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Brassica oleracea var. *capitata:* EXTRAÇÃO, CARACTERIZAÇÃO QUÍMICA E ATIVIDADE BIOLÓGICA DE METABÓLITOS SECUNDÁRIOS

DISSERTAÇÃO DE MESTRADO

Valéria Dal Prá

Santa Maria, RS, Brasil
2013

Brassica oleracea var. *capitata:* EXTRAÇÃO, CARACTERIZAÇÃO QUÍMICA E ATIVIDADE BIOLÓGICA DE METABÓLITOS SECUNDÁRIOS

Valéria Dal Prá

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Mestre em Ciências Farmacêuticas

Orientador: Prof. Dr. Marcelo Barcellos da Rosa

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A Comissão Examinadora, abaixo assinada,
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***Brassica oleracea* var. *capitata:* EXTRAÇÃO, CARACTERIZAÇÃO QUÍMICA E ATIVIDADE BIOLÓGICA DE METABÓLITOS SECUNDÁRIOS**

elaborada por
Valéria Dal Prá

como requisito parcial para obtenção do grau de
Mestre em Ciências Farmacêuticas

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Santa Maria, 22 de julho de 2013.

Aos meus pais, Emilia e Aristides, e minha irmã, Eliza.

Ao meu esposo, Marcio.

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

Brassica oleracea var. *capitata:* EXTRAÇÃO, CARACTERIZAÇÃO QUÍMICA E ATIVIDADE BIOLÓGICA DE METABÓLITOS SECUNDÁRIOS

AUTORA: Valéria Dal Prá
ORIENTADOR: Marcelo Barcellos da Rosa
Data e local da defesa: Santa Maria, 22 de julho de 2013.

O objetivo principal deste trabalho foi avaliar a atividade antioxidante e antibacteriana de extratos de *Brassica oleracea* var. *capitata*, obtidos a partir de extração supercrítica e extração por ultrassom, além de caracterizá-los por cromatografia gasosa acoplada à detector de massas. Para os compostos bioativos apolares de *Brassica oleracea* var. *capitata*, utilizou-se extração com CO_2 supercrítico e avaliou-se o potencial antioxidante dos extratos. Foram realizadas cinco extrações para investigar a influência da pressão (10 - 25 MPa) e temperatura (20 - 60°C) no rendimento da extração, na composição química e na atividade antioxidante frente os radicais peroxila, superóxido e hidroxila. Obteve-se o maior rendimento de extração 0,47% a 60 °C e 25 MPa. Na caracterização dos extratos foi possível a identificação de compostos como sulforafano e iberin nitrila. Todos os extratos apresentaram atividade antioxidante para os três radicais, porém a maior atividade para todos os radicais foi o extrato obtido a 60°C e 25 MPa. Para os compostos bioativos polares, otimizou-se a extração assistida por ultrassom. Os extratos obtidos nas melhores condições de extração foram submetidos a diferentes condições de hidrólise, antes da sua utilização nos ensaios biológicos. Avaliou-se a atividade antioxidante, frente ao radical DPPH, superóxido e peroxila, além da atividade antibacteriana, frente *a S.aureus* e *E.coli*. Tanto os extratos brutos quanto os hidrolisados, foram caracterizados por cromatografia gasosa acoplada à detector de massas. A melhor condição de extração foi a 30ºC e 60% (m/v) de etanol. Todos os extratos apresentaram atividade antioxidante frente aos radicais DPPH, superóxido e peroxila, mas o uso de extratos hidrolisados melhorou consideravelmente a atividade antioxidante. Em relação à atividade antibacteriana, apenas uma amostra, que foi submetida à condição de hidrólise alcalina apresentou ação frente a *E.coli*. Uma das principais contribuições deste trabalho foi que a utilização de extração com CO_2 supercrítico, para obtenção de compostos bioativos de *Brassica oleracea* var. *capitata*, mostrou ser uma alternativa promissora em relação aos métodos convencionais de extração, pois permitiu a extração de compostos com interesse científico e industrial. Além disso, foi demonstrado que a hidrólise dos extratos pode aumentar consideravelmente a atividade antioxidante de extratos vegetais em relação aos extratos brutos.

Palavras-chave: *Brassica oleracea* var. *capitata;* CO_2 supercrítico; Ultrassom; Atividade antioxante; Atividade antibacteriana.

ABSTRACT

Master Dissertation
Graduate Program in Pharmaceutical Sciences
Federal University of Santa Maria

Brassica oleracea var. *capitata*: EXTRACTION, CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF SECONDARY METABOLITES

AUTHOR: Valéria Dal Prá
ADVISER: Marcelo Barcellos da Rosa
Date and place of defense: Santa Maria, July 22, 2013.

The main objective of this work was to evaluate the antioxidant and antibacterial activities of extracts of *Brassica oleracea* var. *capitata*, obtained by supercritical CO_2 and ultrasound-assisted extractions, as well as to carry out the characterization of these extracts using gas chromatography coupled with mass detector. For supercritical CO_2, five extractions were performed to investigate the influence of pressure (10-25 MPa) and temperature (20-60°C) on the extraction yield, chemical composition and antioxidant activity towards peroxyl, superoxide and hydroxyl radicals. The highest extraction yield was 0.47% at 60 °C and 25 MPa. In the characterization of the extracts was possible to identify compounds like sulforaphane and iberin nitrile. All extracts showed antioxidant activity for the three radicals, although the highest activity for all radicals was obtained using the extract obtained at 60 °C and 25 MPa (run 2). For the ultrasound-assisted extraction were evaluated the effects of solvent concentration and temperature. The extracts obtained in the optimized extraction condition, were subjected to different hydrolysis conditions before use in biological assays. It was evaluated the antioxidant activity against DPPH, superoxide and peroxyl radicals, besides the antibacterial activity against *S. aureus* and *E. coli*. Both crude and hydrolyzed extracts were characterized by gas chromatography coupled with mass detector. The best condition for extraction was 30 ° C and 60% (w /v) of ethanol. All extracts showed antioxidant activity towards DPPH, superoxide and peroxyl radicals, but the use of hydrolyzed extracts improved considerably the antioxidant activities. Antibacterial activity was detected only in extracts hydrolysates *Brassica oleracea* var. *capitata*. The main contributions of this work were that the use of supercritical CO_2 extraction to obtain bioactive compounds from *Brassica oleracea* var. *capitata* showed a promising alternative to conventional methods of extraction, since it allowed the extraction of compounds of interest in science and industry. Besides, in this work was demonstrated that the hydrolysis of extracts can increase the antioxidant activity of plant extracts.

Keywords: *Brassica oleracea* var. *capitata*; Supercritical CO_2 extraction; Ultrasound-assisted extraction; Antioxidant activity; Antibacterial activity.

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ABAP - cloreto de 2,2’ – azo-bis (2-metil aminopropano)

CO_2 - Dióxido de carbono

DCFH-DA - 2,7’-diclorofluoresceína

DPPH - 2,2-difenil-1-picril-hidrazila

E. coli - *Escherichia coli*

EDTA - Ácido etileno diamino tetra-acético

ESC - Extração supercrítica

FRAP - Poder antioxidante de redução do ferro

$FeCl_3$. 6 H_2O - Cloreto férrico hexahidratado

CG-MS - Cromatografia gasosa acoplada à espectrometria de massas

GLS - Glicosinolatos

H_2O_2 - Peróxido de hidrogênio

HO•- - Radical hidroxila

HCl- Ácido clorídrico

HPLC - Cromatografia líquida de alta eficiência

HPX - Hipoxantina

MPa- Mega Pascal

LC-MS- Cromatografia líquida acoplada à espectrometria de massas

ORAC- Capacidade de absorção dos radicais oxigenados

O_2•- -Ânion radical superóxido

NaOH- Hidróxido de sódio

NBT - Nitro Blue Tetrazoliun

ROO•- -Radical peroxila

S. aureus - *Staphylococcus aureus*

SFE – Extração por fluído supercrítico

SUS - Sistema Único de Saúde

TBA- Ácido tiobarbitúrico

TCA- Ácido tricloroacético

US - Ultrassom

UV-VIS - Ultravioleta visível

XOD - Xantina oxidase

SUMÁRIO

1 INTRODUÇÃO

Nas últimas décadas, o crescente uso da fitoterapia como uma prática médica, associada à busca intensa por produtos biotecnológicos vem atraindo forte interesse em diferentes setores, como na indústria farmacêutica, cosmética e na indústria de alimentos. O uso de plantas medicinais no Brasil é facilitado pela diversidade de plantas de baixo custo associado à terapêutica, e aliado a isso soma-se o incentivo do uso de produtos fitoterápicos pelo sistema único de saúde (SUS).

O estudo de atividades terapêuticas de extratos obtidos a partir de plantas medicinais é gerado através de avanços científicos envolvendo estudos químicos, farmacológicos e toxicológicos. Esses produtos se apresentam de tal forma que atendem às necessidades básicas de saúde em função da facilidade de acesso, baixo custo e compatibilidade com as tradições populares (WAGNER, 2007).

Dentre as inúmeras espécies com grande interesse farmacológico, se destacam as espécies da família Brassicaceae, em especial a *Brassica oleracea* var. *capitata*, conhecida popularmente como repolho branco, que antes de ser empregado como um alimento era usado na medicina popular para o tratamento de dores de cabeça, gota, diarréia e úlceras pépticas (SINGH et al., 2006). *Brassica oleracea* vem sendo amplamente estudada por apresentar atividade antioxidante e possuir compostos que apresentam mecanismos de desintoxicação, que eliminam e estabilizam a produção de substâncias cancerígenas (BROOKS; PATON; VIDANES, 2001; FARAG; MOTAAL, 2010). Estudos farmacológicos revelam que a *Brassica* apresenta ações como anti-inflamatória, antimicótica, anti-hiperglicêmica, anticarcinogênica, antioxidante e antibacteriana (KATAYA; HAMZA, 2007; LIN; LI; HWANG, 2008; BAPJAI; KANG; BAEK, 2012; JAISWAL; ABU-GHANNAM; GUPTA, 2012).

No entanto, a atividade biológica da *Brassica* está diretamente relacionada ao método de extração, uma vez que isso implica na seletividade de compostos. Paralelamente à extração convencional (hidrodestilação, maceração e soxhlet), a extração supercrítica (ESC) e a extração por ultrassom são métodos alternativos de extração. A ESC tem ganhado destaque, sendo considerada como uma inovação tecnológica, pois utiliza fluidos supercríticos como solventes e seu emprego em processos industriais vem ganhando espaço continuamente, principalmente devido aos fatores ambientais e de qualidade envolvidos. Trata-se de um

processo livre de resíduos tóxicos, que não provoca a degradação térmica dos extratos e não requer grandes gastos de energia, como ocorre em processos de destilação. Além disso, proporciona uma melhor seletividade e eficiência ao processo e o solvente pode ser facilmente removido no final da extração. Assim, o emprego de fluídos supercríticos tem sido considerado uma ótima opção para a extração e fracionamento de produtos naturais, particularmente para as indústrias de alimentos e farmacêuticas (PEREIRA et al., 2004). Já a extração por ultrassom proporciona uma maior recuperação do analito e utiliza uma quantidade de solvente inferior as demais análises, além de proporcionar uma melhor extração em um tempo mais curto (LUQUE DE CASTRO; PRIEGO-CAPOTE, 2007). Além disso, é versátil e pode ser utilizada tanto em pequenas quanto em grandes escalas (DUBIE et al., 2013).

A obtenção e estudo de extratos de *Brassica* usando métodos convencionais são bem reportados na literatura, porém conforme revisão realizada há uma escassez de estudos referente à obtenção desses extratos por métodos alternativos, como os citados anteriormente. Especificamente, sobre uso de ESC para obtenção de compostos bioativos, Pereira e Meireles (2010) descrevem estudos para uma grande diversidade de matérias-primas. Porém, não foram verificados, até o presente momento, trabalhos envolvendo ESC para extração de compostos bioativos de *Brassica oleracea* var. *capitata.* Além disso, a extração de compostos bioativos desta planta por ultrassom é pouco estudada. Consequentemente, o estudo da atividade biológica desses extratos obtidos por métodos não convencionais é também pouco relatado, abrindo assim uma lacuna para sua exploração.

A presente dissertação encontra-se organizada na forma de artigos científicos. Inicialmente é apresentada uma introdução geral, revisão da literatura, objetivos gerais e específicos. Bem como, um artigo de revisão complementando a revisão da literatura. A seguir, são apresentados dois artigos científicos referentes aos resultados experimentais desse trabalho, além de uma seção integrando a discussão do mesmo. Para finalizar, é apresentada a conclusão geral da pesquisa realizada.

2 OBJETIVOS

2.1 Objetivo Geral

O objetivo deste trabalho é avaliar a atividade antioxidante e antibacteriana de extratos de *Brassica oleracea* var. *capitata*, obtidos a partir de extração com dióxido de carbono (CO_2) supercrítico e extração assistida por ultrassom, além de caracterizá-los por cromatografia gasosa.

2.2 Objetivos Específicos

Para atender ao objetivo geral, fez-se necessário o cumprimento das seguintes etapas:

- Otimização da extração de *Brassica oleracea* var. *capitata* por CO_2 supercrítico;
- Otimização da extração de *Brassica oleracea* var. *capitata* por ultrassom;
- Seleção da melhor condição de extração de *Brassica oleracea* var. *capitata* por ultrassom e submissão à condições de hidrólise ácida e alcalina dos mesmos;
- Caracterização dos compostos químicos majoritários dos extratos de *Brassica oleracea* var. *capitata*, tanto obtidos por CO_2 supercrítico quanto por ultrassom, via Cromatografia Gasosa acoplada à detector de massas (CG-MS);
- Investigação da atividade antirradicalar dos extratos de *Brassica oleracea* var. *capitata*, obtidos por CO_2 supercrítico pelos métodos *in vitro* do radical superóxido ($O_2^{-\bullet}$), radical hidroxila (HO•), radical peroxila (ROO•);

- Investigação da atividade antirradicalar dos extratos brutos e dos extratos hidrolisados de *Brassica oleracea* var. *capitata,* obtidos por ultrassom pelos métodos *in vitro* do radical superóxido ($O_2^{-\bullet}$), radical peroxila (ROO•) e pelo método clássico baseado na captura do radical $DPPH^{\bullet}$;

- Avaliação da atividade antibacteriana frente à *Staphylococcus aureus* e *Escherichia coli* de todos os extratos;

3 REVISÃO BIBLIOGRÁFICA

3.1 Brassica

A família Brassicaceae apresenta cerca de 400 gêneros e 4.000 espécies. No Brasil ocorrem 7 gêneros e aproximadamente 50 espécies (SOUZA; LORENZI, 2005). Através do melhoramento genético são encontradas diversas variedades horticulturais, com destaque para *B. oleracea* var. *acephala* (couve-manteiga), *B. oleracea* var. *capitata* (repolho), *B. oleracea* var. *gemmifera* (couve-de-bruxelas), *B. oleracea* var. *borytis* (couve-flor) e *B. oleracea* var. *italica* (brócolis). Outras espécies de destaque são as mostardas (*Brassica nigra* e *Sinapis* spp.), o rabanete (*Raphanus sativus*), a raiz-forte (*Armoracia rusticana*), o agrião (*Rorippa nasturtium-aquaticum*) e a rúcula (*Eruca sativa*) (SOUZA; LORENZI, 2005; PAULINO, 2008).

Algumas espécies são cultivadas como ornamentais, destacando-se o repolho-ornamental (*B. oleracea* var. *acephala*), o alisso (*Lobularia maritima*) e o mussambe (*Cleome hassleriana*). Muitas Brassicaceae são invasoras de cultura, incluindo-se aqui as mostardas-do-campo (*Brassica rapa*, *Rapistrum rugosum* e *Sinapis arvense*), a bolsa-de-pastor (*Capsella bursa-pastoris*), o agrião-bravo (*Cardamine bonariensis*), o mastruco (*Coronopus didymus*), o menstruz (*Lepidium* spp.) e a nabica (*Raphanus raphanistrum*) (SOUZA; LORENZI, 2005; PAULINO, 2008).

A família Brassicaceae tem sido muito estudada devido às propriedades farmacológicas de seus principais metabólitos, os glicosinolatos (GLS). Esses metabólitos, assim como os seus produtos de hidrólise (isotiocianatos e nitrilas) são potentes antioxidantes, anticarcinogênicos e agentes antibacterianos (MÁRTON, et al., 2013). A ação protetora dos vegetais crucíferos também tem sido atribuída à presença de compostos antioxidantes, especialmente vitaminas incluindo ácido ascórbico, α-tocoferol e β-caroteno. Estudos mostram que a maior parte da atividade antioxidante desses vegetais pode ser atribuída a compostos fenólicos, como os flavonóides, isoflavonas, flavonas, antocianinas e catequinas (SINGH et al, 2006).

3.2 Brassica oleracea var. capitata

Brassica oleracea var. *capitata*, conhecida popularmente como repolho (Figura 1), é uma das hortaliças mais importantes cultivadas no mundo. Este vegetal enraizado é cultivado por sua estrutura grande e frondosa. Os diferentes tipos de cultura de repolho mostram uma grande variação no que diz respeito a tamanho, forma e cor das folhas, bem como o tamanho, cor, forma e textura da cabeça. Estudos mostram que a *Brassica* sp. antes de ser empregada como alimento, já era utilizada na medicina popular, no tratamento de cefalgias idiopáticas (dores de cabeça de causas desconhecidas), dores reumáticas, tumores, desnutrição, anemia, úlceras pépticas, hemorróidas, alcoolismo, gota e como cicatrizante (SINGH et al, 2006; CARVALHO et al., 2008).

Muitas pesquisas têm se concentrado nos benefícios dos seus fitoconstituintes. Entre eles, os glicosinolatos (GLS), um dos maiores grupos de metabólitos secundários contendo enxofre, que são encontrados principalmente em vegetais comestíveis. Esses compostos são armazenados nos vacúolos das células vegetais, porém após dano celular e exposição à mirosinase, enzima encontrada nas plantas que contêm GLS, são hidrolisados a isotiocianatos e nitrila. Esses metabólitos, assim como, os seus produtos de hidrólise são potentes antioxidantes, anticarcinogênicos, anti-inflamatórios e agentes antibacterianos (KATAYA; HAMZA, 2007; MÁRTON et al., 2013).

Estudos sugerem que o sulforafano, GLS presente em vegetais *Brassica* oferece proteção contra o desenvolvimento de tumores durante a fase de pós-iniciação, e os mecanismos envolvidos nos efeitos supressores do sulforano incluem parada do ciclo celular e indução de apoptose (GAMET-PAYRASTRE et al., 2000; PARNAUD; LI; CASSAR, 2004).

Embora a maioria das pesquisas aborde a obtenção majoritária dos GLS em *Brassica oleracea*, compostos como vitamina C, vitamina E, β-caroteno e compostos fenólicos com atividade antioxidante, têm sido detectados (SINGH et al., 2006; KORUS, 2011). Ácidos fenólicos, como ferúlico, p-cumárico, cafeico (LEE; BOYCE; BREADMORE, 2011) e triterpenos como lupeol, α e β amirina têm sido encontrados na cera epicuticular da *Brassica oleracea* var. *capitata* e demonstraram efeito gastroprotetor e anti-inflamatório (MARTENLAC; VOVK; SIMONOVSKA, 2007).

Figura 1-*Brassica oleracea* var. *capitata* (repolho).

3.3 Métodos de Extração

A extração tem por objetivo a separação de determinadas substâncias a partir de diversas matrizes, sólidas ou líquidas, através de processos químicos, físicos ou mecânicos. Dentre os métodos de extração há os convencionais, como soxhlet, hidrodestilação e maceração, que são mais empregados na extração de compostos, bem como extração por ultrassom e extração por fluído supercrítico, que são consideradas alternativas aos métodos convencionais de extração (KELLNER et al., 2004, p 404).

A extração supercrítica (ESC) é um método alternativo de extração, considerado como uma inovação tecnológica, pois utiliza fluidos supercríticos como solventes e seu emprego em processos industriais vem ganhando espaço continuamente, principalmente devido aos fatores ambientais e de qualidade envolvidos. Trata-se de um processo livre de resíduos tóxicos, que

não provoca a degradação térmica dos extratos e não requer grandes gastos de energia, como ocorre em processos de destilação. Além disso, proporciona uma melhor seletividade e eficiência ao processo e o solvente pode ser facilmente removido no final da extração. Assim, o emprego de fluidos supercríticos tem sido considerado uma ótima opção para a extração e fracionamento de produtos naturais, particularmente para as indústrias de alimentos e farmacêuticas (BRUNNER, 1994, p.386; KELLNER et al., 2004, p.406; PEREIRA et al., 2004).

Entre os solventes utilizados para esse processo de extração, o CO_2 é considerado adequado para extração de produtos naturais, pois é atóxico, não inflamável, apresenta baixa reatividade, além de ser de fácil obtenção. O CO_2 possui baixa viscosidade e elevados coeficientes de difusão. Sua temperatura (31,1ºC) e pressão crítica (73,8 bar) são facilmente atingidas, o que diminui os custos de compressão (PAVIANI, 2004).

No diagrama pressão *versus* temperatura (Figura 2), a região supercrítica demarca o final da coexistência de fases líquido e vapor. Acima da temperatura crítica um componente puro gasoso não pode ser liquefeito apenas aumentando a pressão aplicada. Da mesma forma, acima da pressão crítica um líquido não pode se vaporizar apenas com o aumento da temperatura. A pressão crítica é a pressão de vapor do gás à temperatura crítica (RODRIGUES et al., 2003).

Especificamente, sobre o uso de ESC para obtenção de compostos bioativos, Pereira e Meireles (2010) em seu artigo de revisão, relatam vários estudos realizados para uma grande diversidade de plantas, como por exemplo, Aloe Vera (*Aloe barbadensis Miller*), Camomila (*Matricaria recutita*), Eucalyptus (*Eucalyptus camaldulensis* Dehn.), Macela (*Achyrocline alata, A. satureioides*), Manjerona (*Origanum majorana* L.), Menta (*Mentha piperita* L.), Alecrim (*Rosmarinus officinalis*), Aquiléia Mil-Folhas (*Achillea millefoliun*), entre outras espécies. Além disso, esses autores descrevem que a ESC é utilizada com sucesso para a recuperação das substâncias extraídas das espécies, além de revelarem melhoria da qualidade, maior rendimento, e na maioria dos casos, maior atividade dos compostos obtidos por esse método de extração. Esse melhor desempenho pode ser atribuído às temperaturas mais brandas utilizadas, que não degradam os compostos extraídos. Porém, através da análise deste artigo de revisão, e de ampla busca na literatura, não foram verificados, até o presente momento, trabalhos envolvendo ESC para extração de compostos bioativos para a espécie *Brassica oleracea* var. *capitata*.

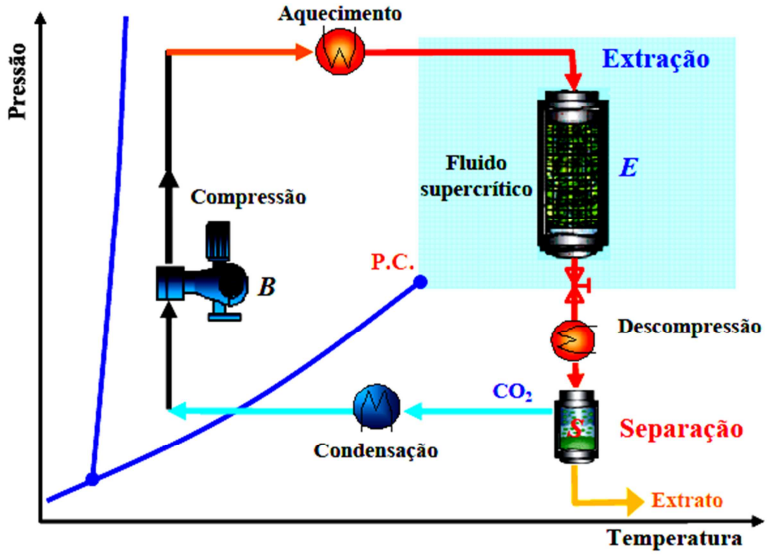

Figura -2. Diagrama de fases para uma substância pura (Fonte: CONDORI, 2005).

Nos últimos anos aumentou-se o uso do ultrassom como uma alternativa aos métodos tradicionais de extração, devido à simplicidade de operação, diminuição no tempo de extração, condições mais seguras para o analista. Esse método possibilita a operação à pressão e temperatura ambiente, além da redução do uso de ácidos e oxidantes, o que também minimiza as perdas de elementos voláteis e gera menos resíduos a serem descartados (LUQUE-GARCIA; LUQUE DE CASTRO, 2003; CAPELO; MADURO; VILHENA, 2005).

O ultrassom em soluções aquosas induz o fenômeno de cavitação acústica no meio líquido, que refere-se à formação, crescimento e implosão de bolhas de gás. A energia liberada durante a cavitação acústica fornece excelentes perspectivas para o preparo e tratamento de amostras, devido às modificações físicas e químicas resultantes deste processo, o que impulsionou novas estratégias de preparo de amostras. Em sistemas heterogêneos, o tratamento é favorecido devido a fenômenos de emulsão nas interfaces de sistemas líquido-líquido, lixiviação na superfície em sistemas sólido-líquido, erosão, fragmentação e aumento da área superficial de partículas sólidas. Esses ocorrem devido às ondas de choque originadas da implosão das micro-bolhas, além da diminuição do gradiente de concentração pelo aumento do transporte de massas ocasionado pela turbulência e micro-jatos (LUQUE-GARCIA; LUQUE DE CASTRO, 2003).

A extração por ultrassom é reconhecida pelo seu potencial de aplicação na indústria fitofarmacêutica para uma grande variedade de extratos de plantas (VILKHU et al., 2008). Esses autores também relataram em artigo de revisão que trabalhos feitos com algumas

plantas, como por exemplo, “hortelã”, “funcho” e “calêndula” apresentaram um maior rendimento de extração quando comparado a métodos convencionais de extração. Hojnik, Skerget e Knez (2007) avaliaram técnicas para extração de compostos da *Urtiga dioica* L. e concluíram que o ultrassom é uma técnica alternativa e promissora por possibilitar maior eficiência na extração.

Kataya e Hamza (2007) reportam a obtenção de extratos de “repolho roxo” por ultrassom ao invés de “repolho branco”. Ainda, Carvalho et al. (2008) citam o uso de ultrassom para a obtenção de extratos de *Brassica oleracea* var. *capitata*, porém os autores não avaliaram os efeitos da concentração de solvente, temperatura, tempo de extração, os quais foram mantidos constantes em 10% (m/V), 28°C e 60 minutos, respectivamente.

3.4 Obtenção e caracterização de extratos de *Brassica*

Existem diferentes métodos de extração e caracterização de compostos bioativos de *Brassica.* Cada um deles com suas especificidades, sendo utilizados em função da aplicação dos extratos obtidos, conforme pode ser visto na Tabela 1.

Tabela 1- Literatura acerca da obtenção e caracterização de extratos de *Brassica.*

Planta	**Método de extração**	**Método de caracterização**	**Referência**
Brassica oleracea L. cv. (Brócolis)	Maceração	HPLC-UV	(ROSSETTO et al., 2013)
*Brassica napus B. juncea (*mostardas amarela e marrom)	Microextração em fase sólida	CG-MS	(WEI et al., 2012)
Brassica alba e (mostarda amarela) e *B.juncea*	Decocção	HPLC-UV	(HERZALLAH; HOLLEY, 2012).
Brassica oleracea L. var. *capitata* f. *rubra* DC	Extração com metanol	LC/MS	(KOO et al., 2011)
Brassica oleracea L. Curvar. *acephala* Var. *sabellica*	Extração por ultrassom	HPLC-DAD-ESI-MS	(OLSEN; AABY; BORGE, 2009)
Brassica oleracea var. *capitata*	Extração por ultrassom	Espectrofotometria UV-VIS, perfil fitoquímico	(CARVALHO et al, 2008)
Brassica oleracea L. var. *capitata f. rubra* (repolho roxo)	Extração com líquido pressurizado	HPLC-DAD e ESI-MS/MS	(ARAPITSAS; SJOBERG; TURNER, 2008).

Entre os métodos de extração para compostos de *Brassica*, têm-se desde os métodos mais simples como decocção e maceração, até métodos alternativos aos convencionais, como microextração em fase sólida, extração por ultrassom e extração por líquido pressurizado. O método escolhido, normalmente reflete o que se deseja obter, bem como a tecnologia que se tem disponível para tal função.

Em relação aos métodos de caracterização, existe também uma variedade de métodos, desde os mais preliminares como análise fitoquímica (reações gerais de caracterização), até métodos mais sofisticados como cromatografia liquída acoplada à detector de massas (LC-MS) e cromatografia gasosa acoplada à detecção de massas (CG-MS). A escolha do método normalmente está associada ao objetivo principal do trabalho.

3.5 Atividade Biológica em *Brassica*

O gênero *Brassica* tem sido amplamente estudado, devido a gama de atividade biológicas que seus metabólitos secundários apresentam. A tabela 2 apresenta alguns estudos científicos acerca das atividades do gênero em questão.

Embora existam vários trabalhos na literatura, a tabela 2 apresenta um apanhado geral das principais atividades do gênero *Brassica*, como por exemplo, antioxidante, anticarcinogênica, anti-inflamatória, fotoprotetora, antiulcerogênica, antibacteriana.

Especificamente sobre a espécie *Brassica oleracea* var. *capitata*, serão abordados alguns trabalhos à respeito de atividade antioxidante e antibacteriana, uma vez que tais atividades são foco do presente trabalho.

Jaiswal, Abu-Ghannam e Gupta (2012) avaliaram a atividade antioxidante de extratos de *Brassica oleracea* var. *capitata* frente ao radical sintético DPPH, apresentando ação inibitória frente ao mesmo. Ahmed et al. (2012) também avaliaram a ação antioxidante dessa espécie frente ao radical DPPH. Ou et al. (2002) determinaram a capacidade antioxidante do repolho pelos métodos ORAC (*Oxygen Radical Absorbance Capacity*), e FRAP (*Ferric Reducing Antioxidant Power*), em que a espécie apresentou ação sobre ambas técnicas. Jacob et al. (2011) investigaram a capacidade antioxidante de extratos de repolho frente aos radicais superóxido e hidroxil, os quais apresentaram ação inibitória sobre os mesmos.

Tabela 2- Atividade biológica de diferentes espécies de *Brassica.*

Planta	**Atividade biológica**	**Referência**
Brassica sp	Quimioprevenção, supressão no crescimento e indução da apoptose de células cancerígenas	(LAMY et al., 2013)
Brassica oleracea var. *capitata*	Antimicótica	(BAJPAI; KANG; BAEK, 2012)
Brassica oleracea (brócolis, repolho branco e couve de Bruxelas	Antibacteriana e Antioxidante	(JAISWAL; ABU-GHANNAM; GUPTA, 2012)
Brassica oleracea var. *acephala* DC	Antiulcerogênica	(LEMOS et al., 2011)
Brassica oleracea L. (repolho roxo)	Toxicidade aguda e subcrônica negativa	(THOUNAOJAM et al., 2011)
Brassica oleracea var. *capitata* (repolho)	Anticarcinogênica	(FARAG; MOTAAL, 2010)
Brassica oleracea L. var. (repolho roxo)	Anti-inflamatória	(LIN; LI; HWANG, 2008)
Brassica oleracea var. capitata (repolho branco)	Fotoproteção	(ROSA et al, 2008)
Brassica oleracea (repolho roxo)	Antioxidante e anti-hiperglicemiante	(KATAYA; HAMZA, 2007)

Em relação à atividade antibacteriana, Jaiswal, Abu-Ghannam e Gupta (2012) testaram a atividade antibacteriana de extratos de *Brassica oleracea* var. *capitata*, frente à *Salmonella abony*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, e *Enterococcus faecalis*, os quais apresentaram ação inibitória sobre as mesmas. Shrivastava e Bhargava (2011) avaliaram a ação antibacteriana frente à *E.coli* de extratos de *Brassica oleracea* var. *capitata.*

3.6 Conclusões acerca do estado da arte

Conforme visto na revisão bibliográfica, existem vários estudos com diferentes métodos de extração para compostos bioativos de *Brassica.* De modo geral, não é comum trabalhos relatando a otimização do processo de extração por ultrassom seguido de hidrólise. Por exemplo, Carvalho et al. (2008) utilizaram o ultrassom para a obtenção de extratos de *Brassica oleracea* var. *capitata*, porém os autores não avaliaram os efeitos da concentração de solvente, temperatura e tempo de extração, os quais foram mantidos constantes, conforme visto anteriormente. No presente trabalho, todos esses parâmetros foram otimizados, bem como a influência dos solventes metanol e etanol na extração de compostos. Além disso, os extratos foram submetidos a processos de hidrólise ácida e alcalina, visando melhorar a atividade biológica dos mesmos. Com relação à extração supercrítica, sabe-se que este processo é considerado uma "tecnologia verde", ou seja, ecologicamente correta, uma vez que trata-se de um processo livre de resíduos tóxicos, não provoca a degradação térmica dos extratos e não requer grandes gastos de energia. Além de proporcionar uma melhor seletividade e eficiência ao processo e o solvente poder ser facilmente removido no final da extração. Especificamente sobre a *Brassica oleracea* var. *capitata,* até o presente momento não foram encontrados trabalhos na literatura científica envolvendo tal método de extração.

Sabe-se que o método de extração escolhido, está totalmente vinculado com o resultado da atividade biológica, uma vez que os compostos bioativos extraídos estão relacionados ao método selecionado. Para atividade antioxidante de compostos de *Brassica*, existe uma quantidade de trabalhos consideráveis, porém a maioria desses utiliza métodos antioxidantes sintéticos, como DDPH. Neste trabalho foi avaliado, além do DPPH, métodos *in vitro* como superóxido, hidroxila e peroxila, os quais também estão presentes na literatura para esta espécie de planta, porém normalmente avaliados de forma isolada, ou seja, poucos trabalhos estudam as três espécies reativas de oxigênio juntas. Tendo em vista, que o extrato é uma matriz complexa e que os radicais apresentam comportamentos distintos e que são fisiologicamente relevantes, é de extrema importância que se avalie os mesmos de forma conjunta. Para atividade antibacteriana, têm-se poucos trabalhos avaliando tal atividade, em relação do potencial biológico que podem vir a oferecer.

4 ARTIGO 1

A review of influence of environment and process parameters on glucosinolate-myrosinase system from *Brassica*

Artigo aceito no periódico Jounal of Applied Pharmaceutical Science

Dear Dr. Marcelo Barcellos da Rosa

I am pleased to inform you that your manuscript entitled "**A review of influence of environment and process parameters on glucosinolate-myrosinase system from *Brassica***" has been provisionally accepted for publication in Journal of Applied Pharmaceutical Science. However, our reviewers suggested few corrections in manuscript (see attachment). You are required to submit duly corrected manuscript along with processing fees **$ 75 USD within next four** days of receiving this mail through Banking/Paypal. Payment details are as follows;

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A review of influence of environment and process parameters on glucosinolate-myrosinase system from *Brassica*

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ABSTRACT

The family *Brassicaceae* has been very studied due to the pharmacologic properties of the glucosinolates (GLS) and their hydrolysis products, which are associated with the action of an endogenous thioglucosidase myrosinase. Factors such as climate, soil, genotype, seasonal variation, processing, extraction quantification can affect the enzyme activity and stability, leading to increase or decrease the hydrolysis of GLS. Based on this aspect, the main objective of this work is present a review concerning the glucosinolate-myrosinase system, influence of climate and genotype to seasonal variation in the glucosinolate-myrosinase system, effect of thermal and high hydrostatic pressure treatments on the GLS content, as well as, the isolation and quantification of GLS from *Brassica*.

Key-words: *Brassica*, environmental parameters, glucosinolates, myrosinase, process parameters,

INTRODUCTION

The family *Brassicaceae* includes about 400 genus and 4,000 species. In Brazil is found 7 genus and 50 species. Through the genetic improvement have several horticultural varieties that present economic interest, mainly *B. oleracea* var. *acephala* (kale), *B. oleracea* var. *capitata* (cabbage), *B. oleracea* var. *gemmifera* (Brussels sprouts), *B. oleracea* var. *borytis* (cauliflower) and *B. oleracea* var. *italica* (broccoli). Other species economically important are *Brassica nigra* and *Sinapis* spp. (mustards), *Raphanus sativus* (radish), *Armoracia rusticana* (horseradish), *Rorippa nasturtium-aquaticum* (watercress) and *Eruca sativa* (rocket). Some species are cultivated as ornamental, mainly *B. oleracea* var. *acephala* (ornamental cabbage), *Lobularia maritime* (Alyssum) and *Cleome hassleriana* (spider flower). Many *Brassicaceae* are weeds, including *Brassica rapa*, *Rapistrum rugosum and Sinapis arvense* (turnip mustard), *Capsella bursa-pastoris* (shepherd's-purse), *Cardamine bonariensis* (wild watercress), *Coronopus didymus* (Lesser swine-cress), *Lepidium* spp. (peppercress) and *Raphanus raphanistrum* (wild radish) (Souza and Lorenzi, 2005).

The family *Brassicaceae* has been very studied due to the pharmacologic properties of its main metabolites, the glucosinolates (GLS). These metabolites, as well as, their hydrolysis products (isothiocyanate and nitriles) are powerful antioxidants and anti-carcinogenic agents (Paulino, 2008). The GLS are one of the greatest metabolic groups containing sulfur, which are found mainly in comestible vegetables. Until the year of 2004, approximately 120 GLS were identified from species of *Brassicaceae* and other families (Falk et al., 2004).

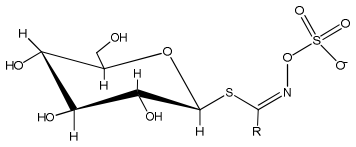
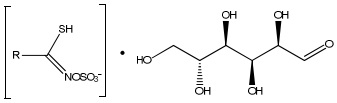
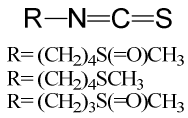
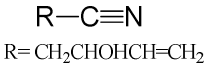
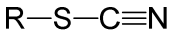
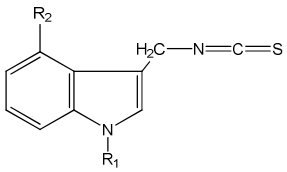
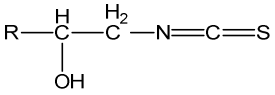
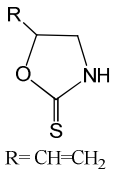
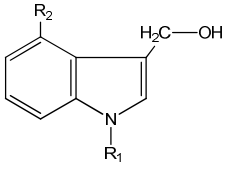
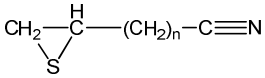
The chemical structure of GLS consists of ester of (Z) hydroxylamine sulphate that had an atom of sulfur bonded to a β-$_D$-glucopyranose that is a side chain derived from an amino acid. The chemical side chain is highly variable and can contain groups aliphatic (alkyl, alkenyl, hydroxy alkenyl, ω-methyl alkyl, ω-sulphinyl and ω-sulphinyl alkyl), aromatic (benzyl, substituted benzyl) or heterocyclic (indolic groups), depending of the amino acid

precursor (Holst and Williamson, 2004; Padilla et al., 2007). The presence of sulphate in the molecule confers properties strongly acid. In this way, the GLS are not volatile and occur as salt (Holst and Williamson, 2004).

The hydrolysis of GLS occurs due to action of an endogenous thioglucosidase myrosinase when the plant tissues are disrupted during processing, chewing, and digestion, since this enzyme is located within the vacuoles of the plant matrix. The hydrolysis generates an unstable aglycone intermediate, thiohydroxamate-O-sulfonate, which is spontaneously converted to different classes of breakdown products including isothiocyanates, thiocyanates, nitriles, epithionitriles, hydroxynitriles, and oxazolidine-2- thiones (Fenwick and Heaney, 1983b). In Table 1 are presented the chemical structures of the main compounds 76 detected in Brassica family.

The extent of hydrolysis of glucosinolates and the nature and composition of the breakdown products formed are known to be influenced by various characteristics of the hydrolysis medium. Intrinsic factors such as coexisting myrosinase and its cofactors ascorbic acid, epithiospecifier protein (ESP), or ferrous ions (Bones and Rossiter, 1996) and extrinsic factors such as pH and temperature (Ludikhuyze et al., 2000) can affect the hydrolysis of glucosinolates, because these factors present influence on myrosinase activity and stability, leading to increase or decrease the efficiency of hydrolysis of glucosinolates. By this reason, processing, extraction and quantification methods are likely to influence the extent of glucosinolate hydrolysis and the ratio of the derivatives produced (Verkerk et al., 1997).

Table 1. Compounds normally observed in *Brassica* family.

Compound	Chemical structure
Glucosinolate	
Thiohydroxamate-sulfonate	
Stableisothiocyanate	
Nitrile	
Thyocianate	
Indolylmethil-Isothiocyanate	
β-OH-isothiocyanate	
5-oxazolidine -2- thione	
Indole-3-carbinol	
Epithioalkylnitrile	

Although the related variables influence the myrosinase activity, the literature is focused mainly on the effects on glucosinolase content. There is a well documented review in the literature (Cartea and Velasco, 2008) that reports the influence of environmental conditions, processing and storage on the glucosinolates content of *Brassica* sp. and their effects on human nutrition and health. Based on these aspects, the present work reports the influence of climate, genotype and seasonal variation in the glucosinolate-myrosinase system, effect of heat treatment and high hydrostatic pressure on the stability of myrosinase as well as in the GLS content, and additionally, this work reports the isolation and quantification of these compounds, focusing in the methods of isolation and quantification of glucosinolates from biological matrices.

INFLUENCE OF CLIMATE AND GENOTYPE TO SEASONAL VARIATION IN THE GLUCOSINOLATE-MYROSINASE SYSTEM

Cultivar, location, and growing conditions play important roles in the production of bioactive compounds in *Brassica* sp. (Rosa et al., 1997). The concentration and composition of GLS, phenolics, and vitamin C in *Brassica* sp. is genotype dependent (Sarikamis et al., 2009). Moreover, climatic factors such as temperature, irradiation, and water supply also have an important influence on the phytochemical content in *Brassica* sp. (Martinez-Villaluenga et al., 2009). According to Schmidt et al. (2010), GLS breakdown product levels are due to the combination of GLS content in the plant and myrosinase activity (Ludikhuyze et al., 2000). The activity of this enzyme depends on the genetic variation (Rask et al., 2000), on some intrinsic (metal ions, ascorbic acid, pH) and on some extrinsic factors (temperature) (Bones and Rossiter, 1996; Ludikhuyze et al., 2000). Therefore, cultivar selection should be tailored to specific environmental factors at each location to achieve optimization in phytochemical

content of *Brassica* sp. In addition, selected white cabbages with an optimized bioactive compound content could be used as raw material for sauerkraut production, enhancing the human dietary intake in health promoting compounds.

Brassicas are economically important crops that show high intra-specific variation in morphological and chemical traits (Hanson et al., 2009). Previous studies have shown that concentrations and profiles of GLS show considerable variation within species and that they vary with environmental conditions and developmental stage (Potter et al., 2000; Rosa and Rodrigues, 2001; Castro et al., 2004; Poelman et al., 2008; Hanson et al., 2009).

Among the cultivated *Brassicaceae*, broccoli attracted attention after the discovery that it contains high levels of the isothiocyanate sulforaphane [1-isothiocyanate-(4R)-(methylsulfinyl) butane], and of other glucosinolate derivatives thought to have anticarcinogenic properties (Beecher, 1994; Zhang et al., 1992; Cover et al., 1998). The ability of sulforaphane or indole-3-carbinol to protect against tumorigenicity is dose and time dependent. Therefore, selection of cultivars accumulating high levels of isothiocyanates may be important. Based on the perceived beneficial effects, broccoli has received widespread attention as a medicinally significant food, its consumption being recommended throughout the year. To meet this requirement, and because it is a very perishable vegetable, producers tend to grow suitable cultivars under mild climatic conditions in spring and summer, principally for the fresh market, although some cultivars are more suited to freezing.

Variation in GLS has been of interest to ecologists and nutritional chemists alike. Ecological studies have investigated the effects of variation in GLS concentrations and profiles of *Brassica oleracea* on above ground plant-insect interactions. Both generalists and specialists herbivores can be influenced by GLS (Gols et al., 2007; Poelman et al., 2008).

Members of the *Brassicaceae* vary considerably in GLS quantity and composition (Rask et al., 2000; Fahey et al., 2002) and insect herbivores specialized on *Brassicaceae* differ

in their performance on different members of this plant family (Fox et al., 1996; Ohsaki and Sato, 1999; Sznajder and Harvey, 2003). Differences in GLS quantity and composition between crucifer species may thus contribute to differences in herbivore performance. *Plutella xylostella* L. (Lepidoptera, Plutellidae) is a specialist herbivore that is known to feed on a number of species in the *Brassicaceae*. Adult females and larval stages use GLS as oviposition and feeding stimulants (Pivnick et al., 1994). However, an increase in GLS concentration does not always positively correlate with larval performance (Li et al., 2000).

Some GLS may have anticarcinogenic effects (Lund, 2003; Moreno et al., 2006) and nutritional studies have mainly focused in the aerial parts of the plants. Root GLS levels, although less studied in the context of human health, are important for resistance against soil pests and may be used for biofumigation (Smetanska et al., 2007). In aerial parts, GLS concentrations depend on a variety of factors including temperature, time of day, water content, and nutrient supply (Rosa et al., 1994; Rosa, 1997; Pereira et al., 2002; Gols et al., 2007). Cole (1980) observed, based on measurements of volatile myrosinase hydrolysis products, a substantial decline in levels of aliphatic glucosinolates over the first few days of development in seedlings of turnips, Chinese cabbage, fodder rape (*B. campestris* L), cauliflower (*B. oleracea* var *botrytis*) and radish (*Raphanus sativus* L). A thorough understanding of glucosinolate metabolism in plants requires intensive studies of their distribution between plant organs and changes during the various development stages. Major differences in the relative amount of individual GLS have been observed between the different parts of developing rapeseed plants McGregor (1988), indicating that them have defined distribution patterns. Furthermore, large differences between seed, leaf and root glucosinolate profiles of several brassicas have been described by Sang et al. (1984). Rosa et al. (1994) reported that under mild conditions, GLS in the leaves of young cabbage plants

showed significant variation throughout a single day. Although the data suggested a rapid metabolism of GLS, the causes of this variation are not fully understood.

Even under controlled greenhouse conditions, GLS concentrations in leaves of Brassica species fluctuate when sown over a time period of several months, which was ascribed to abiotic seasonal changes (Gols et al., 2007). Soil characteristics like pH influenced GLS concentrations in leaves of kale (Petersen et al., 2002; Velasco et al., 2007; Gerendas et al., 2008; Pongrac et al., 2008). Variation on the amount and pattern of GLS has been attributed to genetic and environmental factors, including plant age, temperature, water stress, and soil type (Fenwick et al., 1983; Rosa et al., 1997; Farnham et al., 2004).

Distribution of the GLS varies depending on plant part, with both quantitative and qualitative differences among roots, leaves, stems, and seeds. Van Dam et al. (2009), in his review article reports that the roots usually have a higher concentration of glucosinolates compared with other parts of the plant. Agronomic factors, such as soil type, moisture, and mineral nutrient availability, are known to exert a significant effect on GLS content. Soil fertility has significant effects on levels of specific GLS in the growing plants (Rosa et al., 1997). Total and indolic GLS concentrations have been correlated with climatic factors in several crops of *B. oleracea* (Charron et al., 2005). Winter seasons seem to induce lower GLS levels due to short days and cool temperatures accompanied by frost (Rosa et al., 1997).

In addition, the profile and concentration of GLS are affected by developmental stage of the plant (Petersen et al., 2002) and by biotic interactions. Above ground plant-animal interactions can cause up regulation of specific GLS, depending on the tissues attacked and the identity of the herbivore (Textor and Gershenzon, 2009). In *B. oleracea* herbivory by the generalist *Myzus persicae* up regulates indolic GLS(Kim and Jander, 2007), whereas damage resulting from the specialist *Pieris rapae* also increases aliphatic GLS (Agrawal and Kurashige, 2003). There is especially little information on root GLS.

EFFECT OF THERMAL TREATMENT ON THE GLUCOSINOLATE-MYROSINASE SYSTEM OF *Brassica*

Glucosinolate hydrolysis products, and in particular isothiocyanates and indoles, have received a special interest in food research because of their anticarcinogenic properties (Verhoeven et al., 1997; Wallig et al., 1998). Moreover, glucosinolates and their hydrolysis products are associated with important taste, aroma and flavour attributes in *Brassica* vegetables (Van Doorn et al., 1998; Drewnowski and Gomez-Carneros, 2000; Coogan et al., 2001).

Like other vegetables, most *Brassica* vegetables are heat processed before consumption. This leads to myrosinase inactivation and hence stops the hydrolysis of GLS into beneficial breakdown products. GLS can be hydrolyzed by myrosinases existing in the human gut, but the production level of isothiocyanates is three times greater when GLS are hydrolyzed by plant myrosinase (Conaway et al., 2001). Controlling myrosinase activity during processing is, therefore, of particular interest.

Earlier investigations showed different thermal stability of myrosinase based on *Brassica* source (Table 2). This table shows that broccoli myrosinase has the lowest thermal stability compared to other myrosinase sources, whereas it is the highest in the case of rapeseed myrosinase. However, Matusheski et al. (2004) found that heating fresh broccoli florets to 60 ºC prior to homogenization simultaneously increased sulphoraphane formation, which indicates a much higher thermal stability of broccoli myrosinase than what has been reported by Ludikhuyze et al. (1999). However, some of the differences could be due to the limitation of heat transfer to whole vegetable.

Table 2. Thermal stability of myrosinase from different *Brassica* sources.

Source of myrosinase	Stability (ºC)
Broccoli myrosinase (crude extract)	Up to 30ºC
Broccoli myrosinase (juice)	Up to 40ºC
Red cabbage myrosinase (crude extract)	Up to 60ºC
Red cabbage myrosinase (crude extract)	Up to 40ºC
White cabbage myrosinase (crude extract)	Up to 50ºC
Mustard seed (crude extract)	Up to 60ºC
Rapeseed myrosinase (crude extract)	Up to 65ºC
Rapeseed myrosinase (intact and flaked seeds)	Up to 90ºC

Source: Ghawi et al. (2012)

Earlier work has indicated that the glucosinolate-myrosinase system is modified during the processing of *Brassica* vegetables due to partial or total inactivation of myrosinase, thermal breakdown of GLS and their hydrolysis products, loss of enzymatic cofactors, leaching of GLS and their derivatives into the cooking medium, or volatilization of the derivatives (Dekker et al., 2000). The extent of these losses probably depends on the duration and type of heat treatment, the degree of material disintegration, and the vegetable matrix itself (Rosa and Heaney, 1993).

High hydrostatic pressure (HHP) is a non-thermal technology used in the food industry to inactivate microorganisms and spoilage enzymes without affecting the quality of fresh products (San Martin et al., 2002). Moreover, the application of pressure processing has been found to retard thermal inactivation (Hendrickx et al., 1998). HHP stability has been investigated for broccoli and mustard seed myrosinases (Ludikhuyze et al., 1999; Van Eylen

et al., 2008). However, a very wide difference in pressure stability has been reported. Green cabbage is widely consumed as either cooked or processed products; however, no published data on the thermal and pressure inactivation of myrosinase from green cabbage are yet available in the literature. Vegetables are primarily consumed in the cooked form and are processed by various techniques. Blanching is a short heat treatment that is typically applied to vegetables prior to further processing with the aim of enhancing both safety and quality attributes. Blanching imparts benefits, such as destruction of surface microflora of vegetables and enhancing the colour and texture and also the keeping quality of vegetable products. The quality of blanched product depends significantly on the time and temperature of blanching and also on the size of vegetable to be blanched. Under-blanching speeds up the activity of enzymes and is worse than no blanching. Over-blanching causes loss of texture, colour, phytochemicals and minerals. Industrial blanching processes involve temperatures ranging from 70 to 95 °C and times usually no higher than 10 min (Morales-Blancas et al., 2002) whereas for domestic purposes vegetables are generally blanched for 10–12 min in boiling water (98–100 °C).

A considerable amount of research has been done to understand the effects of blanching on texture, colour, phytochemical content and antioxidante activity of different vegetables. Volden et al. (2008) showed the effects of blanching of red cabbage on the levels of glucosinolates, polyphenols and anthocyanins, as well as for the antioxidant potential by the ferric reducing ability power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Data on the effect of blanching on physicochemical properties of cabbage is scarce (Amin and Lee, 2005; Volden et al., 2008).

Isolation and quantification of glucosinolates from Brassica

Due to their physicochemical properties, the separation and the isolation of GLS is an extremely difficult task. The presence of the sulfate group and of the thioglucose moiety cause the octanol–water partition coefficient of GLS (log Po/w) to fall in the low value domain (−4.30 for glucoiberin to −1.38 for neoglucobrassicin), thus suggesting they are very hydrophilic and always water-soluble entities (Holst and Williamson, 2004). The characterization and quantification of GLS, either in pure state or within mixtures, is possible through the desulpho-glucosinolates technique (Wathelet et al., 1995; Kiddle et al., 2001).

Moreover, many ion pair chromatography based methods have been developed after the initial work published by Helboe et al. (1980) to purify GLS (Prestera et al., 1996; Toribio et al., 2007). They exploit the well-known property of alkyl-ammoniums (tetramethylammonium, tetraoctylammonium, tetradecylammonium, etc.) to form ion pairs with sulfate groups (Prestera et al., 1996). This is the strategy used by plants to transport GLS. Indeed, a GLS anion is sometimes associated with an aromatic choline ester cation, such as sinapine, in sinalbin from mustard or glucoraphanin from broccoli (Butzenlechner, 1996).

Recently, Fahey et al. (2002) successfully resolved glucoraphanin and glucoiberin from crude plant homogenates using high speed counter-current chromatography (HSCCC) in the elution mode and a highly-salted and highly polar biphasic solvent system: 1-propanol/acetonitrile/saturated aqueous ammonium sulfate/water (1:0.5:1.2:1).This protocol was optimized and scaled up by Fisher et al. (2005) where 15 g of enriched glucoraphanin extract were injected in a MIDI-dynamic extraction centrifuge apparatus equipped with a 928mL column. There are different methods of extraction and characterization of glucosinolates in Brassica family. As can be seen in Table 3, extraction with methanol is widely reported, but other methods such as decoction, headspace solid-phase microextraction are also employed.

Among the methods for detection and quantification, there is a wide variation, although liquid and gas chromatography coupled or not the mass detector predominated, since they allowed confirmation of the compounds analyzed. Regarding the isolated glucosinolates, each species has its particularity in relation to them, which can also vary within the same species due to climate change, seasonal variation, aspects that were discussed earlier. Compounds that exhibit prominent, ie appear in a large number of species are sinigrin and sinalbina addition of sulfur compounds, allyl, benzyl isothiocyanates and dimethyl sulfide. Herzallah and Holley (2012), developed a method to reversed phase-high performance liquid chromatography method was to quantify sinigrin, sinalbin, allyl isothiocyanate and benzyl isothiocyanate present in aqueous and freeze-dried yellow and Oriental (brown) mustard extract samples using two pre-treatment methods (autoclaving, boiling) to prevent degradation by myrosinase.

Table 3. List of glucosinolates identified in *Brassica* family, extraction methods and characterization

Plant species	Extraction method	Characterization method	Compounds identified	References
Brassica napus *Brassica juncea*	Headspace-solid phase microextraction	GC-MS	dimethyl sulfide, 3-methyl-3 butenenitrile 1-isothiocyanato-butane 4-isothiocyanato-1-butene	Wey et al. (2012)
Sinapis alba	Decoction	RP-HPLC	sinigrin, sinalbin, allyl- and benzyl isothiocyanates	Herzallah and Holley (2012)
Brassica oleracea L. var. botrytis L. *Brassica* rapa *Ruvo*	extraction with methanol	HPLC-PDA, ESI-MS NMR	sinigrin, glucoiberin, glucoiberverin, gluconapin, glucobrassicanapin and gluconasturtiin	Toribio et al. (2011)
Brassica oleracea L. *var. capitata* f. *rubra* DC	extraction with metanol	LC/MS	Sulforaphane	Koo et al. (2011)
Brassica oleracea L. var. *costata* DC	headspace-solid phase microextraction	GC/ITMS	allyl isothiocyanate dimethyl disulfide dimethyl trisulfide	Pinho et al. (2009)
Brassica oleracea var. *capitata* f. *alba*	extraction with metanol	HPLC-UV	glucobrassicin, sinigrin	Kusznierewiczaet al. (2008)
Brassica oleracea var. itálica	aqueous extract	CPC	sinalbin and glucoraphanin	Toribio et al. (2007)

GC-MS: Gas chromatography coupled with mass detector; RP-HPLC: Reverse phase liquid chromatography; HPLC-PDA: liquid chromatography- photo diode array detector; ESI-MS: electrospray ionization-mass spectrometry; NMR: Nuclear magnetic resonance; LC/MS: liquid chromatography–mass spectrometry; GC/ITMS: chromatography- ion trap mass spectrometry; HPLC-UV: liquid chromatography- ultraviolet detector; CPC: Centrifugal partition chromatography

Toribio et al. (2007) purified the glucosinolates sinalbin and glucoraphanin by strong ion-exchange displacement centrifugal partition chromatography (SIXCPC). The optimized conditions involved the biphasic solvent system ethyl acetate/n-butanol/water, the lipophilic anion-exchanger. Amounts as high as 2.4 g of sinalbin and 2.6 g of lucoraphanin were obtained in starting from 12 and 25 g of mustard and broccoli seed aqueous extracts.

Concluding Remarks

From this review it was seen that the vegetables of the *Brassica* family are rich in glucosinolates, which have a valuable anticarcinogenic action. Glucosinate-myrosinase system may be influenced by factors such as climate, soil, genotype, with seasonal variation. Therefore, cultivar selection should be tailored to specific environmental factors at each location to achieve optimization in phytochemical content of *Brassica* sp. In addition, selected white cabbages with an optimized bioactive compound content could be used as raw material for sauerkraut production, enhancing the human dietary intake in health promoting compounds.

The study of the effects of processing on the concentrations of GLS and the parameters related to their hydrolysis in *Brassica* vegetables has a pivotal role in complementing research on the epidemiology of the consumption of *Brassica* vegetables and chemoprevention. An understanding of the physical and biochemical changes occurring before the ingestion of processed *Brassica* vegetables may help to interpret the metabolic fate of GLS in experimental studies in animals and humans and inform the subsequent formulation of dietary strategies to optimize the uptake of isothiocyanates in vivo. Hydrolysis of vegetables at high temperatures leads to destruction of myrosinase, an enzyme responsible for converting glucosinate in active substances, as well as treatment at high pressures, which is used in the food industry leads to destruction of this enzyme. Therefore, when one wishes to

obtain constant characteristics in a plant of the *Brassica* family all these aspects should be taking in account.

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5 ARTIGO 2

Supercritical CO_2 extraction, chemical characterization and antioxidant potential of *Brassica oleracea* var. *capitata* against $HO^{\bullet}$, $O_2^{\bullet-}$ and $ROO^{\bullet}$

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Supercritical Co_2 extraction, chemical characterization and antioxidant potential of *Brassica oleracea* var. *capitata* against $HO^{\bullet}$, $O_2^{\bullet-}$ and $ROO^{\bullet}$

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Abstract

In this work were extracted bioactive compounds from *Brassica oleracea* var. *capitata* using supercritical CO_2 and evaluated the antioxidant potential of the extracts. Five extractions were accomplished to investigate the influence of pressure (10 – 25 MPa) and temperature (20 - 60°C) in the extraction yield, chemical composition and antioxidant potential towards peroxyl, superoxide and hydroxyl radicals. The highest extraction yield was obtained at 60°C and 25 MPa, which was 0.47wt% (run 2). In the characterization of the extracts obtained was possible the identification of sulforaphane and iberin nitrile that present known biological properties. The extracts of all runs presented antioxidant activity towards the three radicals, but the highest activity for all radicals was using the extracts obtained in the run 2. The use of supercritical CO_2 extraction to obtain bioactive compounds of *Brassica oleracea* var. *capitata* showed to be a promising alternative to a conventional extraction methods, since allowed the extraction of compounds with scientific and industrial interest.

Keywords: *Brassica oleracea* var. *capitata*, supercritical CO_2 extraction, chemical characterization, antioxidant activity.

1. Introduction

In the recent years, there are increased the interest in the use of plant extracts to combat the oxidative stress, which occurs as a consequence of an imbalance between reactants, such as reactive oxygen and nitrogen species, and antioxidants. The increase in reactive species causes damage to lipoproteins, lipids, DNA and proteins and oxidative stress-induced modifications of these molecules have been implicated in many disease pathways (Valentão, Fernandes, Carvalho, Andrade, Seabra, & Bastos, 2002; Strobel, Fassett, Marsh, & Coombes, 2011). Amongst the reactive oxygen species of great interest it can be cited the superoxide, hydroxyl and peroxyl radicals (Carvalho et al., 2013). Superoxide and hydroxyl radicals are associated with the formation of hydroperoxides by autoxidation of unsaturated fatty acids, whereas peroxyl radical is related as an intermediary compound in the lipid peroxidation (Zhao et al., 2006).

The antioxidant activity of extracts obtained from plant species is related to extraction method, since each method affect the selectivity of compounds. The use of alternative extraction procedure has been increased in relation to traditional ones (hydrodystillation, maceration, soxhlet). Among the alternative procedures, the use of supercritical fluid extraction is the preferable and a considerable amount of studies has been carried out using this procedure over the past 10-15 years (Pereira & Meireles, 2010). Supercritical fluid extraction (SFE) is a green method and attends the modern concerns about environmental aspects related to development of processes. The mains advantages of this procedure is the fact that no toxic residues is verified in the extract after the extraction since the solvent is gaseous at room conditions, mild operational temperatures implying in low degradation of thermolabile compounds, the selectivity, yield and efficiency of extraction can be easily modified by manipulating the temperature and pressure of the system. These characteristics make the SFE a promising technique to be used in the extraction and separation of natural

products, mainly for food and pharmaceutical purposes (Kellner, Mermet, Otto, Valcárcel, & Widmer 2004; Pereira, Marques, Barreto, Siani, Fernandes, & Meireles, 2004).

Brassica sp. is one of the plants with pharmacological interest, since several studies revealed that it presents action as antiinflammatory, antimycotic, photoprotective, antihyperglycemic, anticarcinogenic and antioxidant (Kataya & Hamza, 2007; Lin, Li & Wang, 2008; Bapjai, Kang & Baek, 2012). Among the species of *Brassica sp*., special attention has been paid for *Brassica oleracea* var. *capitata* (white cabbage) that is one of the vegetables most important worldwide (Singh, Upadhyay, Bahadur, Singh, Singh, & Rai, 2006). Before its use as food it was used in the popular medicine for the treatment of headache, podagra, diarrhea and peptic ulcers. White cabbage has been very studied due to its antioxidant activity and detoxification mechanisms that eliminate and stabilize the production of carcinogenic substances (Brooks, Paton & Vidanes, 2001; Singh, Upadhyay, Bahadur, Singh, Singh, & Rai, 2006).

The antioxidant activities of extracts from *Brassica oleracea* obtained by conventional extraction methods are well reported in literature. Kusznierewicz, Bartoszek, Wolska, Drzewiecki, Gorinstein, and Namiesnik (2008) evaluated the antioxidant of methanolic extract of *Brassica oleracea* var *capitata f. alba*, whereas Kataya and Hamza (2008) tested the action antioxidant and antihyperglycemic of ethanolic extracts *Brassica oleracea* var. *capitata* for the treatment of diabetes. Jacob, Mahal, Mukherjee, and Kapoor (2011) evaluated the antioxidant activity of methanolic extracts of *Brassica oleracea* var. *capitata.* Extracts showed antioxidant activity against DPPH, hydrogen peroxide (H_2O_2)-scavenging assay.

However, at the best of our knowledge, no works were published reporting the use of SFE for extraction of bioactive compounds of *Brassica* species, including *Brassica oleracea* var. *capitata*. As a consequence, studies referring to the biological activities of these extracts also are not available. In this sense, the main objective of this work was to obtain bioactive

compounds of *Brassica oleracea* var. *capitata* using supercritical CO_2 extraction and to evaluate their antioxidant potential. For these purposes, experiments were carried out at different temperatures and pressures, being evaluated the extraction yield, chemical characterization and antioxidant activities of the extracts obtained.

2. Material and methods

2.1. Materials

The carbon dioxide (99.9% purity) was purchased from White and Martins®. Sodium hydroxide, HEPES, ABAP 2,2'-azobis (2-aminodinopropane hydrochloride), DFCH2-DA (2',7'-dichlorofluorescin), xanthine oxidase (XOD) 25UN, hypoxanthine (HPX), nitro tetrazolium blue chloride (NBT), 2-Deoxy-D-ribose were obtained from Sigma-Aldrich. DMSO (Dimethyl sulfoxide), potassium chloride and magnesium chloride were obtained from Isofar® (Rio de Janeiro, Brazil). Sodium carbonate was obtained from Merck. Ethylenediamine tetraacetic acid (EDTA) was obtained from Nuclear® (Brazil).

2.2. Samples

Brassica oleracea var. *capitata* was dried in an oven with air circulation at 60°C during 72 hours, according to Tanongkankit, Chiewchan, & Devahastin, (2011). Afterwards, the material was ground in a slicer and stored at room temperature under nitrogen atmosphere prior to the extraction.

2.3. Supercritical CO_2 extraction

The experiments were performed in a laboratory scale unit consisting of a CO_2 reservoir, two thermostatic baths, a syringe pump (ISCO 260D), a 0.1 dm^3 jacketed extraction vessel, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer

(Smar, HT 201) with a precision of 0.12 bar, a collector vessel with a glass tube and a cold trap. Amounts approximately 25 g of dried *Brassica oleracea* var. *capitata* leaves were charged into the extraction vessel. The CO_2 was pumped into the bed, which was supported by two 300 mesh wire disks at both ends, and was kept in contact with the herbaceous matrix for at least 1 h to allow the system

stabilization. Afterwards, the extract was collected opening the micrometering valve and the CO_2 mass flow was accounted by the pump recordings. The experiments were accomplished isothermally, at constant pressure using a mass CO_2 flow rate of 2 g/min, as suggested by Mossi et al. (2004) for three hours. The experimental range investigated was 20-60 ºC in temperature and from 100–250 bar in pressure. Triplicate extraction runs were accomplished for all conditions.

2.4. Gas chromatography–mass spectrometry analysis

The extracts were analyzed with a gas-chromatograph (HP 6890) interfaced with a mass selective detector—GC/MS (HP 5973) with automatic injection system (HP 6890), using a capillary column HP-5MS (30m x 0.32mm x 0.25μm); Helium was the carrier gas with a flow rate of 2 ml/min at a pressure of 5.05 psi; electronic impact mode of 70 eV; samples of 1μL were injected at 250°C interface temperature, with the following column temperature gradient programming: 70°C (1 min); 12°C/min up to 280°C.

2.5. Antioxidant activities of extracts

2.5.1. Superoxide radical scavenging activity – Hypoxanthine/Xanthine Oxidase System (HPX/XOD)

The antiradical activity of extracts of *Brassica oleracea* var. *capitata* obtained by extraction using supercritical CO_2 toward $O_2^{\bullet-}$ radical was evaluated by the enzymatic system HPX/XOD (Zhao et al., 2006). For this purpose, 100 μL of EDTA (30 mmol.L^{-1}), 100 μL of HPX (3 mmol.L^{-1}) and 200 μL of NBT (1.42 mmol.L^{-1}) were mixed with 100 μL of extract. After 3 minutes, was added 100 μL of enzyme XOD (0.75 U.mL^{-1}, diluted in phosphate buffer. The final volume of solution was 3 mL filled with phosphate buffer (0.05 mol.L^{-1}, pH 7.4). The blank sample was prepared in the same manner, but without the presence of NBT. Also, was carried out a control test containing all reagents with the solvent employed in the samples, as well as a blank control. After 40 minutes of reaction, was carried out the absorbance of samples in an UV-Vis 8453 Hewlett-Packard spectrophotometer (Agilent Technologies, Santa Clara, EUA) at 560 nm. The antiradical activity towards $O_2^{\bullet-}$ (AA_{O2}•-) was calculated according to the following equation:

$$AA_{O_2^{\bullet-}} = \left(1 - \frac{(A - A_B)}{(C - C_B)}\right) \times 100 \quad (1)$$

where A, A_B, C, C_B are the absorbance of sample, blank sample, control and blank control, respectively.

2.5.2. Peroxyl radical scavenging activity

The antiradical activity of the extracts of *Brassica oleracea* var. *capitata* obtained by extraction using supercritical CO_2 toward $ROO^{\bullet}$ radical was evaluated by the fluorimetric method using DCFH2-DA as substrate (Amado et al., 2009). A plate containing 96 pools was subdivided in two regions: in the first one, corresponding to the lines A, B, C, and D, and the second one, corresponding to lines E, F, G and H. The first three pools of each region were

used to add 10 µl of solvent used in the samples. In the remaining pools of the plate were added 10 µl of extract. Afterwards, was added 127.5 µl of buffer in all pools of plate. In the following, 7.5 µl of ultrapure water was added in all pools of region 1, whereas 7.5 µl of ABAP (4 mmol.L^{-1}) in the pools of region 2. Before the analysis was added 10 µl of DCFH2-DA (16 µmol.L^{-1}). The fluorimeter Vitor 2 (Perkin Elmer, Massachusetts, EUA) was programed to maintain the temperature at 37°C and measure the fluorescence at 485 nm (excitation) and 520 nm (emission) in regular time intervals of 5 minutes for 30 minutes. The antiradical activity towards ROO$^{\bullet}$ ($AA_{ROO\bullet}$) was calculated according to the following equation considering the measure at 30 minutes:

$$AA_{ROO\bullet} = \left(1 - \frac{(F_A - F_{AB})}{(F_S - F_{SB})}\right) \times 100 \qquad (2)$$

where F_A and F_{AB} are the fluorescence of sample containing ABAP and fluorescence of blank without ABAP, respectively. The F_S and F_{SB} are fluorescence of solvent containing BAP and fluorescence of solvent blank without ABAP, respectively.

2.5.3. Hydroxyl radical scavenging activity

The scavenging activity of *Brassica oleracea* var. *capitata* extracts toward hydroxyl radical was determined by using the deoxyribose method with some modifications (Zhao et al., 2006). $FeCl_3 \cdot 6H2O$ and ascorbic acid were prepared in degassed deionized water prior to use. The reaction tube contained 100 µL of *Brassica oleracea* var. *capitata* extract, 100 µL of 1 mM EDTA, 100 µL of 1 mM $FeCl_3 \cdot 6H2O$, 100 µL of 36 mM 2-deoxy-d-ribose, 100 µL of 10 mM H_2O_2, and 100 µL of 1 mM l-ascorbic acid in 25 mM phosphate buffer (pH 7.4), and the total volume was made up to 1.0 mL with the same phosphate buffer. After incubation at 37 °C for 1 h, the reaction was stopped by adding 1.0 mL of 10% TCA (w/v) and 1.0 mL of 1.0% TBA (w/v) in buffer phosphate (pH 7.4). The mixture was heated in a boiling water bath

for 15 min. Once samples were cooled, the final volume was adjusted to 5.0 mL with deionized water, and the absorbance was read at 532 nm. The capability to scavenge the •OH ($AA._{OH}$) was calculated using the equation.

$$AA._{OH} = \left(1 - \frac{(S - S_B)}{(C - C_B)}\right) \times 100 \quad (3)$$

where S, $S_{B,}$ C, C_B are the absorbance of sample, blank sample, control and blank control, respectively.

2.6. Statistical analysis

The statistical analysis was accomplished with the ANOVA test coupled with the Tukey test at a 95% confidence level using the software Statistica® 8.0.

3. Results and discussion

3.1. Obtainment of extracts from Bassica oleracea var. capitata using supercritical CO_2

Table 1 presents the extraction yield obtained in the supercritical CO_2 extraction of *Bassica oleracea* var. *capitata* at different conditions of temperature and pressure. The yield ranged from 0.015 wt% to 0.47 wt% in the runs 4 and 2, respectively. There are no statistical difference ($p<0.05$) in the yield obtained at runs 2 and 5 as well as for runs 1 and 3, whereas the yield obtained in run 4 is statistically different of all runs. Although the extraction yields obtained in this study are low, the values are in good agreement with other studies reported in literature. Mossi et al. (2003) obtained maximum extraction yield of 1.11 wt% using supercritical CO_2 extraction to obtain extracts from *Maytenus ilicifolia*, which justified that the low yield can be compensated by the wide range of compounds extracted. Mazutti, Mossi, Cansian, Corazza, Dariva, and Oliveira (2008) used supercritical CO_2 to obtain extract from

Peumus boldus Molina and obtained a maximum yield of 0.38 wt% corroborating with the results presented above.

Table 1. Extraction yields of *Brassica oleracea* var *capitata* extracts obtained using supercritical CO_2 at different operational conditions

Run	Temperature (ºC)	Pressure (MPa)	CO_2 Density* ($kg.m^{-3}$)	Yield (wt%)
1	20	25.0	970	0.101 ± 0.002^{b}
2	60	25.0	794	0.470 ± 0.012^{a}
3	20	10.0	865	0.159 ± 0.006^{b}
4	60	10.0	286	0.015 ± 0.001^{c}
5	40	17.5	823	0.447 ± 0.022^{a}

[a,b,c] different letters represent a significant difference.*Estimated from Angus, Armstrong, & Reuck (1976).

The increase in temperature at the lowest extraction pressure (10 MPa) led to a sharp decrease in the extraction yield (10.6 times), which is due to decrease of solvent density (865 to 286 $kg.m^{-3}$ for temperatures of 20 and 60°C, respectively), decreasing the solvent power. This led to the lowest yield among the runs. By other hand, opposed behavior was verified for runs carried out at 25 MPa, where the increase of temperature presented significant increase (4.7 times) in the yield even with a lower variation in the solvent density (970 to 794 $kg.m^{-3}$ for temperatures of 20 and 60°C, respectively).

These opposite results are is due to the competition between temperature (vapor pressure of compounds of extract) and extraction pressure (solvent power) in the range investigated. It is known that the solubility of compounds decreases with increasing temperature at low pressure (10 MPa) that, combined with low solvent density, led to low

yields. For pressures higher than 17.5 MPa, the change in the density with temperature is less expressive than for pressures of 10 MPa, in a manner that the increase in the vapor-pressure caused by the increase in the temperature is more important than density of the solvent. This statement is easily proven by comparing the yields of runs 2 and 5 (similar densities, but temperatures of 60 and 40°C, respectively), which presented the highest yield (no statistical difference) with runs 1 and 3 (high densities, but low temperatures), where the yields were 0.101 and 0.159 wt%, respectively.

3.2. Chemical characterization of supercritical CO_2 extracts of Brassica oleracea var capitata

The extracts obtained in the five extractions runs extracts were analyzed with a gas chromatograph interfaced with a mass selective detector. The composition of the extracts obtained in this work was expressed as percentage of normalized peak areas, which are presented in Table 2. It was possible indentify 13 compounds among them derivates of fatty acids, sulfur compounds, phytosterols and triterpenes. Run 2 was the extraction with the highest number of compounds identified (13 compounds), followed by runs 3 (11 compounds), runs 4 and 5 (

8 compounds) and run 1 (7 compounds). The main compounds extracted were the nonacosane and 15-nonacosanone (around 20% of total area). Other compounds as n-hexadecanoic acid, sitosterol, campesterol, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester, heptadecanoic acid methyl ester presented intermediary concentration (3-10% of total area).

Among the compounds extracted that present great pharmacological interest there are the sulforaphane (Zhao, Moore, Redell, and Dash, 2007), iberin nitrile (Fahey, Zalcmann, & Talalay, 2001), sitosterol (Singh, 2013), campesterol (Yoshida & Niki, 2003) and α-amyrin (Vázquez, Palazon, & Navarro-Ocaña, 2012). The sulforaphane is the compound with the

highest scientific relevance in Brassica species, since several studies reported its action as antioxidant (Zhao, Moore, Redell, and Dash, 2007), anticarcinogenic (Zhang, Li, & Tang, 2005; Kensler, Chen, & Egner, 2005; Farag & Motaal, 2010). Another compound with highlighted relevance is iberin nitrile due to the anticarcinogenic activity (Fahey, Zalcmann, & Talalay, 2001). Both compounds were obtained only in the runs 1 and 2, where the extraction pressure was the highest (25 MPa). This finding can be related to the solubility of these compounds on supercritical CO_2 that, possibility, increase with pressure. Sitosterol and campesterol are components of phytosterol contained in vegetable extracts that are known to exert antioxidant effects against lipid peroxidation and also act as a stabilizer in the membranes (Yoshida & Niki, 2003). The sistosterol was obtained in all extraction runs, mainly for runs 3 and 5 (around 9% of total area), which presented similar solvent density (around 850 $kg.m^{-3}$), whereas campesterol was obtained only in runs 1-3 that were the conditions with the highest solvent densities. The α-amyrin is a pentacyclic triterpene that have a number of biological effects (Vázquez, Palazon, & Navarro-Ocaña, 2012). This compound was obtained in run 2, 3 and 5, which present in common a similar solvent density.

Table 2. Chemical profile of compounds present in *Brassica oleracea* var *capitata* extracts obtained using supercritical CO_2

Run	**Compounds - retention time (min.)/ normalized peak areas (%)***												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	8.84/ 0.52	-	-	14.01/4.04	-	15.55/1.8	-	16.06/0.42	21.3/22.8	23.09/0.74	-	25.2/3.43	-
2	8.84/0.41	10.37/0.27	10.49/0.42	14.01/ 0.47	14.5/5.39	15.01/1.8	15.6/6.7	15.9/9.52	21.3/13.37	25.3/3.8	23.3/21.8	25.2/3.8	28.5/0.75
3	-	-	10.49/0.92	14.01/0.53	14.5/5.01	15.01/2.17	15.6/1.33	15.9/2.16	21.3/19.12	26.0/9.0	23.3/20.1	25.2/3.41	28.5/1.15
4	-	-	14.2/4.76	-	14.5/2.0	15.01/3.05	15.55/14.7	15.6/2.45	21.3/25.76	26.7/1.18	23.3/24.7	-	-
5			14.5/3.7	14.02/0.22	14.5/3.7	15.01/0.88	15.55/0.4		21.3/19.8	26.7/9.26			27.5/0.43

1: Iberin nitrile; 2: sulforaphane; 3: hexadecanoic acid methyl ester; 4: 9- hexadecanoic acid methyl ester; 5: n-hexadecanoic acid; 6: heptadecanoic acid methyl ester, 7: 9-Octadecadienoic acid methyl ester; 8: 9, 12-Octadecadienoic acid methyl ester; 9: Nonacosane; 10: sitosterol; 11: 15-nonacosanone; 12: campesterol; 13: α-amyrin;

*Experimental errors in the chemical analysis were lower than 5%.

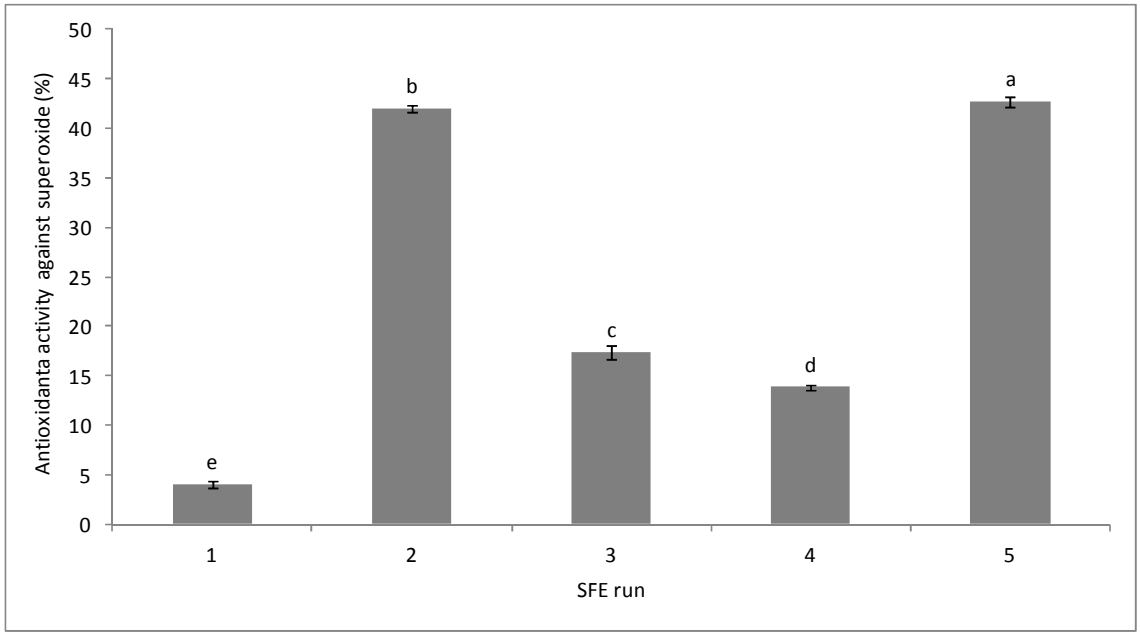
The chemical profile obtained in this study is in good agreement with data published by Peñas, Pihlava, Vidal-Valverde, and Frias, (2012), which analyzed the chemical composition of extracts from *Brassica oleracea* var. *capitata* obtained by conventional extraction and also reported the presence of sulforaphane and iberin nitrile. The method of supercritical fluid extraction use a process free from toxic residues, does not cause thermal degradation of the extracts and did not require large expenditures of energy giving better selectivity and efficiency in the process, once extracted compounds also obtained by conventional methods, which typically use chemical solvents, which besides being toxic and generate waste can degrade the constituents present in plants.

3.3 Antioxidant activity of supercritical CO_2 extracts of Brassica oleracea var capitata

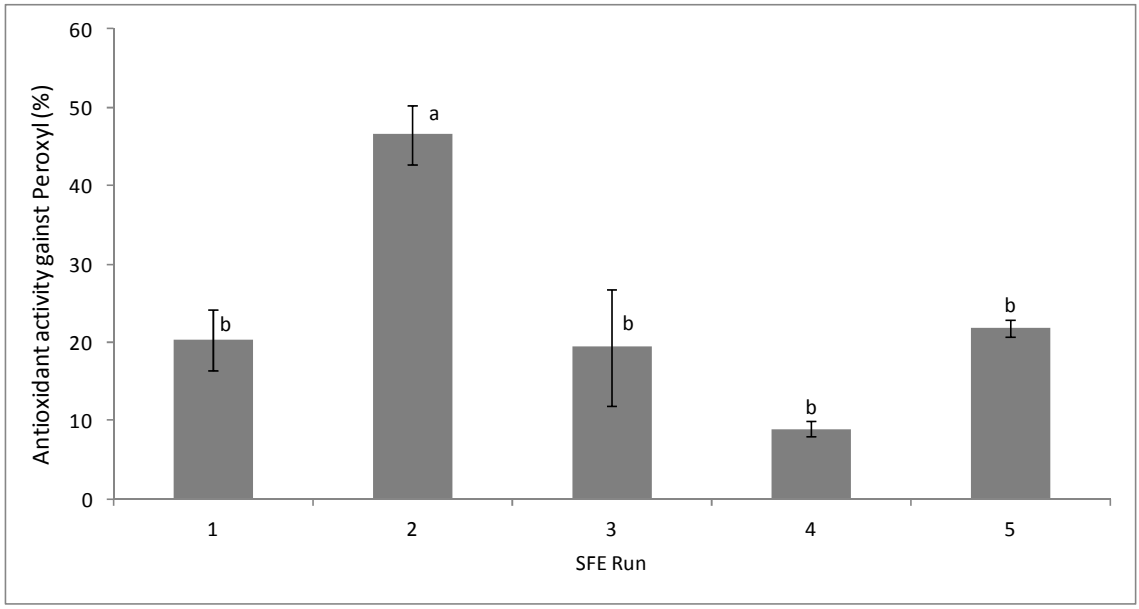
The results concerning the antioxidant activity of supercritical CO_2 extracts of *Brassica oleracea* var. *capitata* are presented in Figure 1, where Figure 1a presents the antioxidant activity towards superoxide radical, Figure 1b towards peroxyl radical and figure 1c towards hydroxyl radical. From Figure 1a is seen that the extracts obtained in the runs 2 and 5 presented the highest antioxidant activities against superoxide radical. Runs 1, 3 and 4 presented lower antioxidant activities in comparison runs 2 and 5. From the statistical analysis was verified that all samples are statistically different ($p<0.05$). In Figure 1b, the extracts obtained in the run 2 presented the highest antioxidant activity towards the peroxyl radical, which is statistically different from the others ($p<0.05$). In Figure 1c, the extracts obtained in the run 2 also presented the highest antioxidant activity towards the hydroxyl radical, which is statistically different from the others ($p<0.05$).

An important aspect to be mentioned from the analysis of results presented in Figure 1 is the fact that depending of the extraction condition used (temperature and pressure) was verified different antioxidant activities towards the three radicals tested, which are a strong

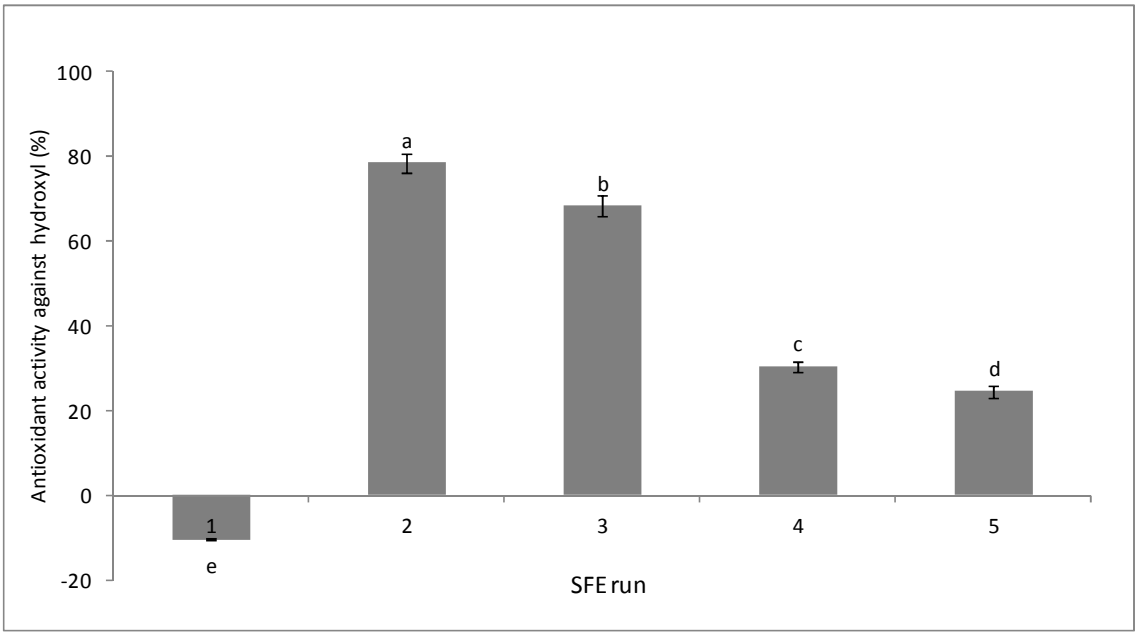
indicative that the differences in the chemical composition led to different antioxidant activities. The highest antioxidant activity of samples 2 and 5 for the superoxide radical in relation to the other can be due to higher extraction yield, extracting a great number of compounds. Another reason would be the fact that the sample 2 is the only condition that extracted the compound sulforaphane, which has high antioxidant activity, already proven in the literature, and despite being at lower concentrations than the other compounds showed higher antioxidant activity (Zhao, Moore, Redell, & Dash, 2007).



a)



b)



c)

Figure 1. Antioxidant activity of supercritical CO_2 extracts of *Brassica oleracea* var *capitata* obtained in the five runs towards superoxide radical (a), peroxyl radical (b) and hydroxyl radical (c). [a,b,c,d,e] different letters represent a significant difference.

Another class of compounds that justifies the higher antioxidant capacity of the samples 2 and 5 is the class of phytosterol (Sitosterol and campesterol), which also have

antioxidant activities (Yoshida & Niki, 2003; Singh, 2013). When compared to the other samples (1, 3 and 4), sample 5 also was verified considerable antioxidant activity, which is consistent with the results previously obtained in this work (yield and chemically). This sample did not present the compound sulforaphane in its constitution, but when compared to other samples presented a greater amount of sitosterol (9.26%), steroid which has proven antioxidant activity in literature Singh (2013), which becomes a reason for its greatest inhibitory capacity along with sample 2.

The sample 1 showed prooxidant activity against the hydroxyl radical probably due to lack of fatty acids, which were present in other conditions of the supercritical extraction. Furthermore, the hydroxyl radical presents half-life so short (about 10^{-9} seconds) which makes hard the scavenging of this radical by the compounds in the extract. In this way, the compounds should have a higher inhibitory action to scavenge hydroxyl radical when compared to other reactive oxygen species.

The data were statistically analyzed by Tukey test, where it can be seen a statistical difference of sample 2 in comparison with the others, but these were not statistically different from each other. Samples 2 and 5 had the best yield and antioxidant activity due to the presence of a greater range of compounds which were characterized by GC-MS, thus more detailed investigation would fit around these conditions of extraction of *Brassica*, since already been proven in the literature anticarcinogenic and antioxidant action of these compounds (Zhang, Li, & Tang, 2005; Kensler, Chen, & Egner, 2005; Farag & Motaal, 2010). As is known, some degenerative diseases are associated with production of free radicals in the body. Once the supercritical extraction showed a good inhibitory capacity of the radicals can come as an alternative to preventing these pathologies.

4. Conclusions

In this work were presented experimental data concerning the supercritical CO_2 extraction of bioactive compounds from *Brassica oleracea* var. *capitata*, as well as the chemical characterization and the antioxidant activities of the extracts towards peroxyl, superoxide and hidroxyl radicals. The highest extraction yield was obtained under 60°C and 25 MPa, which was 0.47wt%. From the characterization of the extracts obtained was possible the identification of 13 compounds, which the presence of sulforaphane and iberin nitrile are the most relevant due to their biological properties already reported in literature. The extracts of all runs presented antioxidant activity towards the three radicals, but the highest activity for all radicals was using the extracts obtained in the run 2, possibly due to the presence of above mentioned compounds. The use of supercritical CO_2 extraction to obtain bioactive compounds of *Brassica oleracea* var. *capitata* showed to be a promising alternative to a conventional extraction methods, since allowed the extraction of compounds with scientific and industrial interest.

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6 ARTIGO 3

Ultrasound-assisted extraction and biological activities of extracts of *Brassica oleracea* var. *capitata*

Artigo submetido ao periódico Ultrasonics Sonochemistry

Ultrasound-assisted extraction and biological activities of extracts of *Brassica oleracea* var. *capitata*

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ABSTRACT

In this work, the antioxidant and antimicrobial activities of *Brassica oleracea* var. *capitata* extracts obtained through ultrasound-assisted extraction were evaluated. The extracts obtained using the best extraction conditions were submitted to different hydrolysis conditions before their use in the biological tests. The crude and hydrolyzed extracts were characterized using gas chromatography coupled with a mass detector. The use of ultrasound enabled a richer extract to be obtained at 30°C with 60 wt% solvent. All extracts presented antioxidant activities toward DPPH, superoxide and peroxyl radicals, but the use of hydrolyzed extracts considerably improved the antioxidant activities. Antimicrobial activities were only detected for the hydrolyzed extracts of *Brassica oleracea* var. *capitata*. The main contribution of this work was demonstrating that the hydrolysis of extracts can enhance the antioxidant activity of extracts from vegetables matrices.

Key-words: Brassica oleracea var. *capitata*, ultrasound-assisted extraction, hydrolysis of extracts, biological activity.

1. Introduction

Brassica sp. are one type of vegetable that are of pharmacological interest because several studies have revealed that they exhibit anti-inflammatory, antimycotic, photoprotective, antihyperglycemic, anticarcinogenic and antioxidant activities [1-3]. Among the various *Brassica species*, *Brassica oleracea L.* var. *capitata* (cabbage) has been widely studied because of its biological activities [4].

Several authors have reported the antioxidant activities of extracts of *Brassica* against different radicals. Among the methods for testing radicals, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay provides basic information about the antiradical activity of extracts. This procedure has been widely used to evaluate the antiradical activity of several plants because it is considered to be an easy, precise and reproductive test for evaluating the antioxidant activities of extracts, including extracts of *Brassica sp* [5-7]. The antioxidant potential of *Brassica* extracts against superoxide radicals ($O_2^{\bullet-}$), which can cause indirect damage because they lead to the formation of hydroperoxides through the autoxidation of unsaturated fatty acids, was also investigated [5, 8-9]. However, the use of *Brassica* extracts against the peroxyl radical ($ROO^{\bullet}$), which is an important intermediary in lipid peroxidation, has not been remarkably considered. Specifically, Zhou and You [10] evaluated the scavenging activities of vegetables, including broccoli and kale, against DPPH, ($ROO^{\bullet}$) and ($O_2^{\bullet-}$), whereas Ou et al [11] determined the antioxidant activity of white cabbage and broccoli extracts against peroxyl radicals.

Extracts of *Brassica* are also used as antimicrobial agents. The antibacterial activity of ethanol, methanol and acetone extracts obtained from *Brassica oleracea* was investigated against *Salmonella abony*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Enterococcus faecalis* [12]. Blazevic et al. [13] tested the antibacterial activity of aqueous extracts of *Aurinia sinuate* (L.) *Griseb.* (genus of flowering plant family *Brassicaceae)*

against *Staphylococcus aureus, Bacillus cereus, Clostridium perfrigens, Enterococcus faecalis, Micrococcus luteus, Aeromonas hydrophila, Chryseobacterium indologenes, Enterobacter sakazakii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas luteola* and *Vibrio vulnificus.*

However, the biological activity of extracts obtained from plant species is related to the extraction method because each method affects the selectivity of the compounds. In this way, novel extraction techniques, such as the ultrasound-assisted method, have been used to obtain plant extracts. The use of ultrasound can increase cell wall destruction, cause leakage of cellular material, enhance the penetration of solvent into plant cells, facilitate hydration and swelling, and improve mass transfer. These phenomena can increase the extraction of antioxidants while significantly reducing the extraction time, thus improving overall efficiency. Ultrasound-assisted extraction (UAE) is versatile and can be performed on small and large scales [14-16].

Therefore, the objective of this study was to evaluate the antioxidant and antimicrobial activities of *Brassica oleracea* var *capitata* extracts obtained using ultrasound-assisted extraction. First, the extraction conditions were optimized, and then the extracts were submitted to different hydrolysis conditions before their use in the biological evaluations. The crude and hydrolyzed extracts were characterized using gas chromatography coupled with a mass detector (GC-MS).

2. Material and methods

2.1. Chemicals

Sodium hydroxide, HEPES, ABAP 2,2'-azobis (2-aminodinopropane hydrochloride), DCFH2-DA (2',7'-dichlorofluorescin), xanthine oxidase (XOD) 25UN, hypoxanthine (HPX), nitro tetrazolium blue chloride (NBT) and DPPH (1,1-diphenyl-2-picrylhydrazyl) were

obtained from Sigma-Aldrich. Dimethyl sulfoxide (DMSO), potassium chloride and magnesium chloride were obtained from Isofar® (Rio de Janeiro, Brazil). Sodium carbonate was obtained from Merck. Ethylene diamine tetra acetic acid (EDTA) was obtained from Nuclear® (Brazil). Ultrapure water was obtained from a Milli-Q system UV Synerg (Millipore SA, Molsheim, France). The solvents methanol, acetonitrile and anhydrous ethanol, all of HPLC grade, were obtained from the Tedia Company (USA).The microorganisms used in the tests (*Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 14948) were obtained from the Laboratory of Immunobiology and Molecular Glycobiology, Federal University of Viçosa - MG.

2.2. Samples

Brassica oleracea var. capitata was dried in an oven with air circulation at 60°C for 72 hours, according to Tanongkankit et al. [17]. Next, the material was ground in a slicer and stored at room temperature under a nitrogen atmosphere prior to extraction.

2.3. Ultrasound-assisted extraction

Experiments were performed in a reactor with a thermostatic water bath (temperature accuracy of ±1.0°C). The experimental setup consists of an ultrasonic bath (Unique Inc., model USC 1800A, Brazil, BR) equipped with a transducer with longitudinal vibrations. The ultrasonic unit has an operating frequency of 40 kHz and a maximum-rated ultrasound power output of 132 W. The ultrasonic transducer (surface area of 282.2 cm^2) is fitted at the bottom of the bath horizontally along the length of the bath.

For the optimization of the extraction conditions, a central composite rotational design (CCRD) for two independent variables was conceived to evaluate the influence of the ethanol concentration (wt%) and temperature on the extraction [18]. Table 1 presents the range

investigated for each independent variable. The extractions were performed using an Erlenmeyer flask containing 2 g of dry material and 10 mL of an aqueous solution of ethanol in the ultrasonic bath for 120 minutes. The same experiment was performed in duplicate in the presence and absence of ultrasound irradiation for comparison. In this paper, the response evaluated was the sum of the area of all compounds identified by HPLC. For comparison, one extraction was performed by substituting ethanol with methanol in the optimized experimental conditions because there is a difference in the polarity of these two solvents.

2.4. Hydrolysis of the crude extracts

The hydrolysis of the crude extracts (methanolic and ethanolic) obtained using the best experimental conditions of the previous step were performed following the method proposed by Robbins et al. [19] with some modifications. Acid hydrolysis consisted of treating 2 mL of the crude extract with 1 mL of HCl (2M) and then heating the mixture to 95°C for 1.5 hours. Alkaline hydrolysis consisted of treating 5ml of the extract with 5 mL of an alkaline solution (2 M NaOH, 10 mM EDTA, and 1% ascorbic acid). The reaction mixture was maintained at 30°C under stirring for 30 minutes. Next, the pH was adjusted to 3.0 using an 8M HCl solution, and extraction with ethyl acetate (two 5-mL portions) using sonication for 20 minutes was performed. Organic solvent was removed using a rotary evaporator. The solid residue was re-suspended in 2 mL of methanol and filtered (particle size, 0.22 µm; polyvinylidene fluoride). Prior to testing for antioxidant and antibacterial activities, these extracts were neutralized.

2.4. Chromatographic analysis

In the extraction step, high performance liquid chromatography (HPLC) was employed to separate the compounds according to the method proposed by Leoni et al. [20]

with some modifications. For this purpose, a HPLC Dionex (model P680, UV-Vis detector UVD-170) equipped with a column ODS-18(250X5 µm,) using a CH_3CN/H_2O 15:85 mixture as the eluent was used. A flow rate of 1.0 mL/ min, wavelengths 240 and 254 nm and a total run time of 15 min were employed.

The identification of compounds present in the crude and hydrolyzed extracts was performed only for the optimized run of the experimental design (methanolic and ethanolic extracts) using gas chromatography coupled with a mass detector (GC-MS). The extracts were analyzed with a gas chromatograph (HP 6890) interfaced with a mass selective detector (HP 5973) with an automatic injection system (HP 6890) using a capillary column HP-5MS (30 m x 0.32 mm x 0.25µm).Helium was the carrier gas with a flow rate of 2 mL/min at a pressure of 5.05 psi and electronic impact mode of 70 eV. Samples of 1µL were injected at a 250°C interface temperature with the following column temperature gradient programming: 70°C (1 min), 12°C/min up to 280°C.

2.5. Antioxidant activities of extracts

2.5.1. Radical DPPH scavenging activity

The analytical method to measure the radical-scavenging antioxidant activity of crude and hydrolyzed extracts of *Brassica oleracea var. capitata* against DPPH radical was based on the addition of 1500 µL of extract to a 1480 µL of a DPPH solution plus 20 µL of hydroethanolic solution. A blank assay was performed using 1500 µL of a hydroethanolic solution instead of the extract. The resulting solution was maintained at rest for 30 minutes. Then, the absorbance of samples was determined at 522 nm in an UV-Vis 8453 Hewlett-Packard spectrophotometer (Agilent Technologies, Santa Clara, USA). The antiradical activity toward DPPH (AA_{DPPH}) was calculated according to the following equation:

$$AA_{DPPH} = \left(\frac{A_{DPPH-}(A - A_B)}{A_{DPPH}} \right) \times 100 \quad (1)$$

where A_{DPPH}, A and A_B are the absorbance of DPPH solution, sample and blank, respectively.

2.5.2. Superoxide radical anion ($O_2^{\bullet-}$) scavenging activity

The antiradical activity of *Brassica oleracea* var. *capitata* extracts obtained by extraction using supercritical CO_2 toward $O_2^{\bullet-}$ radicals was evaluated by the enzymatic system HPX/XOD. For this purpose, 100 μL of EDTA (30 mmol.L^{-1}), 100 μL of HPX (3 mmol.L^{-1}) and 200 μL of NBT (1.42 mmol.L^{-1}) were mixed with 100 μL of extract. After 3 minutes, 100 μL of enzyme XOD (0.75 U.mL^{-1}, diluted in phosphate buffer) was added. The final volume of the solution was brought to 3 mL with phosphate buffer (0.05 mol.L^{-1}, pH 7.4). The blank sample was prepared in the same manner but without the presence of NBT. Additionally, a control test was performed containing all reagents with the solvent employed in the samples and a blank control. After 40 minutes of reaction, the absorbance of the samples was measured in an UV-Vis 8453 Hewlett-Packard spectrophotometer (Agilent Technologies, Santa Clara, EUA) at 560 nm. The antiradical activity toward $O_2^{\bullet-}$ ($AA_{O2\bullet-}$) was calculated according to the following equation:

$$AA_{O_2^{\bullet-}} = \left(1 - \frac{(A - A_B)}{(C - C_B)} \right) \times 100 \quad (2)$$

where A and A_B are the absorbance of the sample and blank, respectively, and C and C_B are the absorbance of the control and blank control, respectively.

2.5.3. Peroxyl radical anion ($ROO^{\bullet}$) scavenging activity

The antiradical activity of *Brassica oleracea var. capitata* extracts obtained by extraction using supercritical CO_2 toward $ROO^{\bullet}$ radicals was evaluated using the fluorimetric method withDCFH2-DA as a substrate. A plate containing 96 wells was subdivided into two regions: the first region corresponded to the lines A, B, C, and D, and the second region corresponded to lines E, F, G and H. The first three wells of each region were used to add 10 µl of solvent to the samples. In the remaining wells of the plate, 10 µl of the extract was added. Then, 127.5 µL of buffer was added to all wells of the plate. Subsequently, 7.5 µl of ultrapure water was added to all wells of region 1, whereas 7.5 µl of ABAP (4 $mmol.L^{-1}$) was added to the wells of region 2. Before the analysis,10 µl of DCFH2-DA (16 $\mu mol.L^{-1}$)was added. A Vitor 2 fluorimeter (Perkin Elmer, Massachusetts, USA) was programmed to maintain the temperature at 37°C and to measure the fluorescence at 485 nm (excitation) and 520 nm (emission) in regular time intervals of 5 minutes for 30 minutes. The antiradical activity toward $ROO^{\bullet}$ ($AA_{ROO\bullet}$) was calculated according to the following equation considering the measurement at 30 minutes:

$$AA_{ROO\bullet} = \left(1 - \frac{(F_A - F_{AB})}{(F_S - F_{SB})}\right) \times 100 \qquad (3)$$

where F_A and F_{AB} are the fluorescence of the sample containing ABAP and the fluorescence of the blank without ABAP, respectively, and F_S and F_{SB} are the fluorescence of the solvent containing ABAP and the fluorescence of the solvent blank without ABAP, respectively.

2.6. Antibacterial activities (S. aureus and E. coli) of extracts

The antimicrobial activity of crude and hydrolyzed extracts of *Brassica oleracea* var. *capitata* was determined using the antibiogram with solid disks methodology. The extracts were resuspended in the same extraction solvent (methanol or ethanol) at a concentration of

100 mg/mL. *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 14948) were inoculated in ágar mueller hinton liquid medium (tryptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L) and kept in an oven maintained at 36 ± 2ºCfor 24 hours. As a positive control for *S. aureus* and *E. coli*, erythromycin (10µg/µL) and ciprofloxacin (0.1 µg/µL), which were applied in the same plate, were used, respectively. The antimicrobial activity of the solvents (negative control) was also determined. The degree of sensitivity or resistance of a microorganism was determined by measuring the size of the zones of the antimicrobial effect (size of halo formed). Samples were examined in duplicate against each bacterium.

2.7. Statistical analysis

The statistical analysis was accomplished using ANOVA coupled with the Tukey test at a 95% confidence level using the software Statistica® 8.0.

3. Results and discussion

3.1. Ultrasound-assisted extractions

The process variables studied in this work were temperatures in the range of 30-70°C and ethanol concentrations in the range of 20-100 wt%. All experiments were performed in the presence and absence of ultrasound irradiation. Table 1 presents the results obtained in the CCRD concerning the total HPLC area of all compounds separated in the sample, which ranged from 35.44 to 237.90 unit area and from 46.71 to 494.25 unit area in the absence and presence of ultrasound, respectively. Considering the mean value obtained in the 11^{th} run, significant differences ($p<0.05$) were detected between the amounts obtained in the absence and presence of ultrasound because the mean values were 132.9 and 194.3 unit area, respectively.

Table 1

Experimental conditions used to compare the presence and absence of ultrasound in terms of HPLC areas after 120 minutes.

Run	Temperature (°C)	Ethanol (wt%)	HPLC $Area_{US}$ (mAu min)	HPLC Area (mAu min)	Area increasing (%) using US $\lvert 100 - \{[(Area/Area_{US})] \cdot 100\}\rvert$
1	36	32	177.81	107.75	39.4
2	64	32	198.99	105.90	46.8
3	36	88	174.51	73.81	57.7
4	64	88	158.17	67.57	57.3
5	30	60	494.25	237.90	51.9
6	70	60	231.89	186.87	19.4
7	50	20	118.87	74.62	37.2
8	50	100	46.71	35.44	24.1
9	50	60	167.33	126.86	24.2
10	50	60	184.39	142.43	22.8
11	50	60	164.59	137.44	16.5

Fig. 1 presents the extraction profiles in presence and absence of ultrasound in terms of the normalized HPLC area. The right y-axis shows the normalized HPLC area for both experimental sets, black for ultrasound extraction and gray without ultrasound, whereas the left y-axis indicates the increase or contribution (white dotted column) in percentage when ultrasound was applied. For all runs, a positive effect of ultrasound in comparison with conventional extraction (maceration) is clearly observed, which presented a general mean of approximately 36.1±15.5% considering the 11 runs.

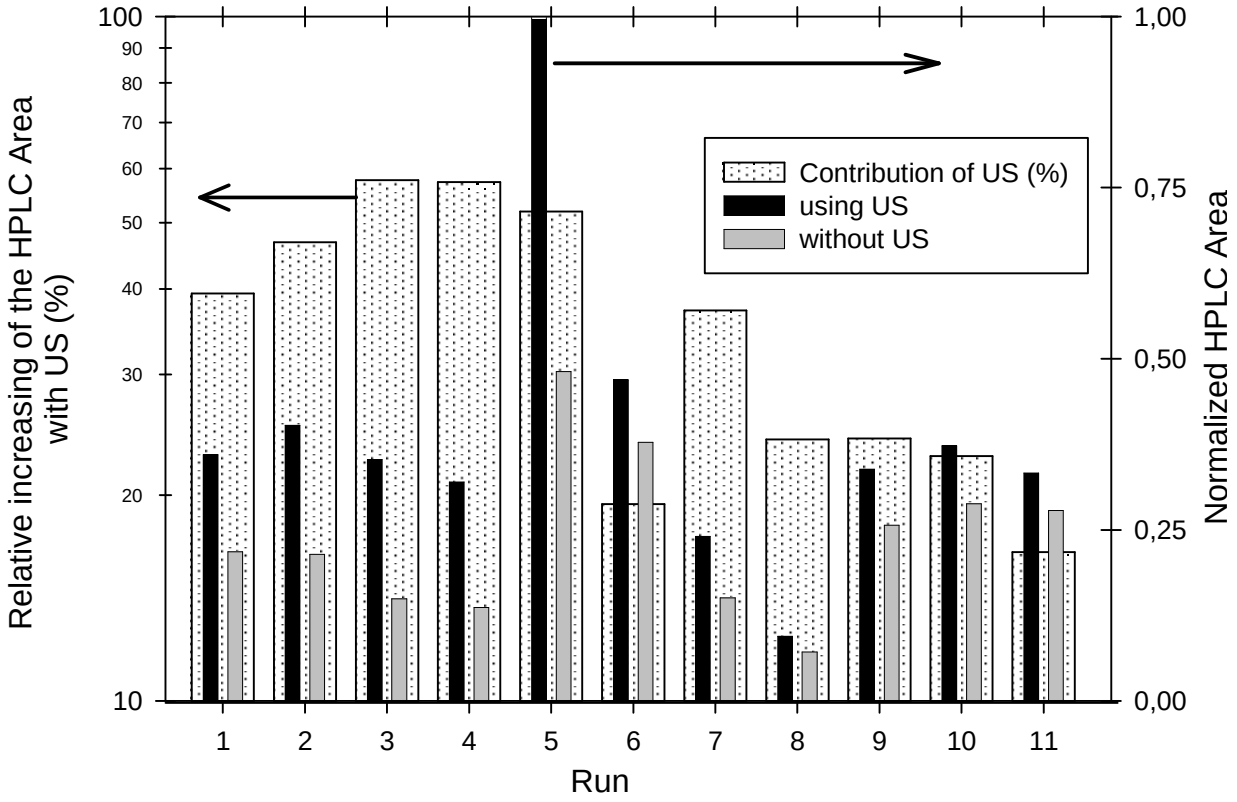

Fig. 1. Comparison between use and absence of US for *Brassica* extraction after 120 minutes (right Y axes) and the contribution/increasing of the US (left Y axes).

Note that the ultrasound effect can be associated with an increase in the mass transfer process during the reaction through the formation of cavitation bubbles, which provide an important benefit of opening up the surface of solid substrates. Consequently, at lower temperatures, the energy provided by the ultrasound process is responsible for the improvement in the extraction rates, but at higher temperatures, the thermal energy is sufficient, the ultrasound exhibits a positive effect on the extraction runs.

Based on the results presented in Table 1, the experimental ultrasound conditions for the extraction of bioactive compounds of *Brassica oleracea* var. *capitata* were verified in run

5 (30°C and 60 wt% of ethanol) in the presence of the ultrasound. Considering this result, one additional extraction was performed in which ethanol was substituted with methanol, and the unit area of the extracted sample was 330.5 unit area. For the following steps, only the crude and hydrolyzed extracts obtained using methanol or ethanol were considered.

3.2. Gas chromatography–mass spectrometry analysis of Brassica oleracea var capitata extracts

The crude and hydrolyzed extracts of *Brassica oleracea var capitata* obtained by ultrasound-assisted extraction (at 30°C and 60 wt% of ethanol or methanol) were analyzed with a gas chromatograph interfaced with a mass selective detector. The composition of the extracts obtained in this work was expressed as the percentage of the normalized peak areas, which are presented in Table 2. It was possible to identify 10 compounds, including derivatives of fatty acids, sulfur compounds and glycosides. The crude ethanol was the sample with the highest number of identified compounds. Desulfosinigrin and octadecanoic acid are among the compounds of interest.

Table 2

Chemical profile of crude and hydrolyzed extracts of *Brassica oleracea var capitata* obtained by ultrasound-assisted extraction.

Solvent	extract	Compounds- retention time (min.)/ normalized peak areas (%)									
		pentanoic acid	2-furancarbox-Aldehyde	L-glucose	phenol	ethyl α-d-glucopyranoside	desulphosinigrin	β-D-glucopyranose	D-manose	Hexadecanoic Acid	Octadecanoic acid
Ethanol	crude	-	-	7.78/49.00	10.32/9.43	-	11.77/3.00	12.24/1.05	12.41/2.00	-	19.84/11.64
	H^+	5.15/5.75	7.16/61.10	-	-	11.37/8.52	-	-	-	-	-
	OH^-	-	-	11.82/13.00	-	-	10/18.95	-	-	14.45/3.35	-
Methanol	crude	-	-	7.47/0.50	-	-	-	-	12.5/0.75	-	
	H^+	-	7.16/35.10	-	-	10.9/41.50	-	11.2/2.50	-	-	-
	OH^-	-	-	-	-	-	-	-	-	14.45/4.77	-

H^+ :acid OH^- : alkaline

Although flavonoids, which are usually found in *Brassica* sp., were not determined in the extracts, a significant amount of glycosides, which are associated with flavonoids and phenolic acids, were found. The presence of these glycosides can be indicative of the presence of flavonoids in the sample. The hydrolysis of the extracts led to differences in the chemical profiles of the samples. For example, in both of the acid hydrolyzed extracts, the presence of 2-furancarboxaldehyde, which is a degradation product formed during the acid hydrolysis of glucose, was detected. In addition, in these same samples, the presence of ethyl α-D-glucopyranoside, which is a compound with a positive effect on the prevention of skin barrier disruption [21], was detected. In the crude and alkaline hydrolyzed ethanolic extracts, the presence of desulfosinigrin, which is a glucosinolate, was observed. Considering the content of fatty acids present in the extracts, hexadecanoic acid was the compound with the highest concentration, 3.33 and 4.77% in samples 5 and 6, respectively. Fatty acids present antioxidant [22] and antimicrobial activities [23], which were also evaluated in this study.

3.3. Antioxidant activity of ultrasound extracts of Brassica oleracea var capitata

Table 3 presents the results concerning the antioxidant activities of the crude and hydrolyzed extracts of *Brassica oleracea var capitata* obtained using ultrasound-assisted extraction (at 30°C and 60 wt% of ethanol or methanol) toward DPPH, $O_2^{\bullet -}$ and $ROO^{\bullet}$ radicals. All samples presented antioxidant activities, which ranged from 13.0 to 80.0, 35.2 to 63.1% and 89.3 to 99.5% toward DPPH, $O_2^{\bullet -}$ and $ROO^{\bullet}$radicals, respectively.

Table 3

Antioxidant activities of crude and hydrolyzed extracts of *Brassica oleracea* var *capitata* obtained by ultrasound-assisted extraction.

Solvent extraction	Extract	DPPH (%)	$O_2^{\bullet-}$ (%)	$ROO^{\bullet}$ (%)
Ethanol	Crude	13.0 ± 0.4 ^{ef}	35.6± 4.0^{ef}	98.6±0.1 ^{a}
	Acid hydrolysis	74.0 ± 3.7^{abcd}	45.3± 2.9^{d}	98.5±0.3^{a}
	Alkaline hydrolysis	70.8± 2.1^{cd}	55.1± 4.1^{c}	99.5±0.1^{a}
Methanol	Crude	14.0± 0.6^{ef}	61.1± 5.0^{ab}	97.4±0.2^{a}
	Acid hydrolysis	78.9± 3.1^{abc}	35.2± 3.9^{ef}	97.8 ±0.2^{a}
	Alkaline hydrolysis	80.0± 4.0^{abc}	63.1± 3.2^{ab}	89.3±2.8^{b}

^{a,b,c,d,e,f} (Significant difference at 95% ($p<0.05$ – Tukey Test))

Concerning the antioxidant activities toward DPPH radicals, both the methanolic and ethanolic hydrolyzed extracts presented higher activities than the crude extracts. These results were statistically evaluated using the Tukey test ($p<0.05$), and the hydrolyzed extracts for both methanolic and ethanolic fraction did not present significant differences, even though the hydrolysis process was different.

For the $O_2^{\bullet-}$ radical, the crude methanolic extract presented a 1.7-fold higher antioxidant activity than the crude ethanolic extract, which was significantly different ($p<0.05$). This result indicates that the solvent used in the extraction has an influence on the antioxidant activities of the extracts, consistent with the results obtained by Zhao et al. [24], who reported that extracts from the barley variety using 80% methanol possessed the highest

O2•-scavenging activity, followed by water, 80% acetone, and 80% ethanol extracts. The hydrolysis of ethanolic extracts presented higher activities than the crude extract; however, the acid hydrolysis extracts presented a lower activity than alkaline hydrolysis extracts, with the results being statistically significant ($p<0.05$). For the methanolic extracts, the acid hydrolysis extract presented a lower activity than the crude extract, but the alkaline hydrolysis extract presented a similar activity as the crude extract, where no significant difference was detected for the crude and alkaline hydrolyzed extracts.

The crude and hydrolyzed extracts of *Brassica oleracea var. capitata* obtained by ultrasound-assisted extraction toward $ROO^{\bullet}$ radicals presented the highest antioxidant activities, which ranged from 89.3 to 99.5%. Only the alkaline hydrolyzed methanolic extract presented a significant difference over the other extracts. The high inhibitory activity against $ROO^{\bullet}$radicals verified in all the tested extracts can be attributed to the fact that this radical is less reactive than the other extracts that have been studied [25]. Another possibility is related to the high concentrations of the extracts (20%).

Vrchovska et al. [8] evaluated the antioxidant activities of aqueous extracts of *Brassica oleracea var. costatata* toward DPPH and $O_2^{\bullet-}$radicals and found inhibitory activity for these radicals. The antioxidant activities of *Brassica oleracea* species (white cabbage and broccoli) obtained using different extraction methods, such as acetone/water 50:50 v/v [11], water extraction+hexane extraction [26] and hexane/dichloromethane (1:1 v/v)+acetone/water/acetic acid (70:29.5:0.5 v/v/v) [27], also presented activities toward $ROO^{\bullet}$ radicals. Podsedek [28] reported that the contribution of *Brassica* vegetables for better health may be related to their antioxidant capacity.

Although several works have reported on the antioxidant activities of extracts from different species of *Brassica* obtained using different extraction methods, there is no common ultrasound method to obtain hydrolyzed extracts; however, this paper demonstrated that the

hydrolysis process can considerably improve the antioxidant activities of the extracts [5, 7]. For example, the antioxidant activity toward DPPH radicals increased from 13% to approximately 72.0% after the hydrolysis of the extracts. A similar trend was observed for the $O_2^{\bullet-}$ radical, which increased from 35.6 to 56.1 after the alkaline hydrolysis of the extracts. In this sense, the main contribution of this work was demonstrating that the hydrolysis of extracts can considerably enhance the antioxidant activity of extracts from vegetable matrices.

3.4. Antimicrobial activity of crude and hydrolyzed extracts of Brassica oleracea var capitata

The antimicrobial activity of the crude and hydrolyzed extracts (methanolic and ethanolic) was determined against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli.* Table 4 presents the experimentally determined antimicrobial activities of the extracts obtained in this work. Only the hydrolyzed extracts presented antimicrobial action, where the alkaline-hydrolyzed extracts presented action against *Escherichia coli*, whereas for *Staphylococcus aureus,* only the acid hydrolyzed methanolic extract presented action; however, the inhibitory halo cannot be considered as antimicrobial activity because the activity is considered only for a halo≥8mm [29].

The result obtained in this study is in good agreement with the literature; Gram-positive bacteria are more resistant than Gram-negative ones because they have thicker cell walls, presenting a thickness ranging from 20-80 nm, whereas Gram-negative bacteria present a maximum value of 20 nm [30]. Other authors have also evaluated the antimicrobial activity of *Brassica* vegetable extracts. Ayaz et al. [31] evaluated the antimicrobial activity of methanolic extracts of *Brassica oleracea* L. var. *acephala* same antimicrobial procedure and microorganisms employed in this work and verified that the extract presented better action against *Staphylococcus aureus* than *E. coli.*

Table 4

Antimicrobial activities of crude and hydrolyzed extracts of *Brassica oleracea var capitata* obtained by ultrasound-assisted extraction.

Solvent Extraction	Extract	Halos for *S. aureus* (mm)				Halos for *E. coli* (mm)			
		1:1	1:2	1:4	1:8	1:1	1:2	1:4	1:8
Ethanol	Crude	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Acid hydrolysis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Alkaline hydrolysis	0.0	0.0	0.0	0.0	6.3±0.3	0.0	0.0	0.0
Methanol	Crude	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Acid hydrolysis	4.5±0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Alkaline hydrolysis	0.0	0.0	0.0	0.0	8.5±1.4	5.3±0,3	0.0	0.0
No solvent	Positive control	25.0	nd	nd	Nd	18.0	16.0	nd	nd

nd – not determined

As verified from the antioxidant activities of the extracts, the hydrolyzed extracts of *Brassica oleracea* var *capitata* presented higher antimicrobial activities than the crude extracts. This result can be attributed to a difference in the chemical profiles of the samples of the crude and hydrolyzed extracts, as shown in Table 2. For example, alkaline hydrolysis enabled the identification of hexadecanoic acid, which presents antimicrobial activity [23], justifying the highest antimicrobial activity of this extract. Another possibility for the better performance of the hydrolyzed extracts is because the extraction procedure used in this work can extract many glycoside compounds, which can present greater activity than the crude extracts after hydrolysis.

4. Conclusions

In this work, we presented experimental data concerning the optimization of the ultrasound-assisted extraction of *Brassica oleracea var capitata*, the chemical characterization of crude and hydrolyzed extracts and the determination of antioxidant and antimicrobial activities of crude and hydrolyzed extracts. All extracts presented antioxidant activities toward DPPH, $O_2^{\bullet -}$ and $ROO^{\bullet}$ radicals, but the use of the hydrolyzed extracts considerably improved the antioxidant activities. Antimicrobial activities were only detected for the hydrolyzed extracts of *Brassica oleracea* var *capitata*. The main contribution of this work was demonstrating that the hydrolysis of extracts can enhance the antioxidant activity of extracts from vegetables matrices.

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7 DISCUSSÃO INTEGRADA

A atividade biológica de extratos, obtidos a partir de espécies de plantas, está diretamente relacionada com o método de extração utilizado, uma vez que cada método afeta a seletividade dos compostos extraídos. Em relação aos resultados referentes à atividade antioxidante dos extratos de *Brassica oleracea* var. *capitata* obtidos por extração supercrítica, de maneira geral, todos os extratos apresentaram ação antioxidante frente aos três radicais testados. Embora, tenham tido comportamentos distintos, ou seja, maior e menor inibição da ação radicalar. Tal fato está relacionado com reatividade de cada radical, aliado a isso, a composição química de cada extrato é fundamental para ter maior ou menor atividade.

A utilização deste método alternativo de extração possibilitou a obtenção de uma composição química diferenciada em relação à extração por ultrassom, uma vez que o primeiro conseguiu extrair compostos como sulforafano, que possui elevada atividade antioxidante já comprovada na literatura (ZHAO et al., 2007), além de compostos da classe dos fitoesteróis como sitosterol e campesterol. Os quais também possuem atividade antioxidante reportada na literatura (YOSHIDA; NIKI, 2003; SINGH, 2013). Resultados já discutidos, de forma mais embasada, no capítulo 2 da presente dissertação.

Na extração de *Brassica oleracea* var. *capitata* assistida por ultrassom todos os extratos apresentaram atividade antioxidante frente aos radicais testados. Porém, a submissão a condições de hidrólise ácida e alcalina dos extratos melhorou consideravelmente essas atividades. Em relação à composição química, conseguiu-se extrair um total de 10 compostos incluindo derivados de ácidos graxos, compostos de enxofre e glicosídeos. Desulfosinigrina e ácido octadecanóico estão entre os compostos extraídos de maior interesse. A hidrólise dos extratos levou a diferenças nos perfis químicos das amostras, e consequentemente na atividade biológica, conforme descrito acima.

Em relação à atividade antibacteriana, as amostras extraídas por ESC não apresentaram atividade, já em relação às amostras obtidas por ultrassom, somente uma amostra, que foi submetida à condição de hidrólise alcalina apresentou ação frente a *E. coli*. Embora os dois artigos tenham abordado aspectos distintos da planta, ou seja, ESC para compostos apolares e extração assistida por ultrassom para compostos polares. Fica evidente que a utilização de extração com CO2 supercrítico mostra-se como uma alternativa promissora em relação a métodos de extração convencionais, uma vez que permitiu a extração

de compostos com interesse científico e industrial. Este trabalho demonstra que o tipo de extração teve uma grande influência no contéudo fitoquímico, capacidade antioxidante e atividade antibacteriana para a espécie de *Brassica oleracea* var. *capitata.*

8 CONCLUSÕES

- Otimizou-se a extração de *Brassica oleracea* var. *capitata* com CO_2 supercrítico, sendo que a melhor condição de extração foi na temperatura de 60º C e pressão de 25 MPa, obtendo-se um rendimento de 0,47% (m/m).

- Otimizou-se a extração de *Brassica oleracea* var. *capitata* por ultrassom, e a melhor condição de extração foi de 30ºC e 60% (m/v) de etanol, os quais foram submetidos a condições de hidrólise ácida e alcalina.

- Os extratos obtidos com CO_2 supercrítico e por ultrassom foram caracterizados por CG-MS, e obteve-se para a primeira extração compostos de enxofre como sulforafano e iberin nitrila, fitoesteróis como sitosterol e campesterol, terpenos como α-amirina, além de ácidos graxos. Para os extratos obtidos por ultrassom, obteve-se compostos de enxofre como desulfosinigrina, ácidos graxos como ácido octadecanóico e glicosídeos.

- Os extratos de *Brassica oleracea* var. *capitata*, obtidos com CO_2 supercrítico apresentaram atividade antioxidante frente aos radicais superóxido ($O_2^{-\bullet}$), hidroxila (HO•), e peroxila (ROO•).

- Os extratos brutos e hidrolisados de *Brassica oleracea* var. *capitata*, obtidos por ultrassom apresentaram atividade antioxidante frente aos radicais superóxido ($O_2^{-\bullet}$), radical peroxila (ROO•) e radical $DPPH^{\bullet}$, porém os extratos hidrolisados apresentaram ação consideravelmente superior em relação aos extratos brutos.

- As amostras extraídas por ESC não apresentaram atividade antibacteriana, em relação às amostras obtidas por ultrassom, apenas uma amostra, que foi submetida à condição de hidrólise alcalina apresentou ação frente a *E. coli*.

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