO e CARRASCO, 1987) em associação com o bloqueio da síntese protéica celular (VRIJSEN *et al*., 1987). Devido ao mecanismo de ação, à atividade antiviral pronunciada de amplo espectro, e à falta de indução de resistência pelas 3-metóxi-flavonas, esta última verificada por NINOMIYA e colaboradores (1985), diversos estudos relacionam a atividade antiviral com a presença da 3-*O*-metilquercetina em extratos vegetais.

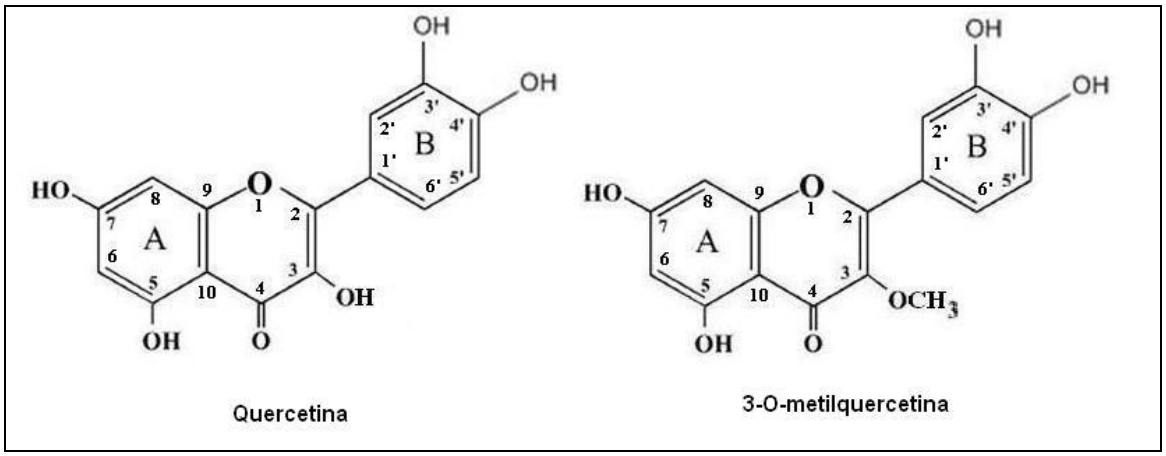

Figura 1. Estrutura química dos flavonóides quercetina e 3-*O*-metilquercetina.

RODA e colaboradores (2003) descreveram a presença de quercetina e de 7 derivados metilados nos tricomas glandulares de *Nicotiana attenuata*, estando a 3-*O*-metilquercetina em maior quantidade nas extrações da superfície da folha. Os extratos da superfície de folhas jovens apresentaram mais quercetina do que as folhas mais velhas, indicando uma possível diluição pelo crescimento, sem suficiente síntese nova ou com conversão em um metabólito diferente. Estudos anteriores mostraram que os tricomas excretam flavonóides, e que 3-*O*-metilquercetina e outros derivados metilados da quercetina são majoritários em exsudatos de *Nicotiana* (YANG *et al*., 1960; WOLLENWEBER e DÖRR, 1995).

A 3-*O*-metilquercetina, isolada e sintetizada, foi testada *in vitro* em linhagens de células e *in vivo* em camundongos para atividade antipicornaviral (VAN HOOF *et al*., 1984). Em culturas de células de rim de macaco verde africano (células VERO), foi observada uma inibição de 90% do Poliovírus Tipo I e Coxsackie B4, em concentrações de 0,01 µg/mL; o efeito permaneceu inalterado com o aumento da concentração da 3-*O*-metilquercetina para 25 µg/mL. Em concentração de 5 µg/mL, também apresentou atividade antiviral contra o vírus da estomatite vascular. A 3-*O*-metilquercetina foi bem tolerada (*in vitro*) pelas células VERO e fibroblastos de pele humana por mais de 5 dias de exposição, sendo a concentração citotóxica para 50% (TC_{50}), 40 µg/mL (VAN HOOF *et al*., 1984).

I.3. Ciclodextrinas

A eficácia clínica de um fármaco aplicado topicamente depende das suas propriedades biológicas, disponibilidade e concentração no sítio de ação da pele. Frequentemente a permeação intrínseca de substâncias bioativas é insuficiente, sendo necessário o uso de estratégias de formulação para alcançar o aumento da penetração tópica na pele. Neste sentido, substâncias promotoras de permeação têm sido empregadas, destacando-se as ciclodextrinas.

As ciclodextrinas são oligossacarídeos cíclicos com variável número de unidades de glicose, possuindo relativa solubilidade em água. São moléculas cristalinas de estrutura tronco-cônica com capacidade de auto-organização, sendo a β-ciclodextrina (βCD) – com 7 unidades de glicose, uma das mais empregadas na indústria farmacêutica (SALTÃO e VEIGA, 2001), por se encontrar com o prazo de vigência da patente expirado, além de ser a de menor custo. Por possuírem uma cavidade hidrofóbica, as ciclodextrinas tendem à formação de complexos de inclusão com substâncias que possuam uma maior afinidade por elas do que pelo meio onde estão inseridas, em geral o meio aquoso. Este sistema proporciona inúmeras vantagens tecnológicas para as formulações, dentre as quais se destaca a alteração da hidrossolubilidade dos fármacos e a melhora da sua estabilidade (BACKENSFELD *et al*., 1991; LOFTSSON e BREWSTER, 1996; TOROS DE ILARDUYA *et al*., 1998; OZDEMIR e ORDU, 1998; PERDOMO-LOPEZ *et al*., 2002; DEL VALLE, 2004; ODA *et al*., 2004; TOMMASINI *et al*, 2004).

Entre as ciclodextrinas naturais, a βCD apresenta a menor solubilidade (1,85 g/100mL), porém esta é, freqüentemente, suficiente para melhorar consideravelmente a solubilidade e, conseqüentemente, a biodisponibilidade de fármacos muito pouco solúveis (DUCHÊNE e WOUESSIDJEWE, 1990). Sua cavidade, com diâmetro de 6,0 a 6,5 Å, tende a complexar compostos aromáticos e heterocíclicos. Uma propriedade interessante das ciclodextrinas está relacionada com a sua capacidade de promover a liberação de fármacos em membranas biológicas agindo como verdadeiros carreadores, pois este efeito pode ocasionar aumento da disponibilidade do fármaco na superfície dessas barreiras biológicas (pele e mucosa, por exemplo) e a promoção de sua penetração (DEL VALLE, 2004).

As ciclodextrinas também podem interagir com alguns componentes lipídicos da pele, provocando a sua extração e conseqüente desorganização do extrato córneo, atuando como promotor de penetração cutânea de certas substâncias (LEGENDRE *et al*., 1995; MATSUDA e ARIMA, 1999). Neste caso, a ciclodextrina pode ser simplesmente adicionada à formulação, sem prévia complexação com a substância de interesse.

SCHWINGEL e colaboradores (2008) relataram estudos da associação de 3-*O*-metilquercetina com β-ciclodextrina, na forma de complexo e de simples mistura, verificando um aumento da hidrossolubilidade do flavonóide quando associado à β-ciclodextrina. Também realizaram estudos sobre a influência das associações na permeação cutânea deste flavonóide, utilizando células de Franz e modelo de pele de orelha de suíno. Em relação a estes estudos, foi verificado que a complexação com β-ciclodextrina promove um aumento bastante significativo na permeação cutânea. Ainda neste sentido, incorporou o flavonóide e as associações, individualmente, em hidrogel para investigar a influência da utilização de hipromelose (HPMC) na permeação cutânea. Traçando-se um comparativo entre a 3-*O*-metilquercetina e as formulações estudadas, verificou que a permeação ocorreu, de forma crescente, na seguinte ordem: 3-*O*-metilquercetina < mistura < complexo em gel < 3-*O*-metilquercetina em gel < mistura em gel < complexo.

PETRY e colaboradores (2007) compararam os incrementos de hidrossolubilidade de diferentes formas polimórficas encontradas em amostras comerciais de quercetina, sendo estes determinados pela complexação com β-ciclodextrina. Ainda, investigaram os efeitos da β-ciclodextrina ou de hidróxipropil-β-ciclodextrina, na ausência ou presença de HPMC, sobre a hidrossolubilidade de uma das amostras. Os resultados obtidos demonstraram diferentes capacidades de complexação dos diferentes polimorfos encontrados em amostras comerciais de quercetina, características provavelmente relacionadas com suas diferentes solubilidades intrínsecas. A associação da amostra com β-ciclodextrina resultou em incremento de hidrossolubilidade de 4,6 vezes, e de 6,5 vezes quando associada com β-ciclodextrina e HPMC. A associação com hidróxipropil-β-ciclodextrina, uma ciclodextrina mais solúvel, resultou em incremento de hidrossolubilidade de cerca de

38 vezes, e de cerca de 62 vezes quando o complexo foi associado ao HPMC. A simples associação da quercetina com HPMC resultou em incremento de hidrossolubilidade de apenas 2,8 vezes, o que claramente evidencia um efeito sinérgico entre as ciclodextrinas e HPMC sobre a hidrossolubilidade da quercetina.

Tendo em vista a necessidade de alternativas para a utilização do tabaco em função de questões econômicas e de políticas de saúde pública, a extração e purificação do flavonóide 3-*O*-metilquercetina a partir da planta demonstra ser de extrema relevância, pois este, além de ser de difícil obtenção e possuir alto custo, vem apresentando importante atividade biológica. O presente trabalho está pautado no desenvolvimento de hidrogéis contendo 3-O-metilquercetina (extraído das folhas de tabaco) ou quercetina visando à aplicação tópica sobre a pele e na investigação da influência de β-ciclodextrina sobre a permeação/retenção dos flavonóides nas camadas da pele.

I.4. OBJETIVOS

Considerando a necessidade de alternativas para a utilização do tabaco e a busca por novas moléculas bioativas para o tratamento de afecções de pele ocasionadas pelo HSV-1, o objetivo do presente trabalho é extrair e purificar o flavonóide 3-*O*-metilquercetina a partir das folhas de *Nicotiana tabacum* com posterior incorporação do flavonóide em hidrogéis visando ao uso tópico comparativamente à aglicona análoga, quercetina, com subseqüente investigação da influência de β-ciclodextrina na retenção dos flavonóides nas camadas da pele.

Assim, os seguintes objetivos específicos foram propostos:

1. realizar o cultivo de *Nicotiana tabacum* L. (Solanaceae) sem o auxílio de defensivos químicos;

2. extrair e isolar o flavonóide 3-*O*-metilquercetina a partir das folhas de *Nicotiana tabacum* L. (Solanaceae), caracterizando-o e analisando o grau de pureza;

3. desenvolver e caracterizar hidrogéis de hipromelose ou quitosana contendo quercetina ou 3-*O*-metilquercetina;

4. avaliar a permeação/retenção dos flavonóides nas camadas da pele a partir dos hidrogéis de hipromelose e quitosana;

5. verificar o efeito da *β*-ciclodextrina sobre a permeação da 3-O-metilquercetina e quercetina em pele de orelha de suíno, *in vitro*, a partir de hidrogéis de hipromelose e quitosana;

6. realizar cálculos de mecânica quântica para modelagem molecular das agliconas 3-*O*-metilquercetina e quercetina, bem como de suas associações com β-ciclodextrina.

II.1. CAPÍTULO 1

II.1. ARTIGO 1 – Liege C. Schwingel, Valquiria L. Bassani. Potential alternative use of tobacco in the pharmacotherapy

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Potential alternative use of tobacco in the pharmacotherapy

Liege C. Schwingel and Valquiria L. Bassani*

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil

* Corresponding author: Tel.: +55 51 33083602; fax: +55 51 33085437.

E-mail address: valquiria.bassani@ufrgs.br (V.L. Bassani).

Abstract

The world imminent crisis regarding the consumption of tobacco as cigarettes has been awakening the attention for the search of alternative employ for the plant. As the tobacco culture is well established and has remarkable importance at the southern Brazil economy, this review presents and discuss some perspectives regarding the plant application for pharmacotherapy purpose. In this context, the extraction of nicotine and various tobacco leaf flavonoids using low costed solvents and procedures is a promising reality. The aim of the present work is to point out an healthy alternative use of tobacco and promote the sustainability and the economic development of the tobacco cultivation regions.

Keywords: Nicotiana tabacum; Tobacco; flavonoids; nicotine.

1. Introduction

The cultivation of tobacco is well established and widely practiced worldwide. The plant culture plays an important role in the economic development, especially of southern Brazil. The global health policy for reducing the use of tobacco as cigarettes raised the urge for alternatives to its employ. This review reflects over the potential use of tobacco for pharmaceutical application. Amongst the leaf components, nicotine and tobacco flavonoids are suitable for this purpose.

The major tobacco alkaloids are nicotine, conitine, nornicotine, myosmine, nicotyrine, anabasine and anatabine. Amongst these alkaloids, the purification of nicotine for pharmacotherapy purpose seems to be of remarkable interest, since there is a great number of patents regarding this subject. The nicotine content can range in concentration from 0.5 to 8% in the major cultivated tobacco species, *N. tabacum* and *N. rustica*. Major polyphenolics found in tobacco include chlorogenic acid, rutin, scopoletin and scopolen, along with quercetin and kaempferol (Leffingwell, 1999).

Flavonoids are generally present as water-soluble glycosides in cell vacuoles, but when present in the epicuticular wax on the leaf surface, flavonoids are nonglycosylated, are very often *O*-methylated, and are lipophylic (Gould and Lister, 2006). In 1960, Yang and coworkers described the occurrence of monomethyl and dimethyl ethers of quercetin containing a O-methyl group at the 3-position which, at that time, have been obtained only by laboratory synthesis. They performed the isolation and identification of quercetin-3,3'-dimethyl ether from tobacco flowers. This research group have also found other related flavonol ether tentatively identified as 3-O-methylquercetin (3-O-MQ).

Trichomes from *Nicotiana* excrete flavonoids such as 3-*O*-methylquercetin, 3,7-*O*-dimethylquercetin and 3,3'-*O*-dimethylquercetin (Wollenweber and Dörr, 1995; Yang et al., 1960). The majority of quercetin produced by a leaf is conjugated at R3, either with rutinose as rutin and stored in the mesophyll or with a methyl group and externalized, possibly as a means of decreasing the reactivity of the aglycone (glycosylation of flavonols may render the molecule less reactive towards free radicals in addition to being more water-soluble, which also facilitates storage). According to Roda et al. (2003), leaf surface extracts from younger leaves presented more quercetin than older leaves, indicating a possible dilution by growth without sufficient new synthesis or its conversion to a different metabolite. Nicotine was found in both trichomes and mesophyll. As expected from polarity, neither rutin nor nicotine was found on the leaf surface.

Flavonols are thought to protect plants against UV-induced damage, pathogen and herbivore attack, as well as oxidative stress, and their accumulation may be elicited by these stresses (Grotewold et al., 1998; Harborne and Williams, 2000; Shirley, 1996). It has been demonstrated that thermal stress induces the production of phenolic compounds, such as flavonoids. This kind of condition also enhances the antioxidant capacity of the shoot extracts, indicating that this may be the primary function of these cold-inducible flavonoids (Gould and Lister, 2006). The flavonoid *O*-methylation tends to shift the UV absorption properties to shorter wavelengths so that they typically absorb significantly in the 250 to 320 nm region. Thus, *O*-methylated flavonoids are better able to protect plant leaves from UV-B damage (Gould and Lister, 2006).

Roda et al. (2003) observed that 3-O-MQ, quercetin 4'-methyl ether, quercetin 3,5-dimethyl ether, and quercetin 3,7-dimethyl ether extracted from the leaf surface

of *N. attenuata* showed a slope of zero considering the flavonoids amount in function of leaf area, suggesting that at an early stage during ontogeny the trichomes cease further production of these metabolites or that their synthesis equaled their loss from the leaf surface.

2. Biological activity of tobacco main compounds for pharmaceutical application

Amongst the plant constituents, tobacco flavonoids and nicotine seem to be of remarkable interest for pharmacotherapy employ. The therapeutic interest on 3-*O*-methylquercetin potential lies on its several *in vitro* and *in vivo* biological activities reports, such as anti-inflammatory, antioxidant, neuroprotective, bronchodilatory, vasodilatory, antinociceptive, immunomodulatory, antitumor and antiviral. Among the mechanisms involved, the inhibition of prostaglandin and phosphodiesterase, inactivation of free radicals and inhibition of viral RNA synthesis are highlighted (Castrillo and Carrasco, 1987; Dok-Go et al., 2003; Hnatyszyn et al., 2004; Ko et al., 2002; Ko et al., 2003; Ko et al., 2004; Küpeli and Yesilada, 2007; Okoko and Oruambo, 2009; Rubio et al, 2006; Sadhu et al., 2006; Vrijsen et al., 1987).

There's a special interest that concerns the antiviral and antitumor activities, considering the molecular conformation and polarity assigned by the *O*-methylation. Antiviral studies have shown that 3-O-MQ inhibits an earlier step of viral replication (between 1 and 1.5 h after the infection), reducing the RNA and viral proteins synthesis (Vanden Berghe et al., 1993; Vlietinck et al., 1986; Vrijsen et al., 1987). A report (Bettega et al., 2004) on a standardized *Achyrocline satureiroides* (Lam.) DC. Asteraceae spray dried powder containing, respectively, 0.933 and 0.70% (w/w) of

quercetin and 3-O-MQ + luteolin, demonstrated that it presents *in vitro* activity against the Herpes Simplex Virus (HSV-1).

Another flavonoid with increased interest is quercetin. Quercetin (3,5,7,3´,4´-pentahydroxy flavone) (Q) is one of the most abundant bioflavonoids, being present in edible fruits and vegetables. It is one of the most potent antioxidants among polyphenols. Quercetin has also been demonstrated to display the antiviral, antibacterial, anticarcinogenic and antiinflammatory effects.It is capable of interacting with and modulating activity of a variety of enzyme systems including cyclooxygenase, lipooxygenase, phosphodiesterase and tyrosine kinase (viral (Kaul et al., 1985; Vrijsen et al., 1988; Formica and Regelson, 1995; Gonçalves et al., 2001). Quercetin presence in plants is often as heterosides forms, especially as rutin.

Rutin presents several pharmacological activities including antioxidant, antiallergic, anti-inflammatory and vasoactive, antitumor, antibacterial, antiviral and antiprotozoal properties. In addition, hypolipidaemic, cytoprotective, antispasmodic and anticarcinogenic are also reported (Chen et al., 2000; Ihme et al., 1996; Deschner et al., 1991; Panasiak et al., 1989; Park et al., 2002; Janbaz et al., 2002; Mata et al., 1997; Webster et al., 1996).

Even the wide occurrence of quercetin in plants, the presence of this molecule in tobacco, together with other flavonoids, could represent a possibility of employ a tobacco flavonoid fraction in phytotherapy/

3. Reports of flavonoids extraction from tobacco

Most of work regarding flavonoid extraction from tobacco refers flowers or leaf as raw material.

Yang and cols. (1960) reported an extraction of the flowers from *Nicotiana tabacum* which were previously oven-dried and further extracted with n-pentane, benzene, chloroform, ethyl acetate (anhydrous) and acetone. Each extract was studied by paper chromatography. The flavonol ethers were mostly in the chloroform fraction, although at least two compounds were present in the ethyl acetate extract. The yield obtained was insufficient to elucidate the identity of the constituents.

Roda and coworkers (2003) reported the extraction of various quercetin methylated flavonoids from the leaf of *Nicotiana attenuate* using acetonitrile. The obtained extracts could be used for further purification process, however the solvent chosen for the extraction procedure presents high cost and important toxicity.

Our research group performed the isolation and purification of the flavonoid 3-*O*-methylquercetin from the leaves of *Nicotiana tabacum* L. (Solanaceae) using ethanol for extraction and subsequent purification performed by preparative thin layer chromatography or column chromatography with a chloroform:methanol system (Schwingel et al., 2013). We also investigated the influence of plant cultivation parameters such as plant variety, cultivation type, leaf size and harvest time on the 3-O-MQ content. It is worth emphasizing that the same flavonoid has been also isolated from inflorescences of *Achyrocline satureioides* (Lam.) DC. Asteraceae (Schwingel et al., 2008) and that its synthesis is very complicated, since the income is low and there is a formation of toxic subproducts of difficult elimination (Krishnamachari et al., 2004).

4. Conclusions

Tobacco is a complex plant material from a chemical composition point of view. No other plant material has been studied more extensively in the history of man. Yet even at the moment, advances in tobacco research are accomplished every day. The reports mainly concern the improvement of the quality of tobacco, especially regarding the nicotine content as well as subjective sensorial aspects, reached through genetic engineering, agricultural practices and harvesting procedures. However, nowadays, against the well proved health deleterious use of tobacco as cigarettes, it is mandatory the search for an alternative use of tobacco in order to promote the sustainability and the economic development of the tobacco cultivation regions. The use of tobacco as a source of molecules presenting therapeutical interest seems to be a promising perspective for the pharmaceutical industry.

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II.1. ARTIGO 2 – Liege C. Schwingel, Geraldo O. Schwingel, Nirlei Storch, Fabiano Barreto, Valquiria L. Bassani. 3-*O*-Methylquercetin: from organic *Nicotiana tabacum* L. trichomes: influence of the variety, cultivation and extraction parameters.

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3-*O*-Methylquercetin from organic *Nicotiana tabacum* L. trichomes: influence of the variety, cultivation and extraction parameters.

Liege C. Schwingel[a], Geraldo O. Schwingel[b], Nirlei Storch[b], Fabiano Barreto[a], Valquiria L. Bassani[a,*]

[a]Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[b]Conselho Regional de Engenharia, Arquitetura e Agronomia do Rio Grande do Sul – CREA-RS

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil

* Corresponding author: Tel.: +55 51 33083602; fax: +55 51 33085437.

E-mail address: valquiria@pq.cnpq.br (V.L. Bassani).

Conselho Regional de Engenharia, Arquitetura e Agronomia do Rio Grande do Sul – CREA-RS

Rua Venâncio Aires 1448, CEP 96810-100, Santa Cruz do Sul, RS, Brazil

Abstract

3-*O*-methylquercetin (3-MQ) is a flavonoid aglycone that presents important biological properties among them antioxidant, antiviral and anticancer. The presence of 3-MQ in *Nicotiana tabacum* L. trichomes is well known as a response to the plant environmental stress or herbivores attack. On the other hand, it has been synthesized, but with low income and toxic sub-products formation of difficult separation. Thus, the present work reports the use of a simple method for extraction and isolation of 3-MQ from organic *N. tabacum* trichomes, in order to turn it available in higher amount and purity for biological tests or pharmaceutical applications. The influence of some parameters related to the plant variety, cultivation, extraction and isolation conditions on the yield of the flavonoid is reported. The highest 3-MQ yield was obtained using ethanol in the primary extraction, a drug:solvent ratio of 1:15 and extraction time of 30 minutes. The isolation of 3-MQ from the primary extract was successfully performed using ethyl acetate extraction followed by normal phase column chromatography, using chloroform and methanol as solvent in different proportions. The small leaves (younger) of the Dark variety, cultivated in vase and collected at night showed the highest 3-MQ yield. Taken together, the choice of the best conditions resulted in the obtention of 3-MQ with over 90% purity and 60% yield. These excellent results may be useful for the alternative use of tobacco.

Keywords: Nicotiana tabacum; Tobacco; 3-*O*-Methylquercetin isolation; alternative use of tobacco.

1. Introduction

The tobacco crop is a well established culture which plays an important role in the economic development of southern Brazil. In contrast with its use as cigarettes, the potential for pharmaceutical application becomes an interesting alternative. Flavonoids are generally present as water-soluble glycosides in cell vacuoles, but when present in the epicuticular wax on the leaf surface, flavonoids are nonglycosylated and very often *O*-methylated (Gould and Lister, 2006).

Trichomes from *Nicotiana* sp. excrete flavonoids such as 3-*O*-methylquercetin (5,7,3,4'-tetrahydroxyflavone) (3-MQ) (Fig. 1), 3,7-*O*-dimethylquercetin and 3,3'-*O*-dimethylquercetin (Wollenweber and Dörr, 1995; Yang et al., 1960). The majority of quercetin produced by a leaf is conjugated at R3, either with rutinose as rutin and stored in the mesophyll or with a methyl group and externalized, possibly as a means of decreasing the reactivity of the aglycone (glycosylation of flavonols may render the molecule less reactive towards free radicals in addition to being more water-soluble, which also facilitates storage). According to Roda et al. (2003), leaf surface extracts from younger leaves presented more quercetin than older leaves, indicating a possible dilution by growth without sufficient new synthesis or its conversion to a different metabolite. Nicotine was found in both trichomes and mesophyll. As expected from polarity, neither rutin nor nicotine was found on the leaf surface.

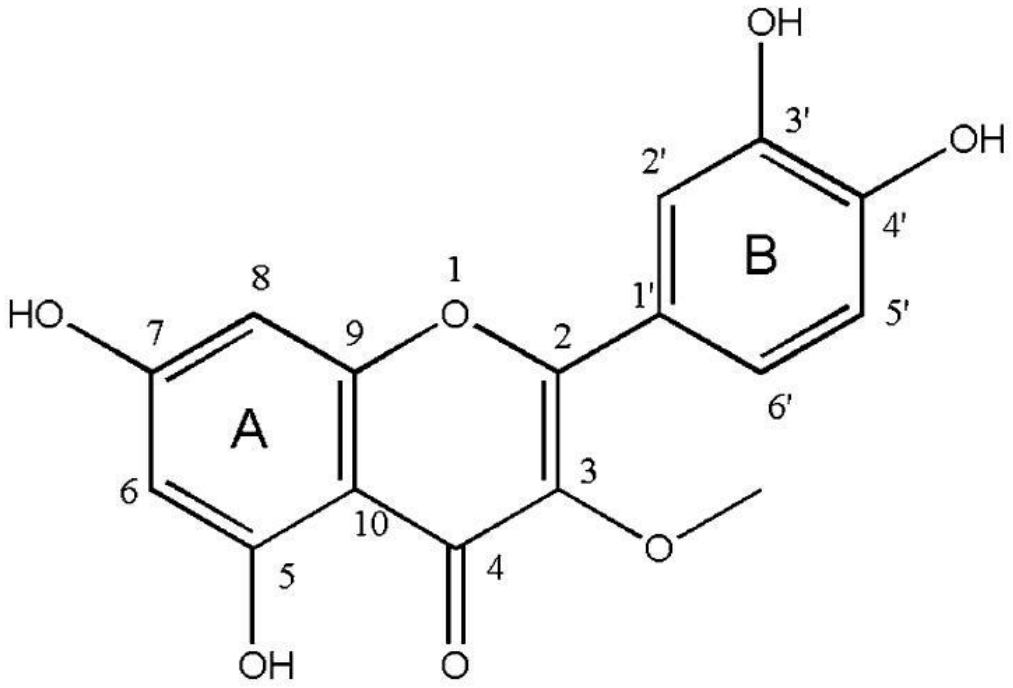

Fig. 1. 3-*O*-methylquercetin molecular structure.

Flavonols are thought to protect plants against UV-induced damage, pathogen and herbivore attack, as well as oxidative stress, and their accumulation may be elicited by these stresses (Grotewold et al., 1998; Harborne and Williams, 2000; Shirley, 1996). Based on these information, and knowing that 3-*O*-methylquercetin is a main constituent from tobacco trichomes exudates, this flavonoid isolation from the leaves of tobacco is an innovative and promising reality.

The therapeutic interest on 3-*O*-methylquercetin potential lies on its several *in vitro* and *in vivo* biological activities reports, such as anti-inflammatory, antioxidant, neuroprotective, bronchodilatory, vasodilatory, antinociceptive, immunomodulatory, antitumor and antiviral. Among the mechanisms involved, the inhibition of prostaglandin and phosphodiesterase, inactivation of free radicals and inhibition of viral RNA synthesis are highlighted (Carini et al., 2013; Castrillo and Carrasco, 1987; Dok-Go et al., 2003; Hnatyszyn et al., 2004; Ko et al., 2002; Ko et al., 2003; Ko et al.,

2004; Küpeli and Yesilada, 2007; Okoko and Oruambo, 2009; Rubio et al, 2006; Sadhu et al., 2006; Vrijsen et al., 1987).

There is a special interest that concerns the antiviral and antitumor activities, considering the molecular conformation and polarity assigned by the *O*-methylation. Antiviral studies have shown that 3-MQ inhibits an earlier step of viral replication (between 1 and 1.5 h after the infection), reducing the RNA and viral proteins synthesis (Vanden Berghe et al., 1993; Vlietinck et al., 1986; Vrijsen et al., 1987). A report on a standardized *Achyrocline satureiroides* (Lam.) DC. Asteraceae spray dried powder containing, respectively, 0.933 and 0.70% (w/w) of quercetin and 3-MQ + luteolin, demonstrated that it presents *in vitro* activity against the Herpes Simplex Virus (HSV-1) (Bettega et al., 2004).

Recently, Carini and cols.(2013) reported a review on the promising 3-MQ anticancer activity related to its cytotoxic/antiproliferative activity against several human cancer cell lines (lapatinib sensitive (SK-Br-3) and resistant (SK-Br-3-Lap R) breast cancer cells, MCF7 breast cancer, Hep2 hepatoma cells, HL60-leukemia, A431-epidermoid carcinoma, HeLa-cervical cancer, SKOV3-ovarian carcinoma and HOS-hosteosarcoma), discussing the involved targets in this activity.

Thus, considering the requirement for new therapeutically active molecules and the potential of 3-MQ, especially for antiviral and anticancer therapies, the aim of the present work is the extraction, the isolation and purification of the flavonoid 3-MQ from the leaves of *Nicotiana tabacum* L. (Solanaceae), in order to obtain this flavonoid in higher amount either for biological tests or pharmaceutical applications. Looking through the socioeconomic prism, an alternative use of tobacco against the imminent economic crisis regarding its consume around the world presents high

interest. To accomplish this objective, the influence of the tobacco variety, the main cultivation and extraction parameters on the yield of 3-MQ was studied.

2. Material and Methods

2.1. Plant material

Four varieties of *N. tabacum* were cultivated: Dark, Maryland, Virginia 1048 and Virginia 2542. The cultivars are respectively registered in the Brazilian Cultivar Protection Service of the Brazilian Agriculture and Environmental Ministry under the numbers 02587, 09894, 06035 and 21095. Tobacco was grown in Santa Cruz do Sul (RS, Brazil) without the use of pesticides or fertilizers (using only black dirt) in either soil or vase (under controlled conditions): the seed pellets (>90% germination) were sown in May 2010 using the float system with polystyrene trays over water containing substrates, and the seedlings were transplanted in September. The voucher specimen was deposited at the HAS herbarium under the no. ICN 174138 (Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, RS, Brazil).The leaves were collected from November to October, at predetermined hours and freshly used.

2.2. Optimization of 3-MQ extraction

Preliminary studies were performed to determine the appropriate solvent for exudates extraction: one entire leaf of the *N tabacum* var. Dark was immersed separately in each of the following solvent, ethanol, acetone, acetonitrile, chloroform, methanol, ethyl acetate, chloroform:methanol 95:5 (v/v), in a 1:10 drug/solvent

proportion for 10 minutes. The supernatant was separated and the leaf was rinsed subsequently for two times generating 3 different extracts for each solvent. The solvent was evaporated under reduced pressure and the 3 residues were dissolved and filtered in methanol for 3-MQ determination. The extraction with each solvent was carried out three times.

After choosing the solvent and the number of subsequent extractions, other extraction parameters were tested: drug/solvent proportion and extraction time, at three different levels (1:10, 1:15 and 1:20 drug/solvent proportions; 10 min, 30 min and 60 min of extraction time). The 1:50 drug/solvent proportion was also tested for 60 min.

2.3. Influence of N. tabacum variety, cultivation and collection of the leaves on the 3-MQ yield

Four varieties of *N. tabacum* were compared, Dark, Maryland, Virginia 1048 and Virginia 2542, regarding the 3-MQ production. The investigation of the influence of the cultivation in soil or vase was carried out for all varieties, in the conditions described at plant material section (2.1). The vases were maintained in an agricultural shed with limited sun exposure. Samples were collected in triplicate for each variety from either soil or vase at the same time, and the 3-MQ extraction was performed with ethanol at 1:15 drug/solvent proportion for 30 minutes. The solvent was evaporated and the residue was dissolved in methanol to determine the 3-MQ concentration by LC.

For the Dark variety, the influence of plant characteristics such as the leaf size and the harvest time was also investigated. In order to determine the flavonoid

concentration over the leaf growth stage, three different leaf sizes were analyzed, 10 cm, 20 cm and 30 cm, approximately. In addition, the leaves were collected at four different times during the same day, at 00:00, 08:00, 11:30 and 18:30. In all samples (n = 3), the 3-MQ was extracted using ethanol at 1:15 drug/solvent proportion for 30 minutes. The solvent was removed under reduced pressure and the residue was dissolved in methanol to determine the 3-MQ concentration by LC.

2.4. Isolation of 3-MQ

The ethanolic extracts from the tobacco leaves were filtrated and dried under reduced pressure and the residue was weighted and resuspended with two subsequent 30 mL aliquots of ethyl acetate, which resulted in a preliminary extraction of 3-MQ. The remaining precipitate (water soluble) was discarded. Then, the ethyl acetate fraction was dried and the residue was weighted. This residue was diluted in 70 mL of methanol and the quantification was performed by LC. The methanol volume was reduced to 5 mL for chromatographic purpose. The isolation of 3-MQ was performed either by preparative thin layer chromatography or by column chromatography.

2.4.1. Preparative thin layer chromatography

The residue dissolved in 5 mL of methanol was applied in silica gel preparative TLC using $CHCl_3$:MeOH (90:10) as mobile phase. After the elution, the corresponding band (R_f 0.4) was collected and 4 successive extractions were performed: the first one with 50 mL of MeOH and the others with 10 mL of MeOH.

Each suspension was sonicated for 30 min and filtered (G4). The last filtration was performed using a 0.2 µm membrane filter. The solution was concentrated in order to obtain purified 3-MQ.

2.4.2. Column chromatography

The isolation of 3-MQ was performed using a normal phase system. Each column was packed with 25 g of silica gel dispersed in chloroform and compacted for 24 h. The 5 ml methanolic extract was uniformly applied on the top of the silica column. The elution was performed with 50 mL of $CHCl_3$, 150 mL of $CHCl_3$:MeOH (98:2, v/v) and 200 mL of $CHCl_3$:MeOH (95:5, v/v). Ten milliliter aliquots from the last fraction were collected in amber vials in a dripping speed of 30 drops per minute.

The flavonoid was characterized by means of LC, mass spectrometry and ^1H NMR.

2.5. 3-MQ quantitation

Samples were analyzed by HPLC based on previous study (Schwingel et al., 2008). Briefly, a Shimadzu SCL-10 equipment with a Shimadzu LC-10AD pump was used and a Shim-pack CLC-ODS (M) RP-18 column (5 µm, 250 mm x 4 mm i.d.), with Alltech refillable pre-column. The mobile phase consisted of a methanol–water (70:30, v/v) mixture acidified with 0.1% of trifluoracetic acid (TFA), filtered and degassed by suction-filtration through a nylon membrane, in isocratic flow. The flow was 0.8 mL min^{-1}, with an injection of 50 µL and 0.05 AUFS of sensitivity at 354 nm.

The LC system was operated at room temperature (23 ± 1 ^{o}C). The LC method was revalidated according to ICH (data not shown).

2.6. LC-MS analyses

A LC system Agilent 1200 series (Agilent Technologies, Palo Alto, CA, USA) coupled to triple quadrupole API 5000 mass spectrometer (Applied Biosystems/Sciex, Foster City, CA, USA) using electrospray ionization interface in negative ionization mode (ESI+) was employed in LC-MS analyses. The software used to spectrometer control, data acquisition and data processing was Analyst 1.4.2 (Applied Biosystems/Sciex). Acquisition data was performed in multiple reaction monitoring (MRM) mode. Nitrogen was used as nebulizer gas, curtain gas, heater gas and collision gas (psi units). Collision gas (CAD) was set at 7 psi. Curtain gas was set 15 psi. Nebulizer gas (GS1) was set at 55 psi. Heater gas pressure and temperature were set at 55 psi and 700 ^{o}C, respectively. Electrospray capillary voltage was set at -3.5 kV.

2.7. Statistical analyses

One-way analysis of variance (ANOVA) with Tukey *post hoc* test was used for statistical comparison. The level of significance was taken as $p < 0.05$.

3. Results and Discussion

3.1. Identification of 3-MQ in the N. tabacum trichomes

Several studies have shown that tobacco trichomes excrete flavonoids, being 3-MQ and other methylated derivatives of quercetin, the main flavonoidic constituents in *Nicotiana* exudates (Roda et al., 2003; Wollenweber and Dörr, 1995; Yang et al., 1960). Based on this knowledge, a preliminary tobacco extract was obtained by the immersion of tobacco leaf in ethanol in a drug:solvent proportion of 1:10 for 10 min. The LC analysis of the extractive solution showed the presence of a peak with retention time of 7 minutes, similar to that shown by the reference compound 3-MQ (Extrasynthèse, France) (Fig. 2).

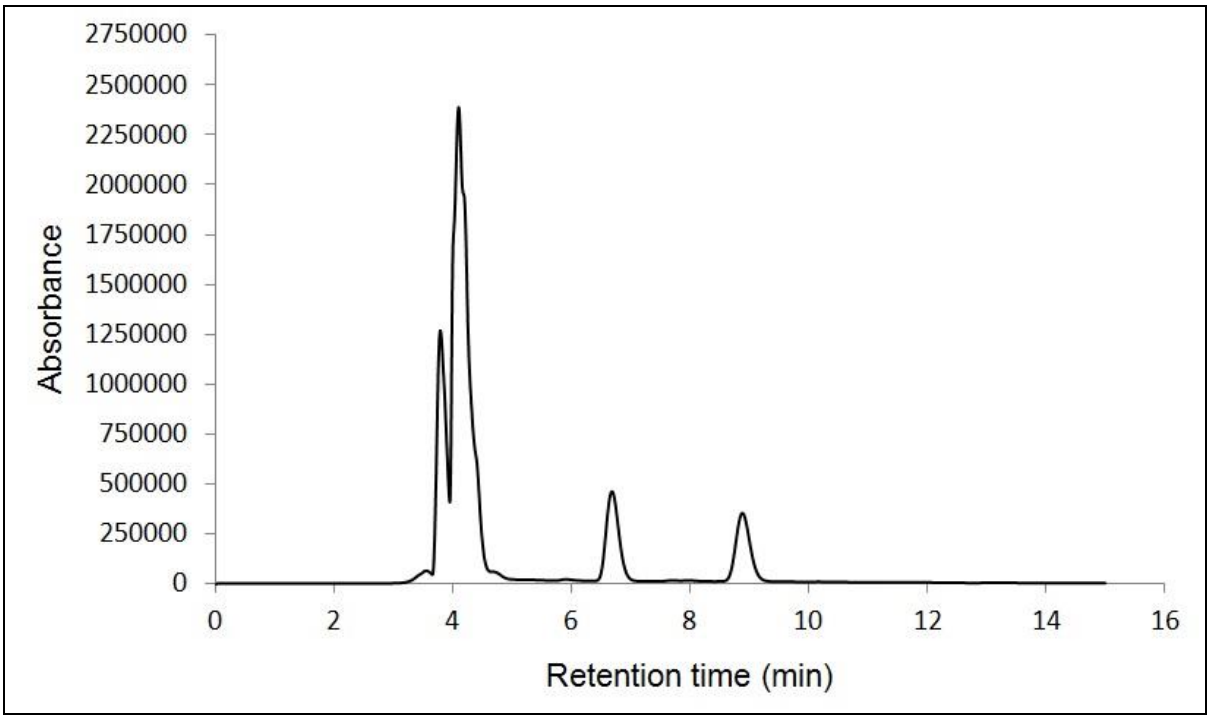

Fig. 2. Tobacco ethanolic extract HPLC profile.

The identity of 3-MQ was also established by the analysis of LC-MS fragmentation patterns (Fig. 3) and ^{1}H NMR.

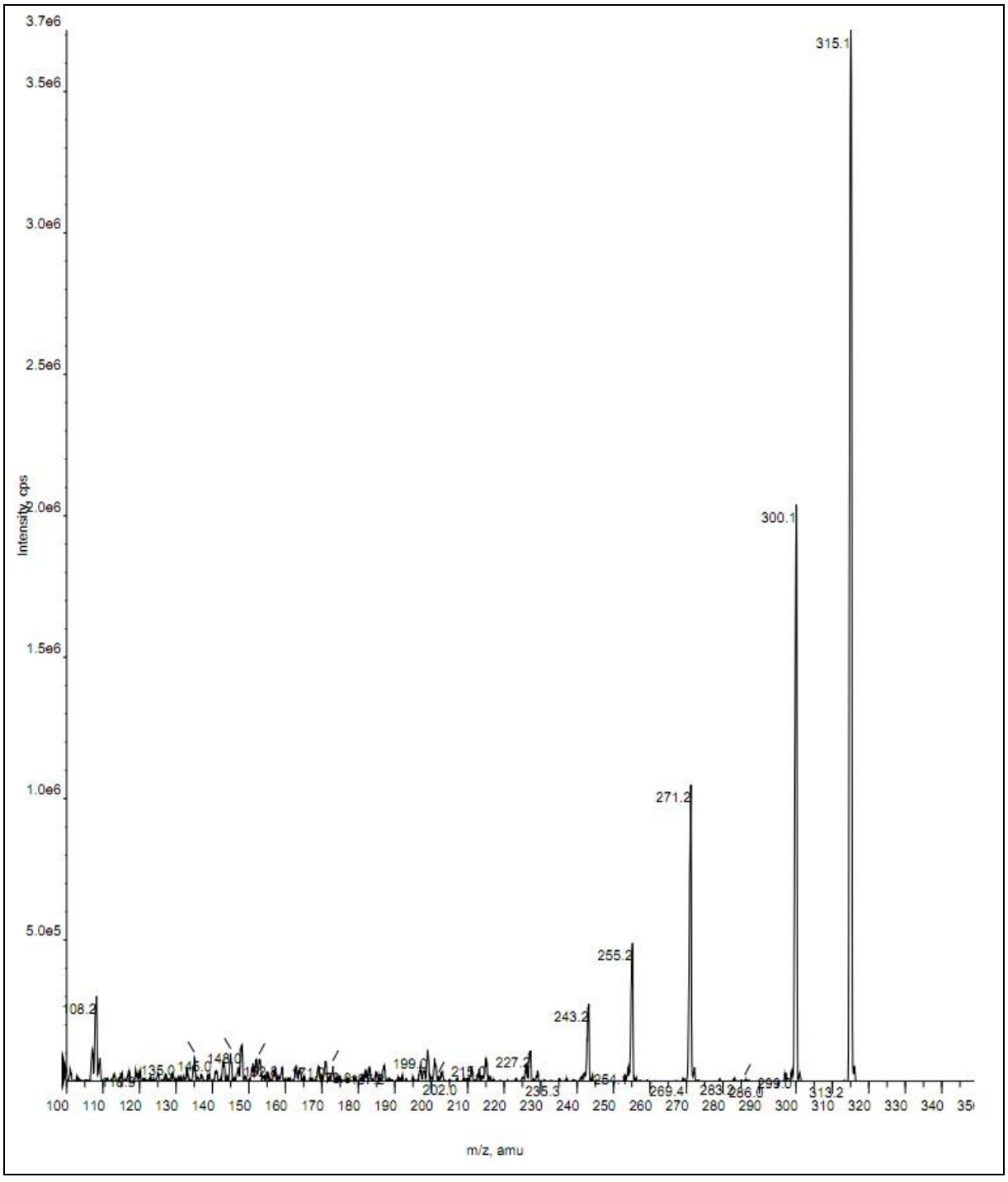

Fig. 3. 3-*O*-methylquercetin LC/MS fragmentation profile.

The presence of the molecular ion 315 (because of the negative ionization) and the M-15 fragment, formed by loss of the methyl group connected to the position 3 oxygen of the C ring, are the main indicative of the monomethylated flavonoid structure (Fig. 4). Other important fragments are *m/z* 273 $[M - 43]^+$ representing the loss of a methyl radical and a carbonyl group, possibly as an acetyl radical confirming the structure of a methoxylated flavonol; *m/z* 153, which shows the A ring dihydroxylation (this fragment is formed by a retro-Diels-Alder fragmentation with hydrogen transfer); *m/z* 137, which shows the B ring dihydroxylation. According to the ^{1}H NMR analysis: δ = 7.57 (d, *J* = 2.02 Hz, H-2'), 6.93 (d, *J* = 8.59 Hz, H-5'), 7.47 (dd, *J* = 8.59 Hz, *J* = 2.02 Hz, H-6'), 6.22 (d, *J* = 2.02 Hz, H-6), 6.43 (d, *J* = 2.02 Hz, H-8), 3.80 (s, O-CH_3).

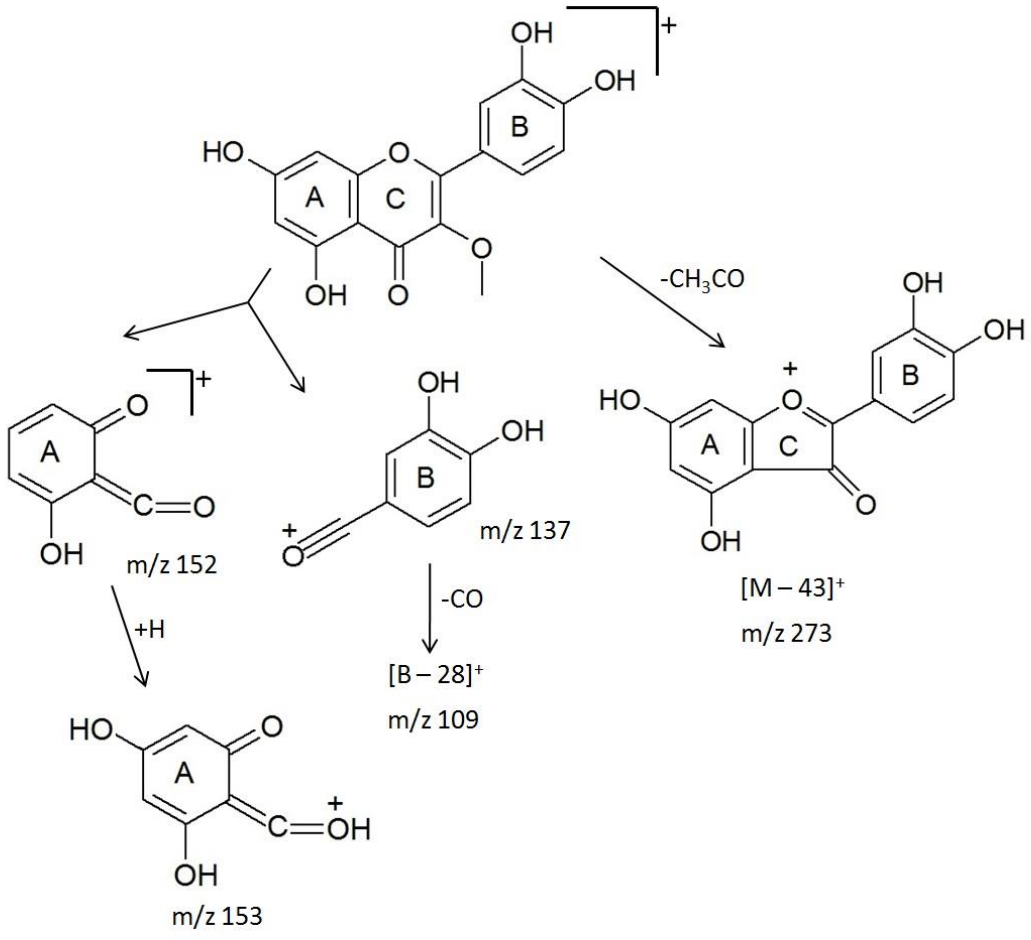

Fig. 4. 3-*O*-methylquercetin fragmentation structures.

The extract was also submitted to hydrolysis in order to determine the presence of heterosides. Extreme hydrolysis conditions were applied (HCl 2M, 80 ^{o}C, 4 h) to ensure the total aglycone release. The 4 min peak was replaced by a 6 min peak, indicating the presence of rutin and its aglycone, quercetin. The identity of these peaks was obtained by co-elution of the respective reference substances followed by LC-MS analyses.

The last peak (9 min) presented identical fragmentation profile to the 3-MQ, indicating that any methylation on the B ring must be discarded. The hypothesis lies on the substitution of the A ring on C5 or C7, resulting, respectively, in 5-*O*-methylquercetin (azaleatin) or 7-*O*-methylquercetin (rhamnetin). This molecule was isolated for further identification.

Knowing that nicotine is the characteristic alkaloid of tobacco, the presence of this substance in the extractive solution was also investigated. A peak with retention time (3.8 min) corresponding to that of the reference substance was detected. The identity of the peak was also confirmed by LC-MS analysis.

3.2. Optimization of the extraction conditions

The results obtained in the selection of the suitable solvent are shown on Table 1. Considering only the first extraction, ethanol presented the highest 3-MQ yield (2.78 ± 0.02 µg/g). The extractions performed with acetone, acetonitrile and methanol presented similar results whereas 3-MQ concentrations in chloroform extracts were under the detection limit. On the other hand, the higher amount of 3-MQ after the third extraction was obtained yet again with ethanol (3.64 ± 0.02 µg/g), followed by ethyl acetate and $CHCl_3$:MeOH (95:5, v/v). Moreover, the extraction purpose is to

obtain a higher 3-MQ concentration associated to a reduced number of other tobacco constituents. Besides the highest yield, this background supported the preference for ethanol and ethyl acetate, being the first one “cleaner”, less toxic and presenting low-cost.

Table 1. Analyses of the solvent selection and number of extractions for 3-MQ extraction from *Nicotiana tabacum* leaves.

	3-MQ concentration (µg/g)			
Solvent	1st extraction	2nd extraction	3rd extraction	Total
Ethyl acetate	1.54 ± 0.04	0.75 ± 0.05	0.53 ± 0.06	2.82 ± 0.05
Acetone	0.92 ± 0.02	0.46 ± 0.01	0.23 ± 0.01	1.61 ± 0.01
Acetonitrile	0.93 ± 0.03	0.71 ± 0.02	0.41 ± 0.03	2.05 ± 0.03
Ethanol	2.78 ± 0.02	0.66 ± 0.01	0.20 ± 0.04	3.64 ± 0.02
Chloroform:methanol 95:5 v/v	2.02 ± 0.06	0.57 ± 0.01	0.20 ± 0.01	2.79 ± 0.03
Chloroform	–	–	–	–
Methanol	0.94 ± 0.03	0.40 ± 0.01	–	1.34 ± 0.02

The drug:solvent proportion was 1:10 and the extraction time was 10 min.

In order to identify the optimal conditions for the extraction, three drug:ethanol proportions were tested at three different times (n = 3). According to Fig. 5, the higher amount of 3-MQ was obtained using ethanol either in a 1:15 drug/solvent proportion for 30 minutes (3-MQ yield: 6.64 ± 0.04 µg/g) or 1:10 drug/solvent proportion for 60 minutes (3-MQ yield: 6.65 ± 0.06 µg/g). The 60 minutes extracts presented a significant number of substances, which difficult the flavonoid isolation. The extraction time has a particular influence over the 3-MQ yield at lower drug/solvent proportions. With higher amounts of solvent, the equilibrium in the extraction process is reached later. For 30 minutes of extraction, there was no statistically significant difference between the 1:10 and 1:20 drug/solvent proportions ($p < 0.05$). The 1:50

drug/solvent proportion presented no positive effect over the 3-MQ extraction, demonstrating that the use of a great amount of solvent is not necessary. On the other hand, when the plant/solvent time of contact is longer, the extraction of the constituents present not only on the leaves surface is probable.

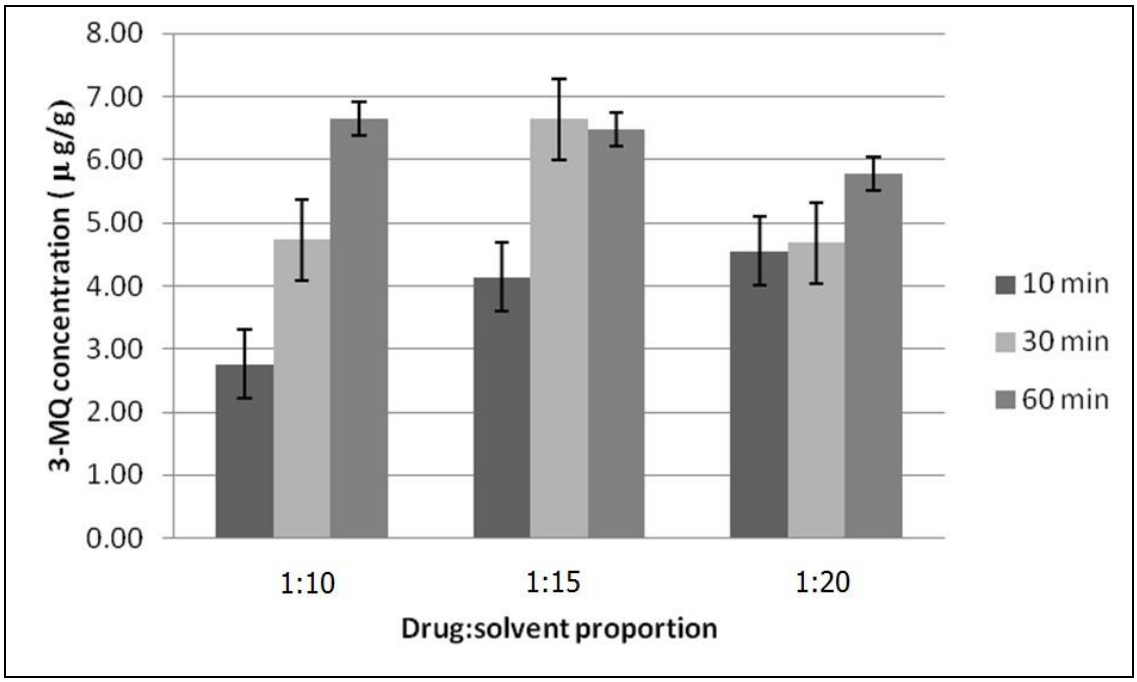

Fig. 5. Optimization of the extraction conditions drug:solvent proportion and extraction time.

3.3. Analyses of the cultivation and harvest conditions

Regarding the tobacco cultivation, the soil seems to increase the 3-MQ concentration in the plant ($p < 0.05$), since it is excreted for defense purpose; whereas the protection provided by the vase from the climatic extreme variations allowed to extend the tobacco growing until the next crop. The Dark variety presented the best 3-MQ yield, followed by Virginia 2542 (Fig. 6). The increased amount of flavonoid obtained by the Dark variety when cultivated in the vase will be

further investigated. In vase, there were no statistical differences among the varieties, except when compared with the Dark variety. In soil, the difference was statistically significant only when compared with the Virginia 2542 variety. The cultivation type presented no difference for the Maryland variety.

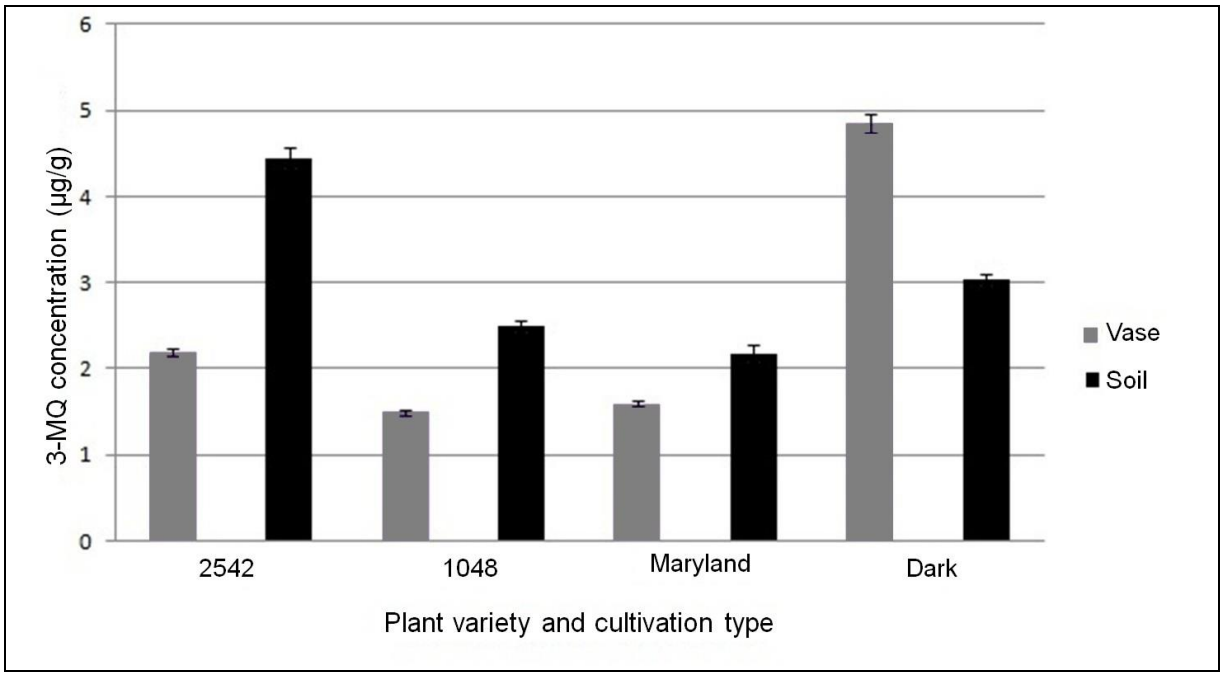

Fig. 6. Influence of plant variety and cultivation type in 3-*O*-methylquercetin yield.

The harvest time also matters at the plant grown: at midnight the plant presented the highest 3-MQ yield, whereas at 8 a.m. the flavonoid concentration was the lowest (Fig. 7). It has been demonstrated that thermal stress induces the production of phenolic compounds, such as flavonoids: the chilling stress promotes the formation of yellow flavonoids. Such conditions also enhanced the antioxidant capacity of the shoot extracts, indicating that this may be the primary function of these cold-inducible flavonoids (Gould and Lister, 2006). The second 3-MQ peak occurred at ca. 12 a.m. The flavonoid *O*-methylation tends to shift the UV absorption properties to shorter wavelengths so that they typically absorb significantly in the 250

to 320 nm region. Thus, *O*-methylated flavonoids are better able to protect plant leaves from UV-B damage (Gould and Lister, 2006). The differences were statistically significant ($p < 0.05$).

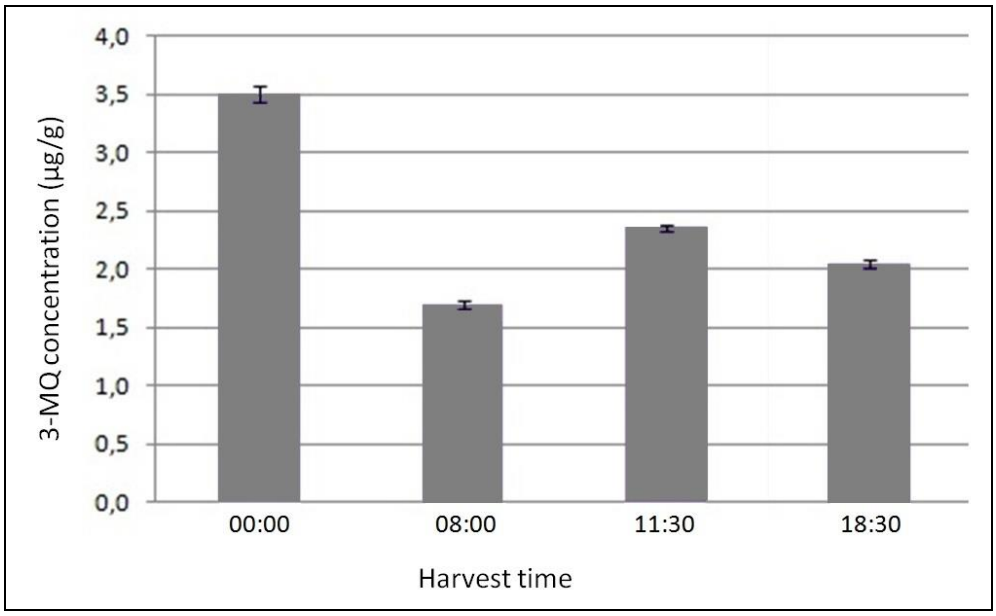

Fig. 7. Influence of the harvest time in 3-*O*-methylquercetin yield.

The results also demonstrate that 3-MQ concentration is inversely proportional to the leaf size (Fig. 8). Roda et al. (2003) observed that 3-MQ, quercetin 4'-methyl ether, quercetin 3,5-dimethyl ether, and quercetin 3,7-dimethyl ether extracted from the leaf surface of *N. attenuata* showed a slope of 0 considering the flavonoids amount in function of leaf area, suggesting that at an early stage during ontogeny the trichomes cease further production of these metabolites or that their synthesis equaled their loss from the leaf surface.

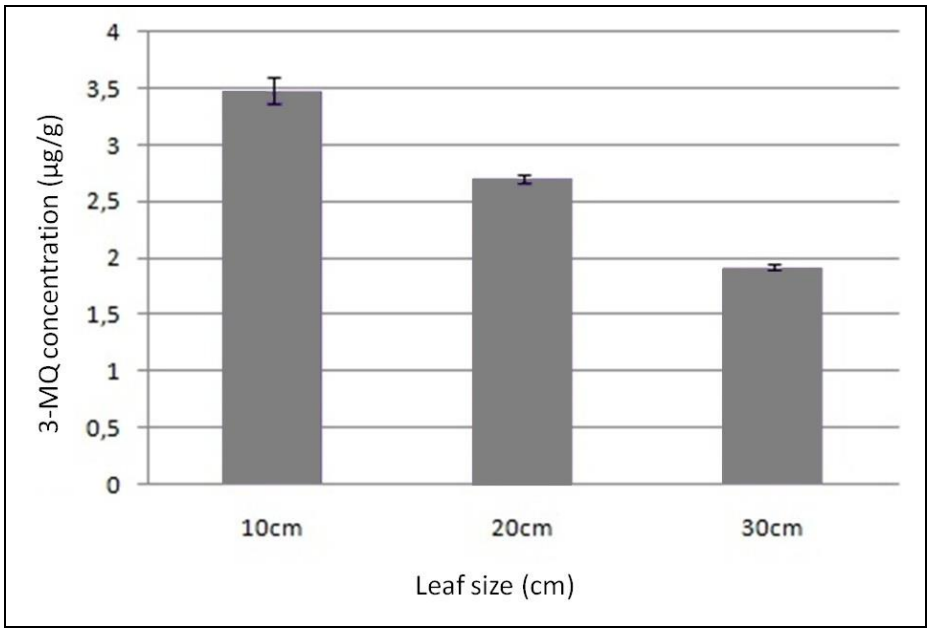

Fig. 8. Influence of leaf size in 3-*O*-methylquercetin yield.

An important data is the 3-MQ concentration in the plant after the harvest: the concentration decreases 24 % after 24 h and 34 % after 48 h at room temperature, without any protection. On the other hand, when the leaves were kept in an insulated container (thermal bag), the flavonoid loss was much lower: 11 % and 19 %, respectively. The differences were statistically significant ($p < 0.05$). These results are encouraging, considering the exceptionally easy and simple extraction method using ethanol and its technical feasibility by the tobacco growers, which can use the fresh leaves for the primary extract production.

3.4. Purification and isolation of 3-MQ

In order to obtain a suitable flavonoid fraction, with lower amount of nicotine, the ethanol extract was evaporated and the residue extracted with ethyl acetate. The residual 3-MQ remaining in the water soluble residue was small (data not shown).

The LC analyses revealed a significant nicotine and rutin reduction in the ethyl acetate fraction. This purification step contributed to obtaining 3-MQ in a higher purity, especially when the final isolation was performed with preparative TLC.

Normal phase preparative thin layer chromatography and column chromatography were suitable for 3-MQ isolation, since it presented good separation using chloroform:methanol system. Preparative TLC presented an 38% yield in relation to the total 3-MQ extraction, whereas the CC yield was 60%, reaching over 90% of purity.

Column chromatography allowed the use of a more concentrated extract (obtained with 60 min) since the separation depends on the silica column size and dripping speed (data not shown). Within this technique, sample previous extraction with ethyl acetate is desirable however not mandatory. In general, the column chromatography is preferable over the preparative TLC for 3-MQ isolation from tobacco ethanolic extracts because of its income and efficiency in addition to its practicality.

It is worth emphasizing that this flavonoid has been isolated from inflorescences of *Achyrocline satureioides* (Lam.) DC. Asteraceae (Schwingel et al., 2008) and from other plants. It has been also synthesized, but with low income and toxic subproducts formation of difficult elimination (Krishnamachari et al., 2004).

4. Conclusions

3-*O*-Methylquercetin was successfully extracted from organic *Nicotiana tabacum* leaves exudates using ethanol (which is a green solvent). Besides the very good 3-MQ yield, the easy plant extraction with ethanol provides a technical

advantage, since it is feasible for the growers to perform and allows the fresh plant employ, without the necessity of previously dry it. The excellent selectivity of the extraction solvent allows further one step 3-MQ purification, reaching over 90% purity.

In summary, the results represent an important contribution for an alternative use of tobacco in order to promote the sustainability and the economic development of the tobacco cultivation regions. Additionally, considering that the plant structures remain practically intact after the extraction, there is yet the possibility of its utilization for paper production as well as other materials.

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II.2. CAPÍTULO 2

II.2. ARTIGO 3 – Liege C. Schwingel, Valquiria L. Bassani. Molecular modeling studies on cyclodextrin associations with flavonoids: a review

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Molecular modeling studies on cyclodextrin associations with flavonoids: a review

Liege C. Schwingel and Valquiria L. Bassani*

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

* Corresponding author: Tel.: +55 51 33083602; fax: +55 51 33085437.

E-mail address: valquiria.bassani@ufrgs.br (V.L. Bassani).

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil

Abstract

Cyclodextrins can form inclusion complexes with many size-suitable guest molecules owing to the unique molecular structure with hydrophobic cavity and hydrophilic exterior. The formation of host/guest complexes effectively protects the compounds against some type of reactions, increases the aqueous solubility and enhances the stability. The inclusion complexes may be characterized by various techniques, however the molecular modeling studies have become a suitable and extensively reported tool to provide additional information about the interaction forces for inclusion complex formation. The software used to process the calculation image provide a greater understanding about the experimental findings, because the detailed visualization of the inclusion phenomena can even allow the possibility to predict the interactions that may occur at the studied system. Flavonoids are a class of constituents widely present in plants, particularly in the form conjugated with sugars. Its therapeutic activity, however, occurs from the aglycone form, which has reduced water solubility. This chraracteristic represents the main limitation for both their incorporation into aqueous vehicles, as well as for absorption. Thus, the association of flavonoids with cyclodextrins has been studied, especially as a way to increase your hdrossolubilidade and improve its absorption in the gastrointestinal tract or skin penetration. This review presents the molecular modeling works found in the literature on the subject and discusses the relationship between the chemical structure of the aglycone and the different behavior observed across the complexation.

Keywords: Cyclodextrin; Flavonoids; Molecular Modeling.

1. Introduction

Flavonoids are natural products found in many plant tissues, inside the cells or on the surfaces of different plant organs. The chemical structures of this class of compounds are based on two hydroxy substituted aromatic rings joined by a three carbon link (a C6-C3-C6 configuration), more specifically on a phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: flavonoids (2-phenylbenzopyrans), isoflavonoids (3- benzopyrans), and the neoflavonoids (4-benzopyrans). They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns (Grotewold, 2006). The flavonoids may be modified by hydroxylation, methoxylation, or O-glycosylation of hydroxyl groups as well as C-glycosylation directly to carbon atom of the flavonoid skeleton (Harborne and Williams, 2000). They are often hydroxylated in positions 3, 5, 7, 3', 4' and/or 5'. Frequently, one or more of these hydroxyl groups are methylated, acetylated, prenylated or sulphated. In plants, flavonoids are also present as *O*- or *C*-glycosides. The O-glycosides (more frequent) have sugar substituents bound to a hydroxyl group of the aglycone, usually located at position 3 or 7, whereas the C-glycosides have sugar groups bound to a carbon of the aglycone, usually C-6 or C-8 (Rijke et al., 2006).

According to the structure of the C-ring, five major subgroups are distinguished: flavonols (with a 2,3-double bond, 3-OH and 4-keto groups), flavones (with a 2,3-double bond and 4-keto group), dihydroflavonols or flavanonols (with 3-OH and 4-keto groups), flavanones (with 4-keto groups) and flavanols (with 3-OH groups). It is

well known that the polyhydroxy substituted flavonoids commonly found in most plants present the highest antioxidant activity, while the most favorable structural characteristic appears to be the *o*-di-OH substitution of the B-ring. Free radicals can abstract the two hydroxyl hydrogens of this ring, producing the corresponding inactive quinones. However, the structure of the C-ring plays an important role in this activity (Tsimogiannis and Oreopoulou, 2007).

The biological applications of flavonoids are remarkably extensive, being the antioxidant, anti-inflammatory, antiviral and the antitumoral activities massively reported (Havsteen, 2002; Grotewold, 2007; Andersen and Markhamm 2006).

The low solubility of the flavonoids and especially their aglycones in aqueous media and body fluids often presents a problem for therapeutic applications of these substances. As the aqueous solubility is known to be one of the main biopharmaceutical drug properties, the development of water-soluble flavonoid formulations is of great importance to transpose this limitation. In this context, the use of cyclodextrins has been an interesting technological strategy for promoting its aqueous solubility.

2. Cyclodextrins

Cyclodextrins are cyclic oligosaccharides with variable glucopyranose units linked by α-(1,4) bonds, with relative water solubility. They are produced as a result of intramolecular transglycosylation reaction from degradation of starch by cyclodextrin glucanotransferase (CGTase) enzyme (Szetjli, 1998).

They are largely used in pharmaceutical technology due to its capacity to entrap a variety of organic compounds in their cavities, which can influence the dissolution

rate, the aqueous solubility of poorly water-soluble substances and their stability in the presence of light, heat and oxidizing conditions, as well as modify physicochemical properties of drugs (Zhang and Rees 1999; Loftsson and Masson, 2001; Del Valle, 2004; Loftsson et al., 2005; Loftsson and Duchêne, 2007; Tommasini et al., 2004)

The cavity of the CDs presents a lipophilic environment which allows the interaction with guest molecules through van der Waals forces and hydrogen bonding. They form inclusion complexes with a variety of guest molecules in aqueous solutions (Boonyarattanakalin et al. , 2011). The size and shape of the guest molecule are determinant for its inclusion onto the CD cavity, as well as its atomic composition (Alvira, 2007). The binding of guest molecules within the host cyclodextrin is not fixed or permanent but rather is a dynamic equilibrium (Del Valle, 2004).

The preparation of cyclodextrin complexes may be performed by various methods. In solution, the complexes are usually prepared by addition of an excess amount of the drug to an aqueous cyclodextrin solution which reaches an equilibrium state and then is filtered or centrifuged to form a clear drug/cyclodextrin complex solution. For preparation of the solid complexes, the water is removed from the aqueous drug/cyclodextrin solutions by evaporation or sublimation (e.g. spray drying or freezedrying). Other methods can also be applied to prepare solid drug/cyclodextrin complexes including kneading and slurry methods, co-precipitation, neutralization, and grinding techniques (Loftsson, 1999).

After the complex preparation, its characterization is mandatory. Amongst the main techniques applied to investigate the interaction of the host-guest molecules, the proton nuclear magnetic resonance spectroscopy provides a detailed profile of

the atomic interactions, especially regarding the specific interactions with the hydrogens located inside the cyclodextrin cavity: H-3 and H-5. Two-dimensional nuclear magnetic resonance spectroscopy is largely used for this purpose. As a complementary technique for acquiring data on the supramolecular configuration of the complexes, the computational chemistry has raised great attention.

Computational studies on host–guest interactions are carried out, in general, to find out the most probable conformation of the complex and to give a meaningful three-dimensional visualization of the complex. In order to gain further insights into the inclusion process, it is important to characterize the orientation of the flavonoids molecules inside the cyclodextrin cavity. The calculations help to explain the experimental observations.

3. Molecular Modeling Studies

One of the great advantages of theoretical computational chemistry is that it can reproduce and predict physical properties of organic molecules such as geometries, relative energies, spectroscopic properties, with reasonable accuracy. The application of computational chemistry to the study of large systems as cyclodextrins has been discussed (Lipkowitz, 1998). Molecular modeling methods are valuable tools for driving information on the geometry and interaction energy of the inclusion compounds (Amato et al., 1992).

Molecular mechanics (MM) or molecular dynamics (MD) methods are unable to fully account for the inherent quantum-mechanical effects, as the molecular mechanics and dynamics methods are based on the classical physics of balls and springs. Nevertheless, in recent years, with increased availability of computer

resources, the number of studies reporting the use of quantum mechanics (QM) for investigating the 3D structure and properties of such complexes has increased dramatically. QM method can provide information about the electronic structure of the system and hence, better understanding of the inclusion structure. There have been a series of QM studies on the complexation of CDs with small organic compounds, and these methods have been successfully applied to these CD systems. There are a great number of reports over the application of molecular modeling on flavonoids/cyclodextrins interaction studies.

Haiyun and coworkers (2003) studied the complexation of rutin and β-cyclodextrin. Rutin (3((6-O-(6-deoxy-α-L-mannopyranosyl)-β –D-glucopyranosyl)oxy)-2-(3',4'-dihydroxyphenyl)- 5,7-dihydroxy-4H-1-benzopyran-4-one), known as vitamin P, is an oral capillary preservation drug, used for the treatment of chronic venous insufficiency. The association with β-cyclodextrin was performed due to its poor solubility. Computer molecular modeling system CS Chem3D Pro7.0 from CambridgeSoft Corporation was used, and four kinds of binding models were intimated separately in respect to binding orientation between host and guest by the molecular dynamic calculation: the flavonoid A ring or B ring was inserted either into the cavity from the primary edge of β-CD or into the cavity from the secondary edge of β-CD. From the results of the four optimized configurations, the total energy for the model with the insertion of the A ring into the cavity from the primary edge of β-CD was the lowest.

A study regarding the flavonoid quercetin (Zheng et al., 2005) was designed to investigate the feasibility of utilizing three CDs: β-CD, hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfobutyl ether β-cyclodextrin (SBE-β-CD) to improve the chemical stability and aqueous solubility of quercetin, and to elucidate the mode of

complexation between quercetin and these CDs. Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a bioactive flavonoid which presents potent antioxidant activity and various biological and biochemical effects including anti-inflammatory, antisclerotic, antineoplastic, spasmolytic, diuretic, and cardio-protective activities (Harborne and Williams, 2000; Havsteen, 2002; Cook and Samman, 1996; Yang et al., 2001). Stability testing, phase solubility measurements, and proton nuclear magnetic resonance (NMR) spectroscopy were employed together with molecular modeling as a complementary tool for characterizing the complexes under investigation. NMR spectroscopic analysis suggested that the B-ring, C-ring, and part of the A-ring of quercetin display favorable interaction with the hydrophobic cavity of the CDs, which was confirmed by molecular dynamics (MD) simulations using a solvated model of the quercetin/β-CD complex. Molecular mechanics and molecular dynamics (MD) simulations were performed in the framework of the AMBER4.0 force field. The quercetin molecule was docked into the CD with the B-ring to C-ring bond coincident with the x axis. Multiple starting positions were generated by movement of the bond along the x axis, and complexes with either the A-ring or the B-ring projecting into the cavity were built. Each complex was energy minimized in vacuum, and the lowest energy complexes were used as the starting geometries for MD simulations. To further probe the mode of interaction between quercetin and β-CD, a molecular modeling (molecular mechanics/molecular dynamics) study was conducted both in vacuo and in water. A proposed energetically favorable structure corresponds to the aromatic B-ring positioned inside the CD cavity. Most importantly, the simulation results are in good agreement with the experimental data.

In a subsequent year, another study of the interaction of quercetin with β-CD was published (Yan et al., 2006) using the Parametric Model 3 (PM3) quantum-

mechanical semiempirical method. All theoretical calculations were performed using GAUSSIAN 03 software package and the initial structures of β-CD and quercetin were constructed with the help of CS Chem3D Ultra (Version 6.0, CambridgeSoft.com). PM3 is a powerful technique which can be applied and performs better than Austin model 1 (AM1) in biochemical systems due to its improved description of the interactions between nonbonded atoms, e.g. hydrogen bond and steric effects. It has high computational efficiency and its precision is comparable to that of *ab initio* with medium-sized basis sets. The statistical thermodynamic calculations at 1 atm and 298.15 K by PM3 demonstrated that 1:1 quercetin/β-CD complex is favored by a negative enthalpy change, and the orientation in which the B ring of the guest molecule located near the secondary hydroxyls of the β-CD cavity was preferred in energy, being one intermolecular hydrogen bond formed. The molecular modeling results were in agreement with the NMR observations and molecular dynamics (MD) simulations.

Afterward, the same research group performed a study on the complexation of quercetin using the association of the PM3 and ONIOM2 methods (Yan et al., 2007). The use of hybrid methods is very important for the study of large molecules or supramolecular systems. Within these schemes the computational cost diminishes considerably and the entire system can be considered without needing to generate simplistic model systems. With an aim toward increasing the knowledge of supramolecular interactions, in this article the authors focused on the O–H---O interactions between host and guest molecules and the conformational changes of the guest molecule. The obtained results clearly indicated that the orientation in which the B ring of the guest molecule located near the secondary hydroxyls of the CD cavity is preferred in the binding energy. Moreover, further information over the

system was acquired: one hydrogen bond between 7-OH of quercetin and 6-OH of β-CD was formed.

Regarding the flavonoid 3-O-methylquercetin, a methylated derivative of quercetin, several studies on *in vitro* and *in vivo* biological activities have been reported for this molecule, such as anti-inflammatory, antioxidant, neuroprotective, bronchodilatory, vasodilatory, antitumor and antiviral (Vlietinck et al., 1986; Vrijsen et al., 1987; Vanden Berghe et al., 1993; Middleton et al., 2000). Schwingel et al. (2008) performed the first study on 3-O-methylquercetin/cyclodextrin complexation, characterizing the inclusion complex using NMR and molecular modeling. The NMR analysis indicated that the inclusion occurred with the flavonoid B-ring into the β-CD cavity. As a complementary tool to increase the information about the supramolecular geometry of the complex, computer molecular mechanics (MM2) method from Chem3D Ultra 9.0 (CambridgeSoft) was used. It was observed that the B-ring of the flavonoid could be included into the CD cavity through the secondary hydroxyl groups edge or through the primary hydroxyl groups edge, although the flavonoid insertion through the wider rim has been more favorable. This orientation and conformation were obtained according to energy characteristics, being the total energy for the first model the lowest.

At the subsequent year, the same research group investigated the complexation of coumestrol with β-CD (Franco et al., 2009). Coumestrol is an estrogenic and antioxidant agent, characterized by its low solubility in aqueous media, once in the aglycone form. In order to improve its solubility in water, coumestrol was associated with β-CD in aqueous media followed by freeze-drying and characterized by SEM, NMR and molecular modeling. The molecular modeling was performed with the Software Chem-Bio Office ChemBio 3D/ChemBio Draw Ultra, v. 9.0. Cambridge Soft

(2005), using the molecular mechanics method (MM2). The simulations were performed by manual insertion of the coumestrol molecule in the vertical position into the cavity in a perpendicular way of its diameter. The dynamic calculations were performed at 300K by molecular mechanics method (MM2). The analysis indicated the inclusion of the coumestrol B-ring into the wider rim of the β-CD molecule. The complexation of two molecules of coumestrol with one of β-CD was energetically more favorable than the complexation of one molecule of each. However, the complex model with the smaller total energy was that obtained by the insertion of the B-rings of two molecules of coumestrol in both sides of the β-CD, being this the most probable complexation pathway according to the molecular modeling. Coumestrol association with cyclodextrin improved its solubility in water up to 4 times at the 1:1 molar ratio. According to the NMR data and molecular modeling, the formation of an inclusion complex showed to be possible through the insertion of the A or B-rings. However, the insertion of the B-ring of one molecule of coumestrol by the wider side of the β-CD is supposed to be the most probable complexation pathway in a 1:1 complex.

Molecular mechanics calculations were performed in order to gain insight into the thermodynamic and structural features of the 1:1 complexes of the flavonols 3-hydroxyflavone and fisetin with β-CD. Judging from the energy of formation values of the inclusion complexes formed and their solvation energies, it is evident that the inclusion complexes with the chromone ring inserted into the β-CD cavity and the phenyl ring exposed are more stable in case of both the flavonols (Banerjee and Sengupta, 2006). By the same methods it was possible to observe that the inclusion complex of robinetin with β-CD also occurred with the chromone ring inserted into the cavity, in agreement with the spectroscopic data (Banerjee et al., 2007).

Genistein is an isoflavone with antioxidant and estrogenic activities. It was found to form a supramolecular, non-covalent inclusion complex with both β-CD and γ-CD, while it did not form a stable complex with α-CD. According to the semi-empirical method PM3, the guest genistein was found to spatially located in the less polar cavity of cyclodextrin (Daruházi et al., 2008).

More recently, Pahari and coworkers (2013), in order to rationalize their experimental data and infer about the mode of inclusion, performed molecular docking studies of fisetin and daidzein with γ-CD by using AutoDock. The lowest energy docked complex of fisetin and daidzein within γ-CD nanocavity revealed a contrasting mode of inclusion for the two flavonoids. For the fisetin- γ-CD inclusion complex, it was observed that the B-ring of fisetin is deeply embedded into the cavity and face toward the primary hydroxyl group of the γ-CD cavity. In contrast to fisetin, daidzein inclusion occurs mainly through A + C ring of the flavonoid and the B-ring is exposed to the secondary hydroxyl rim of the γ-CD cavity.

4. Conclusions

The computational processing evolution allowed the remarkable advance in the computational chemistry field. The use of time-consuming calculations to predict the interactions of such large systems as the cyclodextrins complexes was only a perspective a few years ago. This review points out the importance of molecular modeling studies to provide further knowledge regarding the experimental findings. Furthermore, it is almost possible to infer the possibility of predicting tool for inclusion phenomena studies of flavonoids with cyclodextrins.

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II.2. ARTIGO 4 – Liege C. Schwingel, Paulo Gonçalves, Valquiria L. Bassani. Molecular modeling study of *β*-cyclodextrin complexes with quercetin and 3-*O*-methylquercetin based on RM1 method.

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Molecular modeling study of *β*-cyclodextrin complexes with quercetin and 3-*O*-methylquercetin based on RM1 method

Liege C. Schwingel[a], Paulo Gonçalves[b], Hugo Verli[a], Valquiria L. Bassani[a,*]

[a]Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[b]Departamento de Físico-Química, Instituto de Química, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil

* Corresponding author: Tel.: +55 51 33083602; fax: +55 51 33085437.

E-mail address: valquiria@pq.cnpq.br (V.L. Bassani).

Abstract

Quercetin is a natural occurring flavonoid with potent antioxidant activity and 3-*O*-methylquercetin (5,7,3’,4’-tetrahydroxyflavone) is a quercetin methoxylated derivative with pronounced antiviral activity and moderate anti-inflammatory and antioxidant properties. In this work we have studied the properties of two flavonols, quercetin and 3-*O*-methylquercetin, using the semi-empirical method Recife Model 1 to understand the fundamental difference between the two molecules. Their geometries have been optimized and important molecular properties have been calculated. The conformational analyses of the complexes established that the inclusion of the flavonoid quercetin is preferred over the inclusion of 3-*O*-methylquercetin, demonstrating the effect of the substituent polarity and size in analogous molecules. The flavonoids complexation with ***β***-CD in water occurred preferentially with the insertion of the B ring through the secondary OH rim, whereas the vacuum complexation, with the insertion of the A ring through the secondary OH rim.

Keywords: Cyclodextrin; Quercetin; 3-*O*-Methylquercetin; Molecular Modeling; RM1.

Introduction

Flavonoids are bioactive polyphenolic compounds that occur ubiquitously in plants [1]. Recent interest in flavonoids stems mainly from their high therapeutic activity, acute-high potency, and low systemic toxicity. Quercetin (3,5,7,3',4'-pentahydroxyflavone) (Q) is a natural abundant flavonoid which presents potent antioxidant activity and various biological and biochemical effects including anti-inflammatory, antisclerotic, antineoplastic, spasmolytic, diuretic, and cardio-protective activities [1–4]. 3-*O*-methylquercetin (3-MQ) is a quercetin derivative of great therapeutic interest. There are several *in vitro* and *in vivo* biological activities reports, such as anti-inflammatory, antioxidant, neuroprotective, bronchodilatory, vasodilatory, antitumor and antiviral [5–8].

However, the low solubility of the flavonoids and especially their aglycones in aqueous media and body fluids often presents a problem for medical applications of these substances. As the solubility is known to be one of the main biopharmaceutical drug properties, the development of water-soluble flavonoid composition is of great importance, and the use of cyclodextrins (CDs) is an interesting technological strategy.

Cyclodextrins are cyclic oligosaccharides with variable glucopyranose units linked by α-(1,4) bonds, with relative water solubility. They are largely used in pharmaceutical technology due to its capacity to entrap a variety of organic compounds in their cavities, which can influence the dissolution rate, the aqueous solubility of poorly water-soluble substances and their stability in the presence of light, heat and oxidizing conditions, as well as modify physicochemical properties of drugs [9–14].

CDs possess therefore a lipophilic environment in their cavity and can potentially interact with guest molecules through Van der Waals forces and hydrogen bonding. Consequently, CDs are well suited for forming inclusion complexes with a variety of guest molecules in aqueous solutions [15]. The type of molecules that can be complexed in the hydrophobic cavity of CDs depends mainly on geometric factors such as size and shape, although the host-guest interaction energy finally depends on the composition of guest molecules [16].

Studies of NMR and/or molecular modeling regarding the complexation of Q and 3-MQ, flavonoids which present similar molecular structure, with *β*-CD indicate that the complexation may occur by the insertion of B ring inside the *β*-CD cavity [17–21].

Computational studies on host–guest interactions are carried out, in general, to find out the most probable conformation of the complex and to give a meaningful three-dimensional visualization of the complex. In order to gain further insights into the inclusion process, it is important to characterize the orientation of the flavonoids molecules inside the cyclodextrin (CD) cavity. The calculations help to explain the experimental observations.

One of the great advantages of theoretical computational chemistry is that it can reproduce and predict physical properties of organic molecules such as geometries, relative energies, spectroscopic properties, with reasonable accuracy. The application of computational chemistry to the study of large systems as CD has been discussed [22]. Molecular modeling methods are valuable tools for driving information on the geometry and interaction energy of the inclusion compounds [23].

Computational modeling studies on CDs have been performed for a better understanding of the behavior of CDs at the molecular level. Molecular dynamics

(MD) simulations are appropriate to investigate the dynamics of molecular systems using more realistic boundary conditions that could not be acquired by quantum mechanics calculations. On the other hand, these calculations require high computational cost and time, which difficult the information access.

Configurational and conformational analysis were performed using semi-empirical molecular orbital method in order to obtain structural, electronic and energetic information about the flavonoids quercetin and 3-MQ. Recife Model 1 has been chosen to study the host–guest interactions between quercetin or 3-*O*-methylquercetin and *β*-CD. Due to the size of molecular systems, this approximate method can be currently applied regarding the time demand of the calculation.

Experimental

Quercetin and 3-*O*-methylquercetin conformational analyses

The molecular structures of the flavonoids were built from scratch using the software Molden: each atom was placed sequentially and the variables bond length, bond angle and dihedral angle were assigned. The Z-matrix editor provided full control over the molecules geometry.

In order to obtain the flavonoids most energetically favorable molecular geometry, conformational analyses through the total rotation of the dihedral angles were performed (Fig. 1). The rotations of the dihedral angles were carried out in 30^{o} steps, resulting in 12 different conformations for each dihedral angle. The minimization calculations were performed either in vacuum or water, by the semi-empirical RM1 method. Consideration of solvent effects was accomplished with the

use of conductor like screening model (COSMO) with the water dielectric constant (78.4).

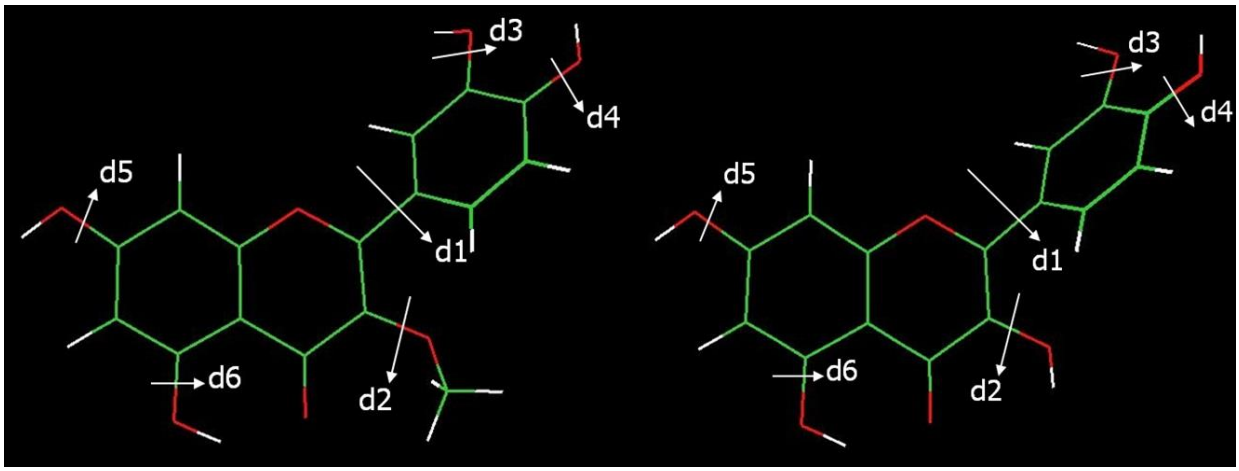

Fig. 1. 3-MQ and Q rotational dihedral angles.

Flavonoid/*β*-CD complexation

Considering the large number of atoms in the studied system, an adequate choice of the model chemistry had to inevitably result from an assembly between the available computer power and the desired level of calculation. Full geometry optimization calculations are computationally expensive at the ab-initio level of theory for such large system, therefore the RM1 approach was chosen to model the inclusion pathway of complexes.

Two distinct inclusion orientations of each flavonol molecules were considered, (I) with the A ring, and (II) with the B ring, respectively inserted into the *β*-CD cavity. To maintain the correct guest molecule orientation, intermolecular restraints between the CD and the flavonoids protons were established.

The study started from optimized structures for the host and the guest molecule, and the calculation of the inclusion process was performed with a full optimization of

every point with the RM1 hamiltonian. In order to examine in details the reaction pathways and seek for a possible global minimum, a controlled reaction coordinate (Z) of the inclusion process was accomplished. The distance between two pseudoatoms, one located at the mass center of the flavonoids A or B ring and the second located right in the center of the *β*-CD cavity, provided the monitoring of the guest insertion coordinate.

The stabilities of the inclusion complexes formed were evaluated from their energy of formation values (ΔE) calculated as the difference in the total energies of the complex and those of the free flavonols (guest) and *β*-CD (host), respectively. The most stable complex among all the configurations corresponds to the greatest negative value of ΔE.

Results and discussion

Quercetin and 3-*O*-methylquercetin conformational analyses

The flavonoids conformational study is graphically represented at Fig. 2 for 3-MQ and Fig. 3 for quercetin, plotting the relative energy (kcal/mol) in function of the dihedral angle. The relative energy values were obtained using the minimum values as reference and the others as relative. In general, the profiles of dihedral rotation in water exhibited lower energy compared to profiles in vacuum.

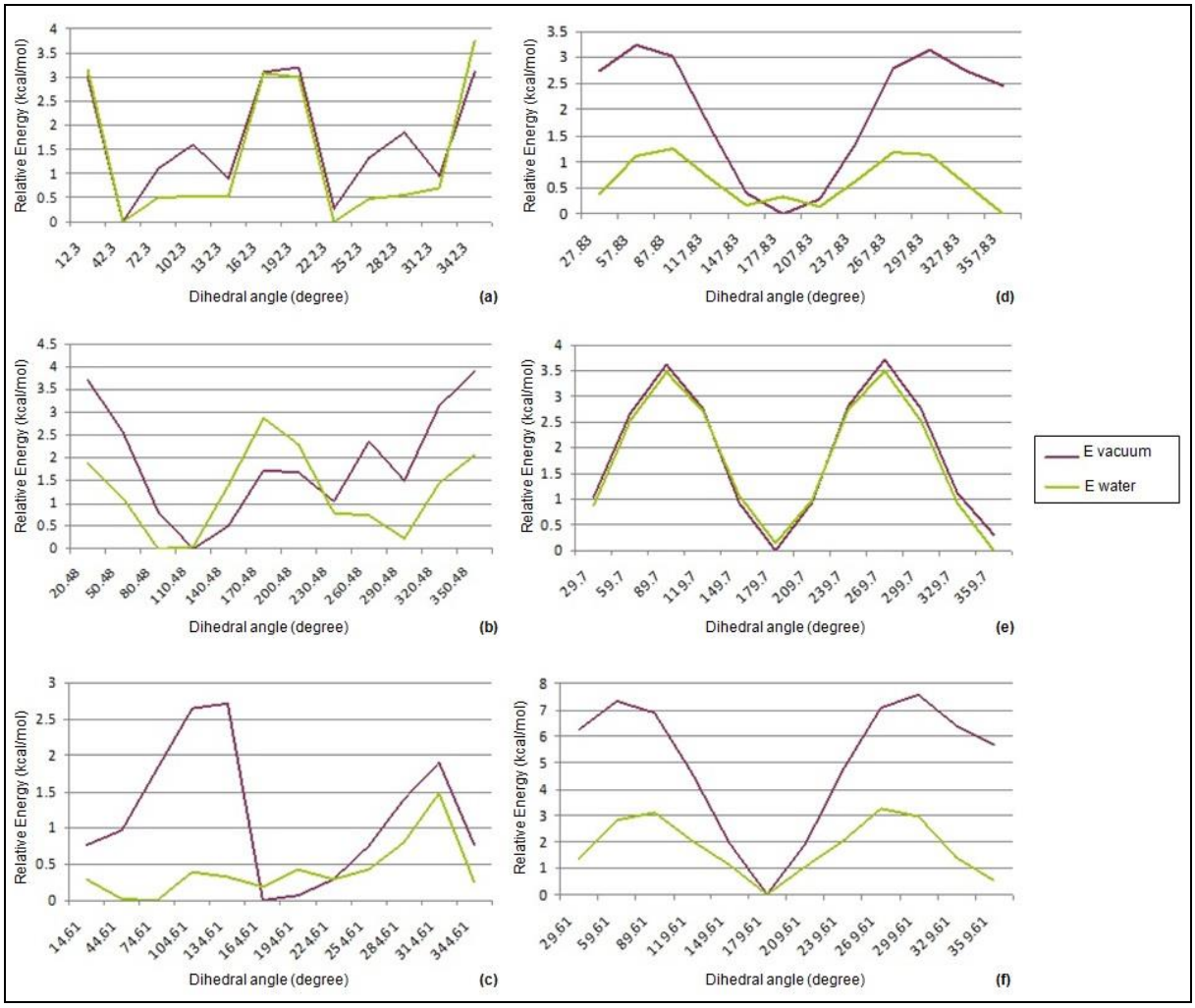

Fig. 2. 3-MQ conformational relative energy in function of the dihedral angle (a) d1, (b) d2, (c) d3, (d) d4, (e) d5, (f) d6. The reference energy was the minimum energy structure.

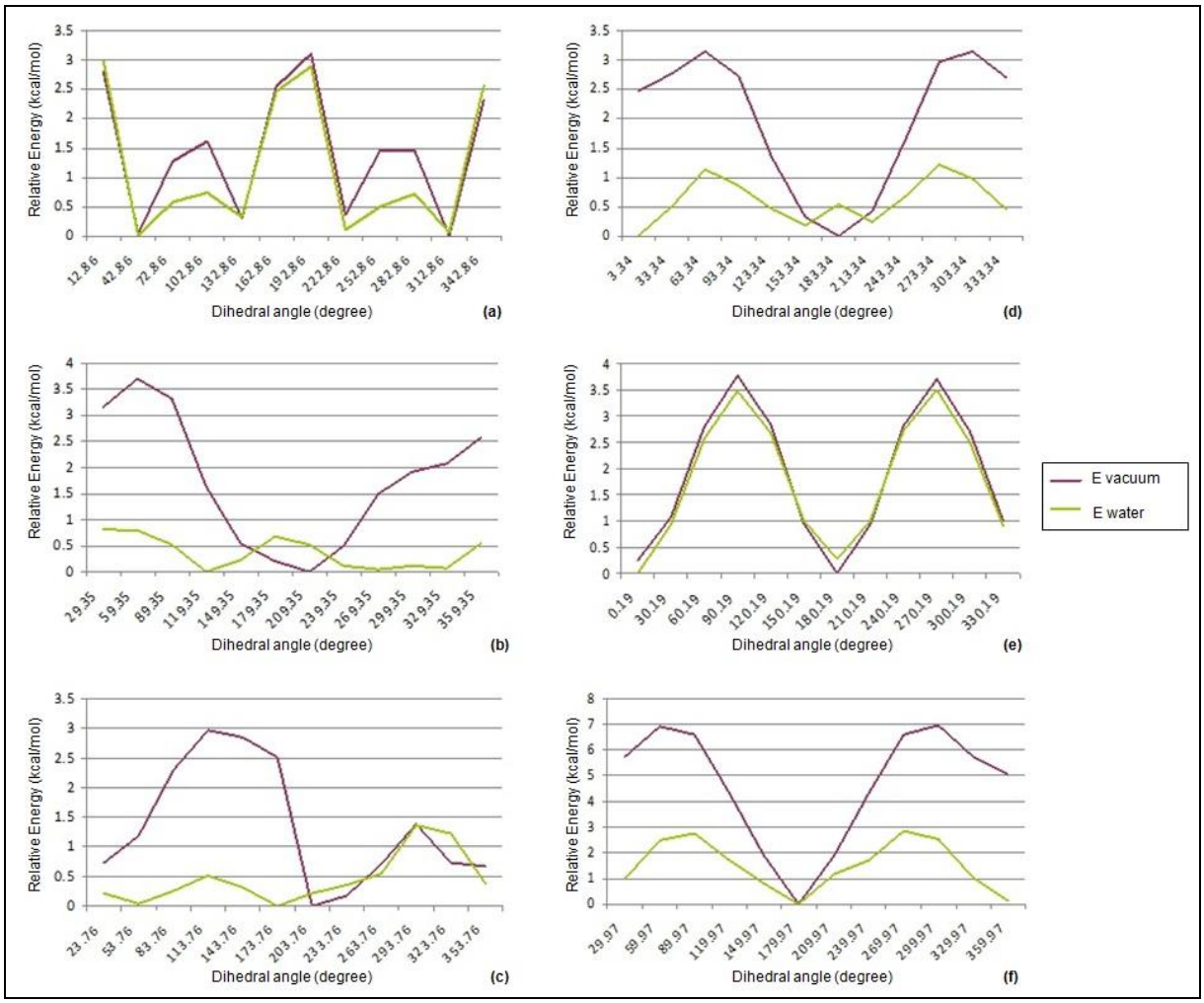

Fig. 3. Q conformational relative energy in function of the dihedral angle (a) d1, (b) d2, (c) d3, (d) d4, (e) d5, (f) d6. The reference energy was the minimum energy structure.

The rotations of 3-*O*-methylquercetin first dihedral angle possess great influence over the methoxyl group conformation. As H2 'and H6' approach, changes in the second dihedral are observed both in vacuum and water. Overall, the global energy minimum occurs close to 75° for both media. The rotation of this dihedral represented the major energy barrier in water compared with the rotation of the other dihedrals. The second local energy minimum can be observed at 237º, which equals to the opposite angle of the first energy minimum. At these positions, the phenolic

hydrogens H2’ and H6’ presented the conformation with greatest distance from the methoxyl group. The highest energy barrier was observed when the phenolic ring achieved the same plan as the A+C rings.

The rotation of the second dihedral, represented by the methoxyl group, promotes the total rotation of the phenolic ring for steric reasons. In water, the global energy minimum was obtained at 95º and 125º whereas, in vacuum, at 125º. The highest energy barrier was verified at 5º and 35º in vacuum, and at 185º in water..

The phenolic hydroxyl group from B ring (3’-OH) rotation induced conformational effects singly over the 4’-OH. This rotary motion was relatively favorable in water, contrasting with the important energy barriers in vacuum (between 120º-150º and at 330º). The energy minimum was obtained from 60º to 90º in water and at 180º in vacuum. The 4’-OH dihedral angle rotation follows the previous profile. Three energy minima were detected in water (at 12º, 162º and 222º) and one in vacuum (192º). Both media presented two energy maxima.

Little or no effect on the flavonoid conformation was observed with the rotation of the 5th dihedral (7-OH from A ring). The profiles in water and vacuum were almost overlapped, generating energy minima at 195º and 15º, and higher energy barriers at 105º and 285º. Despite the similarity between the rotation profiles of the 6th dihedral in water and in vacuum, the last one presented significant energy barriers at 75º and 315º. The global minimum energy occurred when the dihedral angle of 5-OH was at 195º.

The energy barriers generated by the rotation of the quercetin dihedrals in water are similar to those of 3-*O*-methylquercetina, except for the second dihedral, which did not provide steric hindrance toward the conformation of B ring (Table 1).

Table 1. Energy barriers (kcal/mol) of dihedral angles analyses for 3-*O*-methylquercetin and quercetin in vacuum and water.

Dihedral angles	Energy barrier (kcal/mol)			
	3-*O*-methylquercetin		quercetin	
	vacuum	water	vacuum	water
d1	3.20	3.76	3.11	2.88
d2	3.90	2.88	3.71	0.82
d3	2.87	1.48	2.97	1.36
d4	3.23	1.26	3.15	1.22
d5	3.72	3.49	3.78	3.50
d6	7.54	3.27	6.93	2.85

It is worth emphasizing that the more stable energetic conformation is not necessarily the active form, which geometry bonds to the active site.

Flavonoid/*β*-CD complexation

As model studies on the inclusion complexation of CDs with various guests offered important insight into the non-covalent intermolecular interactions and theoretical calculations helped to illustrate the driving forces for the complexation, a theoretical study of the entire process of the formation of 1:1 stoichiometry quercetin/*β*-CD and 3-*O*-methylquercetin/*β*-CD associations was undertaken, in order to help the understanding and rationalization of the experimental results reported in the literature.

All the possibilities of the inclusion process were considered: the orientation in which A or B ring of the flavonoid was inserted toward the narrow side of the *β*-CD cavity (primary OH rim) and the insertion through the wider side (secondary OH rim).

The energy profiles of the complexation process were characterized in Fig. 4 for 3-*O*-methylquercetin and Fig. 5 for quercetin.

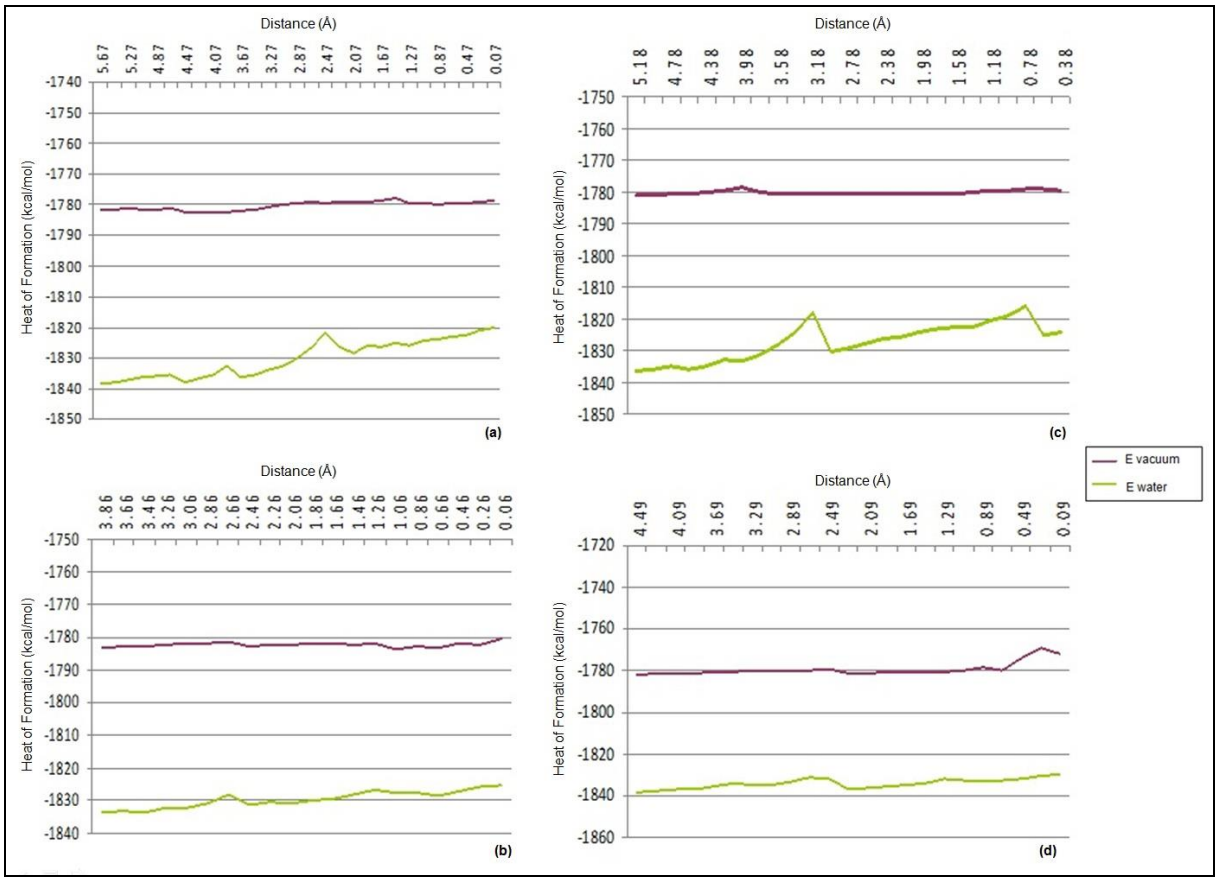

Fig. 4. 3-MQ/*β*-CD complexation energy profile in vacuum and in water. (a) A ring into primary OH rim, (b) A ring into secondary OH rim, (c) B ring into primary OH rim, (d) B ring into secondary OH rim.

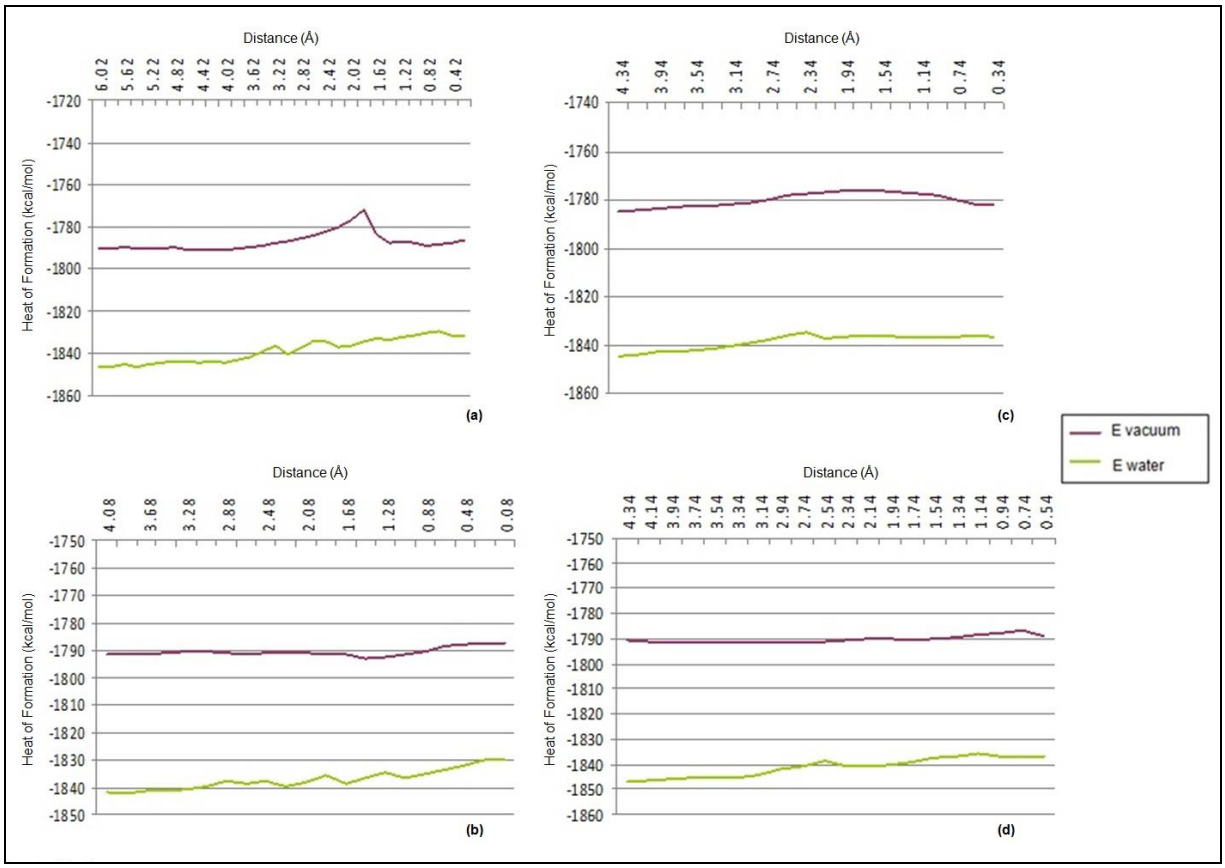

Fig. 5. Q/*β*-CD complexation energy profile in vacuum and in water. (a) A ring into primary OH rim, (b) A ring into secondary OH rim, (c) B ring into primary OH rim, (d) B ring into secondary OH rim.

Table 2 contains the heat of formation of the complexes formed (energy minimum) both in vacuum and water, in addition to the interaction energy (ΔE), which was calculated through the difference between the energies of isolated reactants (host and guest molecules) and their assembly. The heats of formation for 3-MQ were -232.06860 kcal/mol (vacuum) and -246.15283 kcal/mol (water) and for Q were -240.81456 kcal/mol (vacuum) and -254.73441 kcal/mol (water). This evaluation represents the interaction energy provided by the contribution to the total energy that is caused by an interaction between the molecules being considered. The results of negative ΔE values indicate that all the complexes formation are favorable. Despite is

higher polarity, the inclusion of the flavonoid quercetin is preferred over the inclusion of 3-*O*-methylquercetin, demonstrating the effect of the substituent polarity and size in analogous molecules. This is possibly explained by the steric effects produced by the 3-MQ methoxyl group, since the *β*-CD hydrophobic cavity tends to form inclusion complexes with hydrophobic molecules, and the preference for 3-MQ inclusion over quercetin was expected.

Table 2. Stability evaluation of the inclusion complexes formed in vacuum and water from their energy of formation values (ΔE).

	Heat of formation (kcal/mol)		ΔE (kcal/mol)	
Complex conformation	vacuum	water	vacuum	water
3-MQ/*β*-CD				
A ring into primary OH rim	-1782.42	-1838.29	-12.11	-40.18
A ring into secondary OH rim	-1783.53	-1833.44	-13.22	-35.33
B ring into primary OH rim	-1781.38	-1836.45	-11.07	-38.34
B ring into secondary OH rim	-1781.74	-1838.60	-11.43	-40.49
Q/*β*-CD				
A ring into primary OH rim	-1791.20	-1846.71	-12.15	-40.02
A ring into secondary OH rim	-1793.13	-1842.43	-14.08	-35.74
B ring into primary OH rim	-1784.89	-1844.41	-5.84	-37.72
B ring into secondary OH rim	-1791.44	-1847.02	-12.39	-40.33

The conformational analyses of the complexes supplied interesting data. Again, the roll of the solvent was crucial to stabilize the systems, as the energy involved in the vacuum complexation was less favorable and provided different association geometry. It is important to point out that in both media the flavonoids insertion through the secondary OH rim (wide rim) is preferred, although in vacuum the A ring

orientation is preferred. Opportunely, the solvent favored the A ring insertion, and the interaction occurred through the primary OH rim (narrow rim). The less favorable complex conformation in water (A ring insertion through secondary OH rim) matched the most probable conformation in vacuum. Within these associations, the difference of 1 kcal/mol is sufficient to infer that one conformation occurs and not the other.

In order to investigate the possibility of the flavonoid to go from the one side of de *β*-CD to the other, passing through the *β*-CD mass center, a reaction coordinate was executed. This is the best way to study the energy profile of the interaction between two molecules. According to Fig. 6, as the mass center of the flavonoid approaches the mass center of the *β*-CD, there is an increase in the energy barrier which reaches its maximum when the rest of the flavonoid molecule crosses the *β*-CD rim. Disruption of the toroidal structure of the *β*-CD was found in almost every case, indicating that such phenomenon is not energetically favorable, and is unlikely to occur.

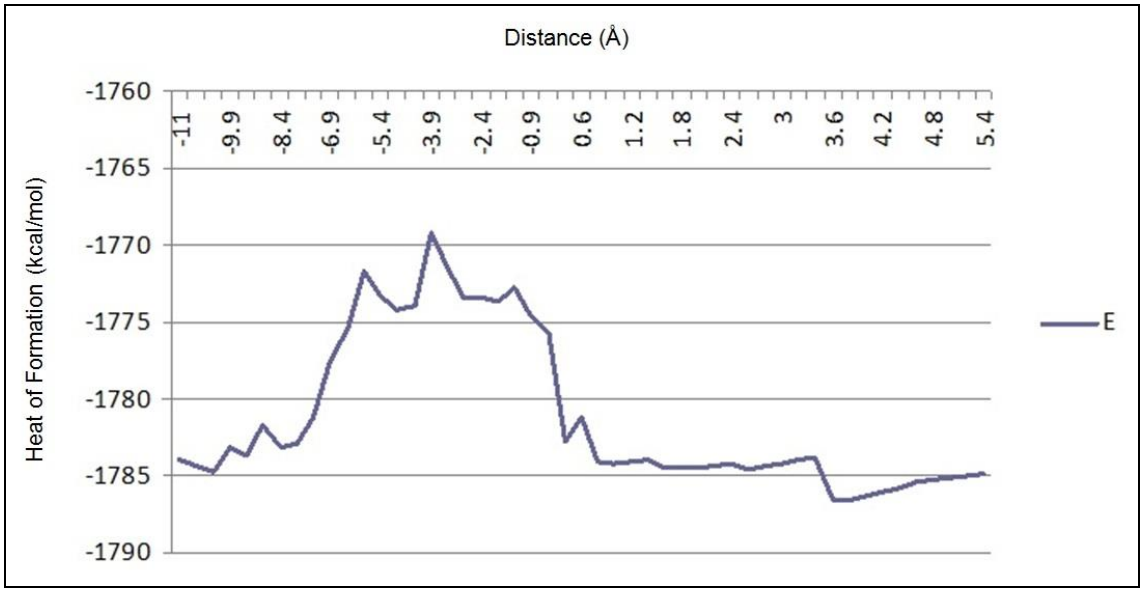

Fig. 6. Reaction coordinate of flavonoid total complexation with *β*-CD.

Using the software Molden, the visualization of the complexation process was possible. Four supramolecular geometries obtained by 3-MQ complexations in water can be visualized at Fig. 7, whereas the Q/*β*-CD conformations stand in Fig. 8. During the screen shots provided by each conformational minimization step, the observation of hydrogen bonds was dominant. The insertion of the flavonoid promoted the formation of hydrogen bonds between the molecule OH groups (and even the carbonyl in C4) with the *β*-CD cavity, while the OH of the flavonoids ring located on the outside of the complex developed hydrogen bonds with the OH in the *β*-CD edges, prevailing the bond formation in the secondary OH rim.

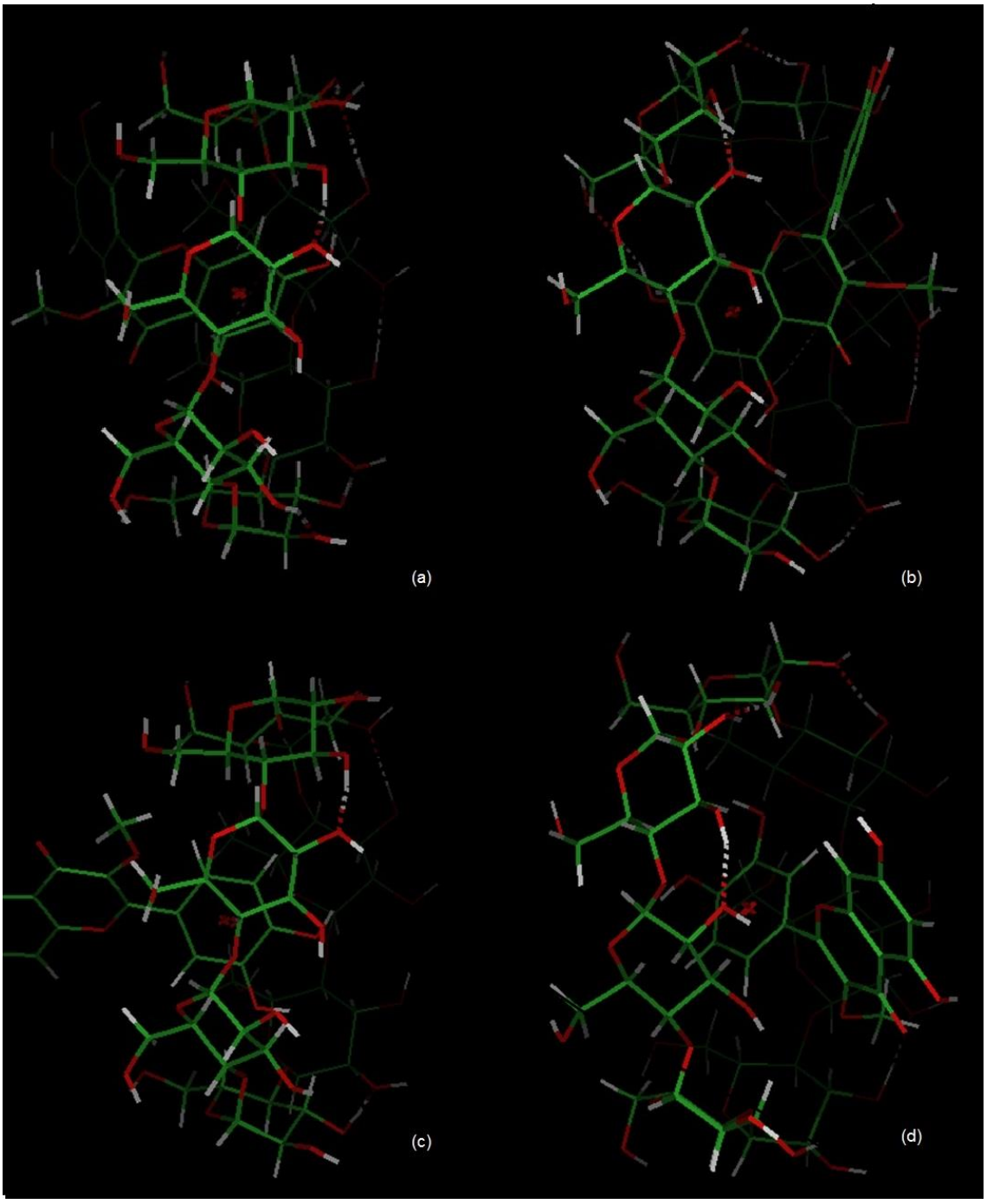

Fig. 7. 3-MQ/*β*-CD complexation in water. (a) A ring into primary OH rim, (b) A ring into secondary OH rim, (c) B ring into primary OH rim, (d) B ring into secondary OH rim.

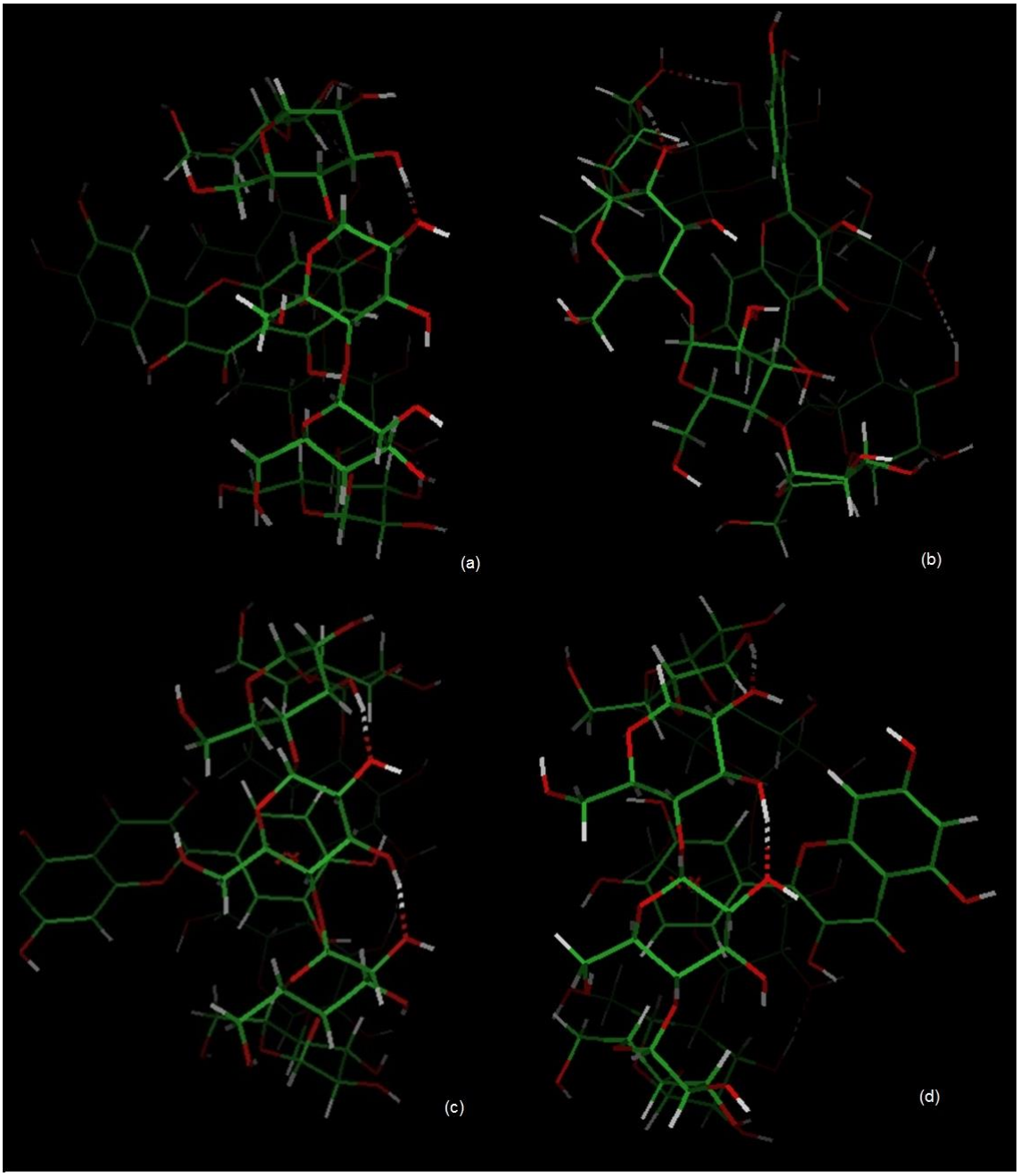

Fig. 8. Q/*β*-CD complexation in water. (a) A ring into primary OH rim, (b) A ring into secondary OH rim, (c) B ring into primary OH rim, (d) B ring into secondary OH rim.

Conclusions

The conformational analyses of the complexes established that the inclusion of the flavonoid quercetin is preferred over the inclusion of 3-*O*-methylquercetin,

demonstrating the effect of the substituent polarity and size in analogous molecules. The solvent effect was critical to stabilize the systems, as the energy involved in the vacuum was less favorable and provided different molecular and complex geometries. The flavonoids complexation with *β*-CD in water occurred preferentially with the insertion of the B ring through the secondary OH rim, which is in accordance with experimental data. In vacuum, the complexation was carried out with the insertion of the A ring through the secondary OH rim.

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II.3. CAPÍTULO 3

II.3. ARTIGO 5 – Liege C. Schwingel, Giovanni K. Zorzi, Helder F. Teixeira, Valquiria L. Bassani. Influence of *β*-cyclodextrin on the quercetin and 3-*O*-methylquercetin *in vitro* skin layers permeation/retention from hydrogels

Manuscrito a ser submetido ao periódico *Carbohydrate Polymers.*

Influence of *β*-cyclodextrin on the quercetin and 3-*O*-methylquercetin *in vitro* skin layers permeation/retention from hydrogels

Liege C. Schwingel, Giovanni K. Zorzi, Helder F. Teixeira, Valquiria L. Bassani*

Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre, RS, Brazil

* Corresponding author: Tel.: +55 51 33083602; fax: +55 51 33085437.

E-mail address: valquiria.bassani@ufrgs.br (V.L. Bassani).

ABSTRACT

Quercetin (Q) is a flavonoid with antioxidant and antiviral activity, and 3-*O*-methylquercetin (5,7,3',4'-tetrahydroxyflavone) (3-*O*-MQ) is a quercetin methoxylated derivative with pronounced antiviral and antitumoral activity. In this work we have studied the evaluation of the permeation/retention of these flavonoids from the hydrogels hydroxypropyl methylcellulose and chitosan in the skin layers. Moreover, the influence of the permeation promoter *β*-cyclodextrin (*β*-CD) on the flavonoids behavior was also evaluated. Q presented a greater skin retention compared with 3-*O*-MQ, and chitosan clearly demonstrated to be a great permeation promoter for Q, since the flavonoid retention in the epidermis was remarkably increased. The association of *β*-CD improved the permeation of the flavonoids, especially regarding the retention in the stratum corneum and epidermis.

***Keywords*:** Cyclodextrin; Quercetin; 3-*O*-Methylquercetin; skin retention; chitosan; hydroxypropyl methylcellulose.

1. Introduction

Flavonoids are bioactive polyphenolic compounds that occur ubiquitously in plants (Harborne & Williams, 2000). Quercetin (3,5,7,3',4'-pentahydroxyflavone) (Q) is a natural antioxidant and antiviral agent,, presenting other important biological activities (Cook & Samman, 1996; Harborne & Williams, 2000; Havsteen, 2002; Yang et al., 2001). 3-*O*-methylquercetin (3-*O*-MQ) is a quercetin derivative of remarkable therapeutic potential, especially regarding the antitumor and antiviral activities (Middleton et al., 2000; Vanden Berghe, 1993; Vlietinck et al., 1986; Vrijsen et al., 1987). However, their low solubility in aqueous media and body fluids are limiting for their pharmaceutical application. To overcome this impairment, the use of cyclodextrins (CDs) is an interesting technological strategy.

Cyclodextrins are cyclic oligosaccharides presenting relative water solubility. They consist of variable glucopyranose units linked by α-(1,4) bonds, which determines their unique toroidal structure conformation. They are largely used in pharmaceutical technology due to their capacity to entrap a variety of organic compounds in their cavities, which presents a more lipophilic environment, whereas the hydrophilic exterior can influence the dissolution rate and the aqueous solubility of poorly water-soluble substances. Also, cyclodextrins can improve the stability of bioactive molecules in the presence of light, heat and oxidizing conditions, as well as modify physicochemical properties of drugs (Del Valle, 2004; Loftsson & Masson, 2011; Loftsson et al., 2005; Loftsson & Duchêne, 2007; Tommasini et al., 2004; Zhang & Rees, 1999).

The topical application of flavonoids demands a suitable vehicle for its purpose, especially regarding the treatment of herpes labial. The formulation must present

good bioadhesion and adequate sensorial properties, as well as allow the bioactive molecules to reach the desired location. Two polymers suit perfectly in this description: hydroxypropyl methylcellulose (HPMC) and chitosan (CS).

Considering the topical application of the flavonoids Q and 3-*O*-MQ, the present work aims the development of hydrogels of chitosan and hydroxypropyl methylcellulose and the evaluation of the β-CD influence on the permeation/retention of the flavonoids in the skin layers.

2. Experimental

2.1. Materials

3-*O*-Methylquercetin with purity of 97% was isolated from *Nicotiana tabacum* leaves (Schwingel et al., 2013). Quercetin, Chitosan (CS), D_2O and DCl were purchase in Sigma-Aldrich (US), *β*-Cyclodextrin was kindly donated by Roquette (Lestrem, France) and HPMC (Methocel F4M, DOW Chemical Company) was supplied by Blanver (São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA), Methanol LC grade was obtained from Merck (Darmstadt, Germany). Trifluoracetic acid and acetic acid of analytical grade were obtained from Nuclear (Diadema, Brazil). Pig ears for permeation/penetration assay were gently donated by Ouro do Sul- Cooperativa dos Suinocultores do Caí Superior Ltda. (Brazil).

2.2. Characterization of chitosan

The degree of acetylation (DA) of CS was determined by means of ^{1}H-NMR spectroscopy, as proposed elsewhere (Hirai et al., 1991). Therefore, CS was dissolved in D_2O with DCl (at pD 3–4; 4 mg/mL) and the sample was freeze dried to allow the exchange of solvation water by D_2O. The resulting powder was resuspended in D_2O. Spectra was recorded on a Bruker-Spectrospin AM 300 spectrometer (300 MHz) at 70°C. Approximately 200–250 scans were acquired.

The weight-average molecular weight (Mw) and the polydispersity index (PDI) were measured by gel permeation chromatography (Novema® columns from PSS 30Å, 3000Å, 3000Å and guard column; I.D.: 8 mm, PSS, Germany) using the universal calibration (Brunel et al., 2013). The equipment was coupled on line with a refractive index detector (Agilent Serie 1200 RID ®) and a viscometer detector (PSS ETA-2010 differential viscometer, PSS, Germany). A standard curve of pullulan (PSS, Germany) was created ranging from 350 to 800.000 Da. A degassed 0.2 M acetic acid/0.15 M ammonium acetate buffer (pH = 4.5) was used as eluent. The flow rate was maintained at 0.7 mL/min.

The water content was determined by water loss upon heating as previously described. Samples were put in an oven at 105 °C until constant weight was achieved. The result was express in % of water.

2.3. Formation and characterization of hydrogels

Physical hydrogels of HPMC and CS were formed with similar rheological profile. CS was solubilized in water with equimolar amount of acetic acid, in relation

to the free amino groups of CS. HPMC was dispersed in water. Both gels were allowed to rest for 24h before the incorporation of the active compound. Q or 3-*O*-MQ was dispersed in propylene glycol (17% - w/w) and incorporated to the preformed gels at a final concentration of 1% propylene glycol (w/w). The polymer final concentrations in the hydrogels were 2.55% (w/w) of CS and 3.0% (w/w) of HPMC, being the flavonoid final concentration 0.17 % (w/w).

The rheological profile of the hydrogels was determined in rotatory viscometer (DV-II+ Viscometer, Brookfield, US). The shear stress and shear rate were plotted and the result was fitted in different rheological models (Ostwald-de Waale, Herschel-Bulkley, Birgham or Casson).

The hydrogels spreadability was determined by means of the parallel plates method. The hydrogels were pressed between sequences of weights, for one minute each, with intervals of 30s between weights. The spreading areas reached by samples were measured. The results were expressed in terms of spreading area (mm^2) as function of the applied mass.

In addition, in order to evaluate the influence of *β*-cyclodextrin on the flavonoids skin permeation/retention, a 5% amount of *β*-CD was diluted in water (corresponding to 10% of the total hydrogel water weight) and added to the preformed hydrogels. Then, the flavonoids (previously disperse in propylene glycol) were incorporated until total homogenization.

2.4. Skin permeation/retention studies

The test was performed in Franz diffusion cells (approximately 2.54 cm^2 of interface area and 10 mL of internal volume). Pig ear skin was used as membrane model, and the permeation lasted 8 h with analysis by HPLC at the 8^{th} hour.

Hairless defrosted pig ears were hygiened with running water and dried with absorbent paper. The skin on the back of the ear was detached with a scalpel and the remaining blood vessels and exceeding fat were removed with a clamp to obtain a homogeneous thickness (approximately 2 mm). Circular cuts of membrane were adhered to the edge of the acceptor phase, creating an interface between the donor and receiver mediums. The cells were hydrated with phosphate buffer pH 7.4 The receptor compartment (10 mL) was supplied with ethanol:PBS (30:70) and the sink conditions were maintained. The system was kept under a controlled temperature (32±1ºC) and constant stirring. The amount of hydrogel applied in each diffusion cell of the permeation study (600 mg) corresponded to exactly 1 mg of 3-*O*-MQ or Q in order to compare its permeation profile with or without associations. After 8 hours, an aliquot of the fluid receptor was withdrawn and the skin was removed from the cell. The skin was cleaned and tape stripped with sixteen adhesive tapes (3 M Scoth nº 750). The first tape was discarded aiming to remove the excess of formulation. Then, the epidermis and dermis were separately cut into small pieces and the flavonoids were extracted with methanol using ultrasound bathing for 30 minutes. Samples were analyzed by HPLC based on previous study (Schwingel et al., 2008). Briefly, a Shimadzu SCL-10 equipment with a Shimadzu LC-10AD pump was used and a Shim-pack CLC-ODS (M) RP-18 column (5 µm, 250 mm x 4 mm i.d.), with Alltech refillable pre-column. The mobile phase consisted of a methanol–water (70:30, v/v)

mixture acidified with 0.1% of trifluoracetic acid (TFA), filtered and degassed by suction-filtration through a nylon membrane, in isocratic flow. The flow was 0.8 mL min-1, with an injection of 50 µl and 0.05 AUFS of sensitivity at 354 nm for 3-*O*-MQ and 368 nm for Q. The LC system was operated at room temperature (23±1 ^{o}C). The LC method was revalidated according to ICH considering the influence of the hydrogels components, skin layers chromatographic profile and the tape retention time used on the stratum corneum stripping (data not shown). Results were expressed as amount of each flavonoid (µg) retained per unit of skin area (cm^2).

3. Results and discussion

3.1. Characterization of chitosan

Because chitosan physical and physicochemical properties are tightly related to its degree of polymerization and degree of acetylation, the knowledge of these parameters is precondition for evaluating the corresponding hydrogels as excipients for semi-solid delivery systems. The correlation between the molecular weight and the viscosity, as expressed by the Mark-Houwink equation, show the relevance of this characterization. As it can be seem in Table 1, the degree of acetylation is in accordance to the specifications of the manufacturer (DA < 25%) and the molecular weight is high enough to form physical hydrogels of CS without adding any chemical.

Table 1. Characterization of chitosan employed as raw material for hydrogel obtantion.

DA (%)	21.7%
Mn (g/mol)	$1.96x10^5$
Mw (g/mol)	$3.83x10^5$
D	1.95
Water content (%)	14.8

DA = degree of acetylation D = polydispersity index

3.2. Characterization of hydrogels

The discovery of an effective active compound is only one step in the development of a new medicine. The attribution of the right dosage form is a critical step to achieve a successful formulation. In case of topical formulations, hydrogels are very popular due to their high viscosity that can control the drug release and increase the residence time, a feature particularly important in case of topical administration. In this context, physical hydrogels of HPMC and CS with similar rheological profile were used to evaluate their influence in the skin permeation of Q and 3-*O*-MQ.

As it can be observed in Figure 1, both hydrogels are pseudoplastic exhibited shear thinning, being this behavior more pronounced in the case of CS hydrogels. Usually, the rheology of HPMC hydrogels varies with the polymer concentration. Concentrations up to 2.0 % result in Newtonian behavior meanwhile concentration above 7.0 % result in pseudoplastic behavior with yield response (Romanski et al., 2011; Chen et al., 2008). The concentration employed in the present work (3.0%) has led to an intermediate behavior of non-newtonian fluid without yield response. In the

same way, physical hydrogels of chitosan solubilized with acetic acid seem to result in pseudoplastic shear thinning fluids that are time-independent (El-Hefian et al., 2010). The importance of the degree of acetylation is to CS as hydroxypropoxyl and methoxyl are to HPMC. Changes in the amout of amino groups can lead to different conformation in the media that result in different interactions among the chains.

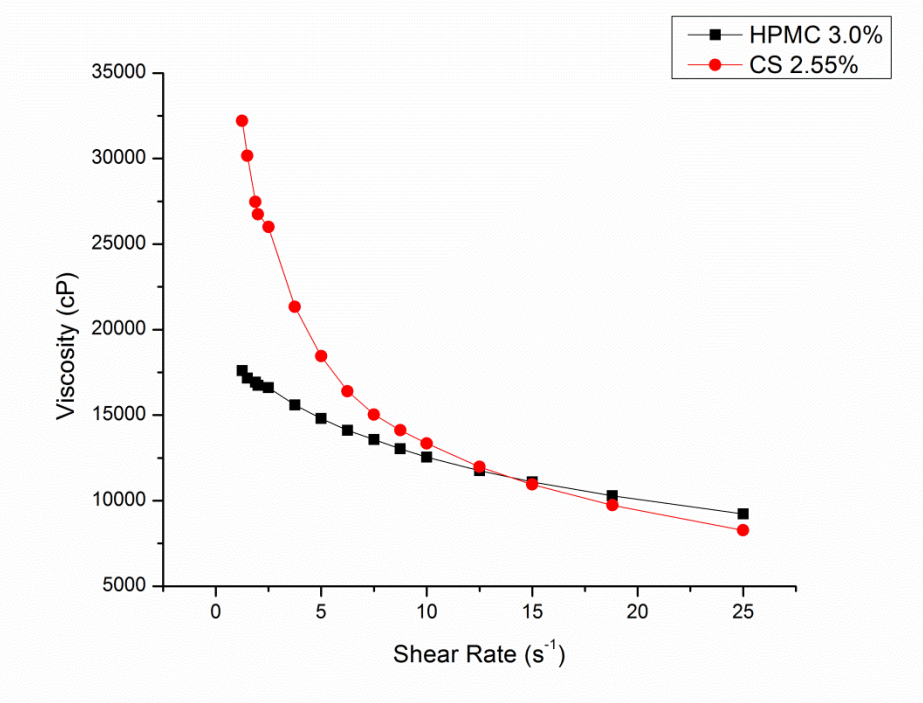

Figure 1. Relationship between viscosity and shear rate of HPMC and CS hydrogels. Both gels are thixotropyc with time-independent shear thinning.

The overlay of ascendant and descendent curves for both hydrogels can be observed in Figure 2. This is an indication of time-independent behavior. It is important to have hydrogels with rheological properties as similar as possible for better comparison of their influence in the permeation profile of Q and 3-*O*-MQ.

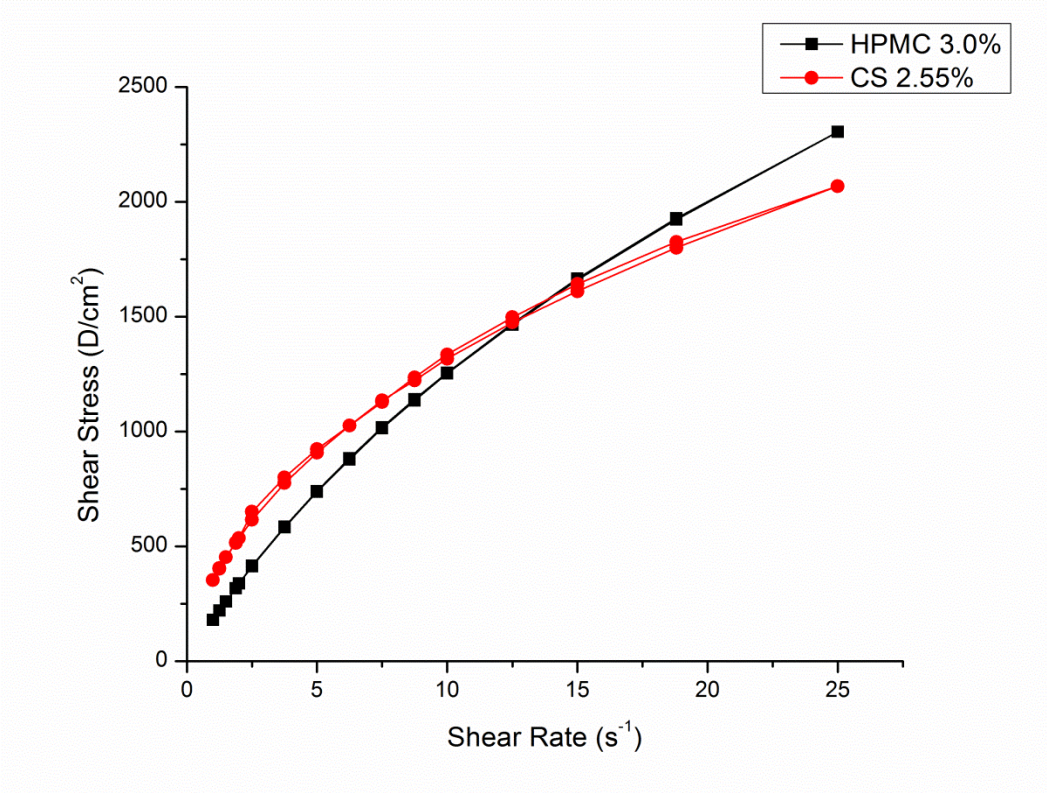

Figure 2. Ascendant and descendent curves of HPMC and CS hydrogels. Both gels are pseudoplastic.

The ascendant curves of both HPMC and CS hydrogels were fitted to different rheological models and the equation that best fitted was the Ostwald-de Waale power law (Table 2), for both cases. The value of flow behavior index (n) below < 1 is also an indication of pseudoplastic behavior, showing a negligible yield response and a varying differential viscosity.

Table 2. Equations describing the hydrogels behavior

	HPMC	CS
Model	Ostwald-de Waale	Ostwald-de Waale
Equation	$\tau = K\dot{\gamma}^n$	$\tau = K\dot{\gamma}^n$
R^2	0.9965	0.9974
K (cP.s^n)	228.57	390.83
n	0.7266	0.5250

The spreadability test performed by means of the parallel plates method. howed no differences between the two hydrogels, as it can be seen in Figure 3. The different nature of the two polymers seems not to have major influence on the spreadability profile of both hydrogels, despite of the slight differences were found in their rheological profile.

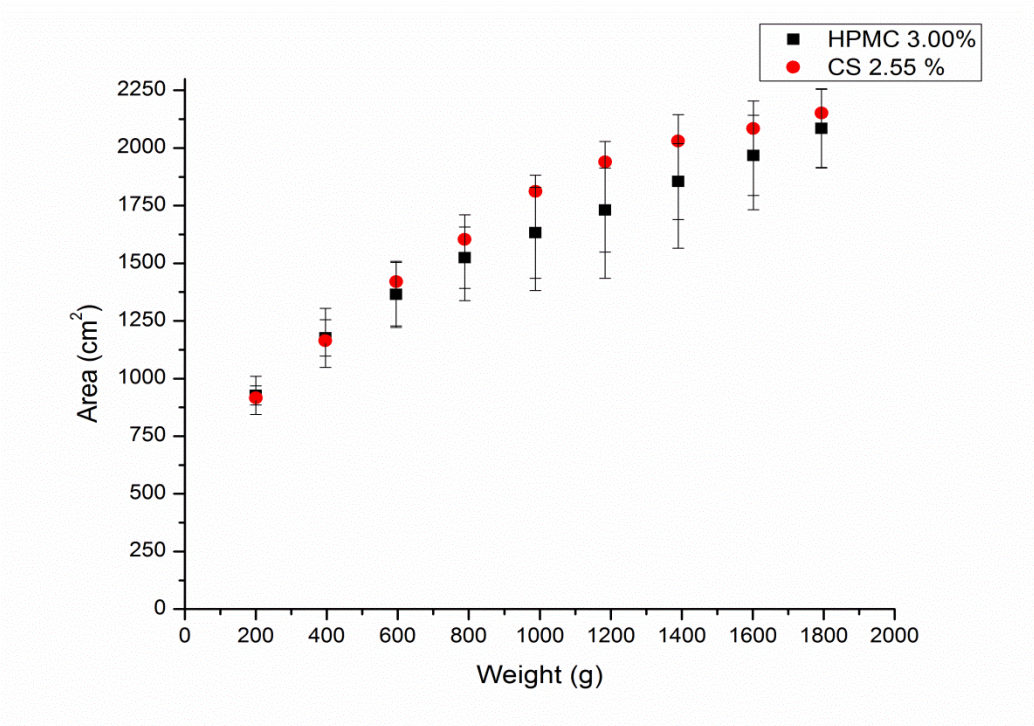

Figure 3. Spreadability of HPMC and CS hydrogels. Both hydrogels exhibit similar behavior under the conditions employed ($p < 0.05$).

3.3. In vitro skin permeation/retention test

The results obtained in the *in vitro* permeation/retention in pig ear skin are presented in the Table 3 and Table 4, respectively. The flavonoid concentrations into different skin layers represent the mean of 5 cells quantification (n = 5). The concentrations are expressed as flavonoid amount per skin area (μg/cm^2). No flavonoid was detected at the receptor media.

Table 3. 3-*O*-methylquercetin retention (μg/cm^2) into skin layers from hydrogels.

	Flavonoid retention (μg/cm^2) ± SD		
	Stratum corneum	Epidermis	Dermis
3-*O*-MQ HPMC	0.16±0.03	ND	0.02±0.01
3-*O*-MQ CD5% HPMC	0.90±0.06	0.14±0.01	0.11±0.01
3-*O*-MQ chitosan	ND	0.06±0.01	0.06±0.02
3-*O*-MQ CD5% chitosan	0.61±0.07	0.12±0.01	0.06±0.01

ND = Not Detected SD = standard deviation
Formulations:
3-*O*-MQ HPMC = 3-*O*-methylquercetin incorporated in 3% hydroxypropyl methylcellulose hydrogel
3-*O*-MQ CD5% HPMC = 3-*O*-methylquercetin incorporated in 3% hydroxypropyl methylcellulose hydrogel added of 5% of *β*-cyclodextrin
3-*O*-MQ chitosan = 3-*O*-methylquercetin incorporated in 2.6% chitosan hydrogel
3-*O*-MQ CD5% chitosan = 3-*O*-methylquercetin incorporated in 2.6% chitosan hydrogel added of 5% of *β*-cyclodextrin

Table 4. Quercetin retention (µg/cm^2) into skin layers from hydrogels.

	Flavonoid retention (µg/cm^2)		
	Stratum corneum	Epidermis	Dermis
Q HPMC	0.47±0.07	0.06±0.03	0.04±0.03
Q CD5% HPMC	1.74±0.13	0.15±0.03	0.06±0.03
Q chitosan	1.17±0.10	0.15±0.03	0.09±0.03
Q CD5% chitosan	1.93±0.14	0.23±0.03	0.05±0.03

ND = Not Detected SD = standard deviation
Formulations:
Q HPMC = quercetin incorporated in 3% hydroxypropyl methylcellulose hydrogel
Q CD5% HPMC = quercetin incorporated in 3% hydroxypropyl methylcellulose hydrogel added of 5% of *β*-cyclodextrin
Q chitosan = quercetin incorporated in 2.6% chitosan hydrogel
Q CD5% chitosan = quercetin incorporated in 2.6% chitosan hydrogel added of 5% of *β*-cyclodextrin

Table 3 shows that when 3-*O*-MQ is incorporated to HPMC hydrogels (3-*O*-MQ HPMC), the release of the molecule to the skin is very low, even null. In CS hydrogel (3-*O*-MQ chitosan), 3-*O*-MQ was detected in the epidermis and dermis, but still in low concentration.

The addition of 5% of *β*-CD results in an increase of the 3-*O*-MQ release and permeation from both hydrogels (HPMC and CS), revealing the permeation promoter effect of the cyclodextrin. The highest 3-*O*-MQ concentration was observed for 3-*O*-MQ CD5% HPMC in the three skin layers, stratum corneum, epidermis and dermis.

The observation of the release and permeation of quercetin from the formulations Q–HPMC and Q-chitosan reveals a clear effect of the hydrogel polymer on this parameter (Table 4). Chitosan showed a promoter effect on the release and permeation of quercetin into the three skin layers, with an retention increase of approximately twofold to the stratum corneum, epidermis and dermis.

The addition of 5% of *β*-CD results in an increase of the Q release and permeation from both hydrogels (HPMC and CS), revealing the permeation promoter effect of the cyclodextrin. The highest Q concentration was observed for Q CD5% chitosan, where the additional effect of chitosan and cyclodextrin could be observed.

Overall, it is possible to observe that Q presented a greater skin retention amount compared with 3-*O*-MQ. Regarding the polymers interactions, the chitosan positive charge seems to facilitate the accumulation of the more polar flavonoid tested (Q), whereas 3-*O*-MQ presented a better permeation when incorporated at the neutral polymeric matrix (HPMC).

In addition, the permeation promotion effect of *β*-CD was significantly higher when the flavonoids are incorporated into the HPMC hydrogel, comparatively to CS hydrogel. The association of *β*-CD improved the permeation of the flavonoids, especially regarding the retention on the stratum corneum and epidermis.

Chitosan clearly demonstrated to be a great permeation promoter for Q, since the flavonoid retention in the epidermis was remarkably increased (1.15 μg/cm^2).

Considering that the experiment was performed aiming a pharmaceutical formulation for topical application in herpes labial infections, the bioactive compounds need to reach the epidermis, more especially, the stratum basal, as this is the main location site of the herpes virus (HUKKANEN et al., 1999). For this purpose, quercetin may be incorporated in chitosan hydrogels without the need of *β*-CD association, however, when HPMC is chosen, the *β*-CD permeation enhancer effect is desired.

The results also reveal that the association of 3-O-MQ with Q in one unique formulation could be an interesting perspective, since both present antiviral activity,

either incorporated in a HPMC or chitosan hydrogel, both containing *β*-CD as permeation promoter.

4. Conclusions

The evaluation of the skin layers retention profile of the flavonoids quercetin and 3-*O*-metilquercetin from the hydrogels hydroxypropyl methylcellulose and chitosan demonstrated that the first one is more suitable for the use of less polar molecules, such as quercetin methylated derivatives, whereas the second one may be employed for a greater polarity range. *β*-cyclodextrin influenced positively the retention of the flavonoids in the skin layers, especially regarding the epidermis. The results point also to the perspective of incorporation of both flavonoids into unique formulation, using hydroxypropyl methylcellulose or chitosan as hydrogel polymer, since that *β*-cyclodextrin is added as permeation promoter.

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III. DISCUSSÃO GERAL

A atividade de interesse apresentada pelo flavonóide 3-*O*-metilquercetina, o custo elevado da substância isolada (U$ 42.00/mg + custos de envio) e a necessidade de sua obtenção em quantidades maiores para o desenvolvimento de produtos tecnológicos e testes biológicos, motivaram a busca de fonte alternativa da substância. Estudos mostraram que os tricomas existentes em *Nicotiana tabacum* excretam flavonóides em resposta ao estresse ambiental e ao ataque de herbívoros, e que 3-*O*-metilquercetina e outros derivados metilados da quercetina são os principais flavonóides encontrados em exsudatos da espécie (YANG *et al*., 1960; WOLLENWEBER e DÖRR, 1995). Esta característica, associada ao cultivo bem estabelecido da espécie e ao desencorajamento ao uso de cigarros, evidencia o potencial do tabaco como matéria-prima para a extração de flavonóides *O*-metilados.

Para o isolamento e purificação do flavonóide 3-*O*-metilquercetina foi necessária a otimização das condições de extração a partir das folhas de tabaco, o que resultou na escolha do solvente etanol na proporção droga:solvente 1:15 com tempo de extração de 30 minutos. O objetivo da extração foi a obtenção de alta concentração do flavonóide associada a uma reduzida presença de substâncias adjacentes, além do uso de solvente de baixa toxicidade. Neste contexto, 60 minutos de extração ocasionou a extração de outros componentes além dos quatro obtidos na extração utilizando o tempo de 30 minutos. Além disso, os demais solventes (exceto acetato de etila) também provocaram a extração de um número superior de substâncias, que dificultam o isolamento do flavonóide. A subseqüente etapa de isolamento a partir da solução extrativa foi realizada com acetato de etila, que selecionou a 3-O-metilquercetina, eliminando grande parte da nicotina e da rutina (solúveis em meio aquoso).

A avaliação da influência de outros parâmetros como variedade da planta, tipo de cultivo (solo ou vaso), tamanho das folhas e horário da colheita sobre a concentração do flavonóide na planta foi fundamental para definição de parâmetros de cultivo da planta e coleta das folhas. De modo geral, todos os fatores supracitados exerceram efeito nos resultados.

Comparando-se o cultivo do tabaco em vaso ou no solo, a concentração do flavonóide na planta foi superior quando cultivado no solo, provavelmente em decorrência da manifestação de defesa mais intensa da planta frente a agentes externos nesse ambiente de menor proteção ambiental; entretanto a proteção provida pelo vaso frente às variações extremas de temperatura permitiu estender o cultivo até a safra seguinte. Entre as variedades e cultivares estudados, a variedade Dark foi a que apresentou a maior concentração do flavonóide. Entre os horários de colheita testados, o que se apresentou mais adequado para a obtenção de maior concentração do flavonóide foi o da meia noite, provavelmente devido ao aumento de sua produção em resposta ao frio e sereno. Além disso, observou-se que a concentração do flavonóide é inversamente proporcional ao tamanho da folha, denotando que sua produção ocorre principalmente nos primeiros estágios de desenvolvimento e se mantém constante à medida que a planta cresce.

A utilização da planta fresca no presente estudo motivou a avaliação da influência de parâmetros como tempo entre a colheita e a extração, bem como o tipo de embalagem utilizada no seu acondicionamento sobre a concentração do flavonóide. Observou-se que após 24 horas de armazenamento em sacola plástica branca em temperatura ambiente, ocorreu diminuição no teor do flavonóide na planta, de 3,5 ± 0,08 µg/g no tempo zero para 2,66 ± 0,05 µg/g após 24 horas, correspondendo a uma redução de 24 % do valor inicial. Após 48 horas, esta diminuição chega a 34 % (2,31 ± 0,09 µg/g). Entretanto, quando a planta é mantida em embalagem em mais baixa temperatura (sacola térmica), a perda foi de 11 % e 19 % após 24 e 48 horas, respectivamente.

3-*O*-metilquercetina já foi sintetizada e isolada a partir de outras plantas estudadas por nosso grupo de pesquisa, como *Achyrocline satureioides* (SCHWINGEL *et al*., 2008). Esta experiência anterior motivou o seu isolamento do tabaco por cromatografia em camada delgada preparativa e por cromatografia em coluna. Um fator que diferencia o isolamento de 3-*O*-metilquercetina a partir de *Achyrocline satureioides* e de tabaco na purificação da substância é a presença de luteolina na primeira espécie, que pela proximidade das bandas no sistema cromatográfico, representava um fator limitante para o isolamento. No caso da extração do tabaco, a extração a partir da planta fresca e a presença do flavonóide

nos tricomas representam fatores facilitadores para a sua extração comparativamente à extração a partir da espécie *Achyrocline satureioides*. Além disso, foram detectadas apenas quatro substâncias interferentes principais nos extratos obtidos a partir do tabaco (nicotina, rutina, 3-*O*-metilquercetina e 5-*O*-metilquercetina ou 7-*O*-metilquercetina), sendo que ao hidrolisar a rutina e retirar a nicotina, a obtenção de uma fração rica em flavonóides (quercetina e seus derivados metilados) revela-se como uma perspectiva do trabalho.

A baixa solubilidade em água das agliconas quercetina e 3-*O*-metilquercetina motivou o estudo sobre a complexação dessas moléculas com *β*-ciclodextrina. Para aprofundar os conhecimentos sobre o processo de complexação dos flavonóides, foi realizado um estudo de modelagem molecular utilizando o método semi-empírico Recife Model 1, que consiste na reparametrização do método de mecânica quântica Austin Model 1 (AM1). Em água, a inserção do anel B na cavidade da *β*-ciclodextrina pela borda das hidroxilas secundárias é a conformação mais provável, enquanto que no vácuo, a conformação mais provável é obtida pela inserção do anel A na cavidade pela borda das hidroxilas primárias.

Estudos de modelagem molecular e ressonância magnética nuclear de complexos de inclusão dos flavonóides quercetina e 3-*O*-metilquercetina, os quais apresentam estruturas moleculares similares, indicaram que o fenômeno de inclusão ocorre pela inserção do anel B dos flavonóides na cavidade da *β*-ciclodextrina pela borda das hidroxilas secundárias (ZHENG, *et al.,* 2005; YAN *et al*., 2006; BERGONZI *et al*., 2007; YAN *et al*, 2007; SCHWINGEL *et al*., 2008). No presente estudo, estas observações foram corroboradas.

As análises conformacionais dos complexos forneceram dados interessantes. Novamente, o papel do solvente foi crucial para estabilizar os sistemas, considerando que a energia envolvida pela complexação no vácuo foi menos favorável e apresentou geometria de associação diferente. É importante salientar que em ambos os meios, a inserção dos flavonóides através da borda de hidroxilas secundárias é preferida, embora a orientação do anel A seja preferida em vácuo. Oportunamente, o solvente favoreceu a inserção do anel A, e esta interação ocorreu com a inserção pela borda das hidroxilas primárias. A conformação menos favorável

em água (inserção do anel A pela borda das hidroxilas secundárias) correspondeu à conformação mais provável em vácuo. Considerando estas associações, a diferença de 1 kcal/mol é suficiente para inferir que uma conformação ocorre e a outra não.

Nos estudos de permeação/retenção nas camadas da pele, foi observada claramente a influência da utilização de hipromelose ou de quitosana como base semi-sólida na incorporação de quercetina ou 3-*O*-metilquercetina. Quercetina apresentou uma maior retenção na pele se comparada com 3-*O*-metilquercetina, sendo que o gel de quitosana contribuiu significativamente para a promoção de sua permeação.

Quando 3-*O*-metilquercetina foi incorporada no hidrogel de hipromelose, a liberação da molécula para a pele foi muito baixa, até mesmo nula. Quando incorporada no hidrogel de quitosana, o flavonóide foi detectado na epiderme e na derme, entretanto, ainda em baixa concentração. A adição de 5% de *β*-CD resultou num aumento da liberação da base e permeação de 3-*O*-metilquercetina a partir dos dois hidrogéis, revelando o efeito promotor de permeação da ciclodextrina. A maior concentração do flavonóide nas camadas da pele estrato córneo, epiderme e derme, foi observada para a formulação de hipromelose contendo 5% de *β*-CD.

No que se refere à quercetina, a observação da sua liberação e permeação a partir de ambos hidrogéis revelou o efeito do polímero neste parâmetro, sendo maior para a quitosana. A adição de 5% de *β*-CD às formulações resultou num aumento da permeação do flavonóide tanto a partir do hidrogel de hipromelose, quanto para o hidrogel de quitosana, revelando, mais uma vez, o seu efeito promotor de permeação.

No geral, foi possível observar que a quercetina apresentou uma maior retenção nas camadas da pele comparativamente à 3-*O*-metilquercetina. Este fenômeno pode ser relacionado com as diferenças estruturais apresentadas pelas moléculas, as quais puderam ser observadas no artigo referente ao estudo de modelagem molecular pelo método semi-empírico RM1. O tamanho da molécula, mas especialmente sua conformação (rigidez da estrutura química) e polaridade são características que podem contribuir de formas diferentes às interações com as

estruturas da pele, com a base polimérica e com a ciclodextrina. Há a possibilidade de que a maior polaridade da quercetina, a qual é superior à da 3-*O*-metilquercetina, tenha contribuído positivamente para a sua difusão através matriz polimérica carregada positivamente (quitosana). A hipótese de que o impedimento estérico e o movimento angular do diedro do grupamento *O*-metila tenha sido um dos fatores limitantes para a sua permeação através das camadas da pele.

Em relação ao efeito promotor da liberação e penetração apresentado pela *β*-CD, é possível observar que este foi significativamente superior quando os flavonóides foram incorporados no hidrogel de hipromelose comparativamente ao hidrogel de quitosana, sendo que o aumento da concentração dos flavonóides ocorreu especialmente na epiderme e estrato córneo. A presença de carga positiva da quitosana é uma característica a ser considerada para explicar a diferença observada entre os hidrogéis testados. A possibilidade de ocorrer interações entre as hidroxilas da ciclodextrina e dos flavonóides com a quitosana, influenciando negativamente a sua difusão no hidrogel e permeação através da pele, não pode ser descartada.

Considerando que o experimento foi desenvolvido com o objetivo de se obter uma formulação farmacêutica de uso tópico para o tratamento de herpes simplex tipo 1, as moléculas bioativas têm necessidade de atingir a epiderme mais profunda, onde ocorre a replicação viral (HUKKANEN et al., 1999). Sob esta perspectiva, a quercetina incorporada em hidrogéis de quitosana sem a necessidade de sua associação com *β*-CD, seria suficiente. Entretanto, optando-se por hipromelose como base para a quercetina, o efeito promotor de permeação da *β*-CD seria necessário.

No que se refere à possibilidade de associação de ambos os flavonóides, quercetina e 3-O-metilquercetina, numa única formulação, esta apresenta-se como uma perspectiva interessante, uma vez que ambos apresentam importante atividade antiviral. Muito embora os resultados obtidos apontem para a formulação de hipromelose contendo 5% de *β*-CD como base para sua veiculação, estudos adicionais são necessários para corroborar esta hipótese.

IV. CONCLUSÕES GERAIS

O flavonóide 3-*O*-metilquercetina foi isolado a partir dos exsudatos das folhas de tabaco orgânico por cromatografia em camada delgada preparativa e também por cromatografia em coluna.

O método proposto para a extração bruta do flavonóide a partir das folhas frescas de tabaco de cultivo orgânico mostrou-se adequado, resultando num rendimento bruto de até 7 µg de 3-*O*-metilquercetina por g de planta fresca. O solvente utilizado é de baixo custo, baixa toxicidade e de reduzido impacto ambiental, denotando seu potencial de utilização industrial.

Após isolamento e purificação do flavonóide 3-*O*-metilquercetina a partir dos extratos brutos por cromatografia em camada delgada preparativa e também por cromatografia em coluna, foi obtido um rendimento de 4,2 x 10^{-4} % em relação à massa da planta fresca, correspondendo em média a 42 µg por folha da planta, e pureza superior a 90 %, medida pelo método de HPLC.

A modelagem molecular do fenômeno de complexação dos dois flavonóides com *β*-ciclodextrina utilizando o método semi-empírico RM1, revelou que a inclusão do flavonóide quercetina é favorecida comparativamente à inclusão de 3-O-metilquercetina. O efeito do solvente foi crítico na estabilização dos sistemas, sendo que as interações determinantes da complexação por inclusão no vácuo foram menos favoráveis.

A complexação dos flavonóides com *β*-CD em água ocorreu com a inserção preferencial do anel B através da borda de hidroxilas secundárias, estando em conformidade com os dados experimentais. No vácuo, a complexação foi realizada com a inserção do anel A através da borda das hidroxilas secundárias.

A avaliação do perfil de liberação e penetração/retenção dos flavonóides 3-*O*-metilquercetina e quercetina nas camadas da pele a partir de hidrogéis de hipromelose ou quitosana demonstrou que, na ausência de ciclodextrina, o primeiro é mais adequado para a liberação e penetração do flavonóide metilado, enquanto que o segundo mostrou favorecer a liberação e penetração do flavonóide de maior

polaridade, a quercetina. A *β*-ciclodextrina influenciou a liberação dos flavonóides da base e a penetração destes nas camadas da pele, especialmente na epiderme mais profunda, local alvo para o tratamento do herpes simplex tipo 1.

Em seu conjunto, os resultados apontam para a utilização da espécie *Nicotiana tabacum* como fonte importante de flavonóides metilados, em especial a 3-*O*-metilquercetina, em rendimento e pureza adequados, utilizando metodologia de baixo custo e impacto ambiental. A associação deste flavonóide e da quercetina com ciclodextrina e sua incorporação em base hidrofílica polimérica, denotaram que esses sistemas apresentam-se adequados para a veiculação dos mesmos na pele visando ao tratamento do herpes simplex tipo 1. A modelagem molecular permitiu a compreensão dos fenômenos estéricos e de dinâmica molecular envolvidos na associação dos flavonóides com *β*-ciclodextrina.

V. PERSPECTIVAS

Tendo em vista a multidisciplinaridade deste trabalho, de modo geral, abrem-se perspectivas interessantes brevemente detalhadas abaixo.

Em relação ao primeiro capítulo, a investigação e aprimoramento da extração e isolamento dos flavonóides e nicotina de *Nicotiana tabacum* ainda se apresenta como uma importante perspectiva. Além disso, a possibilidade de isolamento e purificação da nicotina para comercialização e a obtenção de uma fração rica em flavonóides, metilados e não metilados, juntos ou separados, para aplicação em testes farmacológicos é certamente uma perspectiva de grande interesse.

Ensaios *in vitro* para verificar a atividade dos flavonóides, isoladamente ou associados, frente ao vírus herpes simplex tipo 1, bem como investigar a influência da associação com ciclodextrina nesta atividade, mostram-se como uma etapa subseqüente a ser investigada. A partir dos resultados obtidos nos ensaios *in vitro*, testes das formulações *in vivo* frente ao HSV-1, utilizando camundongos nus imunodeprimidos, representam outra perspectiva.

A determinação de uma dose ótima para aplicação, com o melhor efeito terapêutico e menor conjunto de reações adversas, somente seria alcançada em ensaios clínicos de fase II com humanos. A revisão de literatura não descreve, até o presente, estudos da atividade anti-herpética *in vivo*, em modelo de camundongo BALB/c nu, de formulações de aplicação tópica contendo quercetina e/ou 3-*O*-metilquercetina, associadas ou não à β-ciclodextrina, representando, portanto, esta uma possibilidade para a sequência dos estudos.

V. REFERÊNCIAS

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