UNIVERSIDADE FEDERAL DO PARANÁ

STEPHANE JANAINA DE MOURA ESCOBAR

# EFEITOS DAS CHALCONAS SOBRE PROPRIEDADES OXIDATIVAS DE MITOCÔNDRIAS ISOLADAS DE FÍGADO DE RATO

Curitiba

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# EFEITOS DAS CHALCONAS SOBRE PROPRIEDADES OXIDATIVAS DE MITOCÔNDRIAS ISOLADAS DE FÍGADO DE RATO

Dissertação apresentada ao Programa de Pós-Graduação em Ciências – Bioquímica, Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requesito parcial à obtenção do grau de Mestre em Ciências – Bioquímica.

Orientadora: Profª. Drª. Maria Eliane Merlin Rocha

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2014

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EFEITOS DAS CHALCONAS SOBRE PROPRIEDADES OXIDATIVAS DE MITOCÔNDRIAS ISOLADAS DE FÍGADO DE RATO

Dissertação aprovada como requisito parcial para a obtenção do título de Mestre em Ciências-Bioquímica, no Programa de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas da Universidade Federal do Paraná, pela seguinte Banca Examinadora:

Profª. Dra. Maria Eliane Merlin Rocha (Orientadora)
Departamento de Bioquímica e Biologia Molecular – UFPR

Profª. Dra. Silvia Maria Correia Suter Cadena
Departamento de Bioquímica e Biologia Molecular – UFPR

Profª. Dra. Tatiana Herrerias
Universidade Positivo

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Dedico esse trabalho a minha família e amigos;
Meus pais Margareth e Reinaldo
Meus avós Moura e Gina
Meus irmãos Ariadne, Guilherme e Eduardo e
Ao meu amor Pedro.

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## RESUMO

Os flavonoides têm sido utilizados no tratamento de diversas patologias como alergias, infecções virais, úlceras e câncer. Dentro deste grupo de compostos encontram-se as chalconas, presentes no lúpulo e na maçã, e que possuem várias atividades biológicas conhecidas como: antioxidante, imunomoduladora, anti-inflamatória, antibacteriana, antifúngica, antitumoral entre outras. Além disso, sabe-se que a chalcona (1,3-difenil 2-propenona) induz a morte celular por apoptose utilizando via intrínseca em células tumorais de bexiga. Tamiello *et al*. (2013) verificaram que as nitrochalconas reduzem a viabilidade de células de hepatocarcinoma humano (HepG2) de forma mais intensa que o observado com a chalcona, contudo, os efeitos da chalcona, da 3-nitrochalcona e da 4-nitrochalcona sobre o metabolismo mitocondrial hepático não são bem conhecidos. Sabendo-se do papel central das mitocôndrias na provisão de energia celular e no mecanismo de indução de morte celular por apoptose por via intrínseca e da necessidade de busca por novas moléculas anticarcinogênicas que sejam mais efetivas, porém com maior seletividade contra células tumorais, neste trabalho foram avaliados os efeitos da chalcona (20-200 $\mu mol.L^{-1}$), da 3-nitrochalcona (20 $\mu mol.L^{-1}$) e da 4-nitrochalcona (20 $\mu mol.L^{-1}$) sobre mitocôndrias isoladas de fígado de rato. Os experimentos polarográficos utlizando a chalcona mostraram que houve redução da velocidade respiratória durante o estado 3 de ~26, ~21, ~34 and ~75% nas doses de 20, 50, 100 e 200 $\mu mol.L^{-1}$, respectivamente. Como consequência, os valores de coeficiente de controle respiratório (CCR) foram reduzidos de forma similar. A 3-nitrochalcona (20 $\mu mol.L^{-1}$) diminuiu o CCR em ~10% e a 4-nitrochalcona (20 $\mu mol.L^{-1}$) reduziu a velocidade respiratória durante o estado 3 e o CCR em ~15%. Os resultados sugerem que a adição do grupamento nitro nas posições 3 ou 4 da estrutura da chalcona diminui a ação do flavonoide sobre a respiração mitochondrial. A chalcona inibiu a atividade da NADH oxidase em ~25, ~56, ~78 and ~89%, enquanto, a inibição da succinato oxidase foi de ~30, ~47, ~57 and ~64% nas doses de 20, 50, 100 and 200 $\mu mol.L^{-1}$, respectivamente. A análise das atividades dos complexos enzimáticos demonstrou que a chalcona não inibe outras atividades de enzimas da cadeia respiratória. A chalcona inibiu discretamente a atividade da catalase (~10%), e não afetou a atividade da superóxido dismutase e nem os níveis de $H_2O_2$ durante o estado 3 e 4 da respiração mitocondrial. *In vitro* a chalcona foi capaz de sequestrar radical superóxido. Os resultados acima sugerem que a chalcona reduz a respiração mitocondrial possivelmente por afetar o transporte de elétrons na cadeia respiratória através de reações de oxido-redução diretas com seus componentes sem, contudo, alterar significativamente atividades de enzimas antioxidantes ou níveis de $H_2O_2$.



Palavras-chave: chalcona; nitrochalcona; metabolismo mitocondrial.

## ABSTRACT

Flavonoids have been used in the treatment of various diseases such as allergies, viral infections, ulcers and cancer. Within this group are chalcones, that could be found in hops and apples, and have several known biological activities such as antioxidant, immunomodulatory, anti-inflammatory, antibacterial, antifungal, antitumor, among others. Furthermore, it is known that the chalcone (1,3-diphenyl-2-propen-1-ones) induces apoptotic cell death using the intrinsic pathway of tumor cells in the bladder. Tamiello *et al*. (2013) found that nitrochalconas reduce the viability of human hepatocellular carcinoma cells (HepG2) more intense than that observed with chalcone, however, the effects of chalcone, the 3-nitrochalcona and 4-nitrochalcona on hepatic mitochondrial metabolism are not well known. Given the central role of mitochondria in the provision of cellular energy and the induction of cell death by apoptosis by the intrinsic pathway and the need to search for new anticarcinogenic molecules more effectives but with increased selectivity against tumor cells, this work evaluated the effects of chalcone (20-200 $\mu mol.L^{-1}$) of 3-nitrochalcona (20 $\mu mol.L^{-1}$) and 4-nitrochalcona (20 $\mu mol.L^{-1}$) on mitochondria isolated from rat liver. The polarographic experiments using the chalcone showed that the reduction in respiratory rate during state 3 of ~26, ~21, ~34 and ~75% at doses of 20, 50, 100 and 200 $\mu mol.L^{-1}$, respectively. Consequently, the respiratory control coefficient (RCC) were similarly reduced. The 3-nitrochalcona (20 $\mu mol.L^{-1}$) decreased the CCR in ~10% and 4-nitrochalcona (20 $\mu mol.L^{-1}$) decreased respiration rate during state 3 and CCR at ~15%. The results suggest that the addition of the nitro group in position 3 or 4 of a chalcone structure reduces the action of this flavonoid on the mitochondrial respiration. The chalcone inhibited NADH oxidase activity of ~25, ~56, ~78 and ~89%, whereas inhibition of succinate oxidase was ~30 , ~47, ~57 and ~64% at doses of 20, 50, 100 and 200 $\mu mol.L^{-1}$, respectively. The analysis of the activities of enzyme complexes showed that chalcone does not inhibit others respiratory chain enzymes. The chalcone inhibited slightly the activity of catalase (~10 %), and did not affect the activity of superoxide dismutase nor the levels of $H_2O_2$ during state 3 and 4 of mitochondrial respiration. In vitro chalcone was able to sequester superoxide. The results suggest that chalcone reduces mitochondrial respiration, possibly by affecting the transport of electrons in the respiratory chain through direct reactions with redox components, but without significantly altering antioxidant enzyme activities or levels of $H_2O_2$.



Keywords: chalcone; nitrochalcone; mitochondrial metabolism.

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## LISTA DE ABREVIATURAS E SÍMBOLOS

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**ADP/O** – quociente entre nanomol de ADP e nanoátomos de oxigênio consumidos durante o estado 3

**ADP** – adenosina 5’ difosfato

**ATP** – adenosina 5’ trifosfato

**BSA** - albumina de soro bovino

**CCR** - coeficiente de controle respiratório

**DCPIP** – 2,6-dicloro-fenol-indofenol

**DMSO** - dimetilsulfóxido

**DPH** – 1,6-difenil-1,3,5-hexatrieno

**DPH-PA** – 3-[p-(6-fenil)-1,3,5-hexatrienil]-fenil-ácido propiônico

**EDTA** - ácido etileno diamino tetracético

**EGTA** - ácido etileno glicol bis (éter 2-amino-etil) N, N, N’, N’ tetracético

**ERO** - espécies reativas de oxigênio

**FCCP** - carbonil cianeto p-trifluormetoxifenilhidrazona

**HBSS** - solução salina balanceada de Hanks

**HEPES** - N-(2-hidroxietil) piperazina N’ (ácido 2-etano sulfônico)

**NADH** - nicotinamida adenina dinucleotideo reduzida

**NOS** - NO sintase

**PMS** – fenazina metosulfato

**Tris** - (hidroximetil) amino metano

**$\Delta p$ –** força próton-motriz

**$\Delta\mu_{H+}$ –** gradiente eletroquímico de prótons

**$\Delta\Psi_m$ –** potencial de membrana mitocondrial

**ΔpH –** diferença de pH entre as fases encontradas no espaço intermembranas e a matriz mitocondrial

## ARTIGO CIENTÍFICO

**ADP -** adenosine 5’ diphosphate

**ATP -** adenosine 5’ triphosphate

**BSA -** bovine serum albumin

**Cat -** catalase

**DMSO -** dimethyl sulfoxide

**EDTA -** ethylenediaminetetraacetic acid

**EGTA -** ethylene glycol-bis(β-aminoethylether)-N,N,N’ ,N’-tetraacetic acid

**FCCP -** p-trifluoromethoxy-carbonyl cyanide phenyl hydrazone

**HEPES -** 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

**NADH -** nicotinamide adenine dinucleotide

**NBT -** nitroblue tetrazolium

**PMS -** phenazine methosulfate

**RCC -** respiratory control coefficient

**ROS -** reactive oxygen species

**SOD -** superoxide dismutase

**TRIS -** tri(hydroxymethyl)-aminomethane

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## 1 INTRODUÇÃO

Os flavonoides são compostos fenólicos encontrados em plantas que têm sido analisados como tratamento de diversas patologias como alergias, infecções virais, úlceras e câncer (PADARATZ *et al*., 2009; SIKANDER *et al*., 2011; SITHISARN et al., 2013; XU *et al.,* 2013). Dentro deste grupo de compostos encontra-se a classe das chalconas, que possuem, em sua maioria, coloração amarela e ocorrem em pigmentos de pétalas, lúpulo e maçã (SORGATO *et al*. 2007; TSAO, 2010). As chalconas possuem várias atividades biológicas conhecidas como: hipocolesterolêmica, antinociceptiva, antioxidante e antitumoral, imunomoduladora, anti-inflamatória, antibacteriana, antifúngica e antiviral (CAMPOS *et al.*, 2003; LIMING *et al*., 2004; BHAT *et al*., 2005; NOWAKAWSKA, 2007) entre outras. São conhecidos também alguns efeitos de chalconas hidroxiladas e metoxiladas sobre linhagens de células tumorais e muito pouco sobre seus efeitos no metabolismo mitocondrial (LOU *et al*., 2009; SZLISZKA *et al*., 2010; VOGEL *et al*. 2010; WINTER *et al*., 2010).

Além disso, são conhecidos efeitos da chalcona (1,3-difenil 2-propenona) na indução de morte celular por apoptose utilizando via intrínseca (SHEN et al, 2007), que envolve o metabolismo mitocondrial e por via extrínseca (SZLISZKA *et al*., 2010) em células tumorais de bexiga (T24 e HT-1376) e de próstata (LNCaP) nas doses que variam de 6 a 50 $\mu mol.L^{-1}$ As análises sobre o metabolismo mitocondrial realizadas avaliaram apenas os efeitos da chalcona sobre o consumo de oxigênio (MARTINEAU, 2012), deixando de lado análises aprofundadas sobre o mecanismo de ação deste composto, como: medidas das atividades das enzimas da cadeia respiratória e enzimas antioxidantes e, ainda, interferência sobre a geração de espécies reativas de oxigênio (ERO).

Segundo Chiaradia *et al*. (2008) a modificação estrutural de compostos isolados é um dos métodos mais utilizados para a obtenção de estruturas farmacologicamente ativas ou para a otimização da ação destes compostos. Nitrochalconas sintéticas tem sido descritas por possuírem vários efeitos biológicos importantes como: hipoglicemiante (ALBERTON *et al*., 2008; DAMAZIO *et al*., 2009) e antileishmanicida (BOECK *et al*., 2006). Recentemente, foram analisados os efeitos de nitrochalconas sobre células de hepatocarcinoma humano (HepG2) e pode-se verificar que estes compostos promovem redução da viabilidade destas células e aumento da geração de espécies reativas de

oxigênio (TAMIELLO *et al*., 2013). Contudo, não são conhecidos efeitos destas nitrochalconas ou mesmo do núcleo básico das chalconas sobre células hepáticas normais em especial sobre o metabolismo mitocondrial. O estudo destes compostos sobre mitocôndrias de células normais se justifica porque em muitos casos a interação destes xenobióticos com o metabolismo mitocondrial é fundamental para os seus efeitos biológicos. Além disso, a mitocôndria é um modelo importante por ser a organela responsável pela síntese de quase todo o ATP necessário à função celular, por estar envolvida nos processos de morte celular por apoptose (por via intrínseca), e também na geração de espécies reativas de oxigênio, parâmetros estes que podem ser modificados por chalconas (SHEN *et al*., 2007; TAMIELLO *et al*., 2013).

Outro fator importante a ser considerado é o de que uma das maiores dificuldades para obtenção de sucesso no tratamento contra o hepatocarcinoma humano (HCC) é o fato de os quimioterápicos mais utilizados serem também muito citotóxicos para células normais, o que gera muitos efeitos colaterais. Recentemente, no laboratório de Oxidações Biológicas e Cultivo Celular da UFPR, verificou-se certo grau de seletividade destes compostos (nitrochalconas versus chalcona) quando seus efeitos são comparados com células normais cultivadas de fibroblastos (L929) versus HepG2, o que tornam estas chalconas promissoras para novos estudos. A escolha do grupamento nitro foi baseada no conhecimento da importância da adição deste na estrutura de outros flavonoides para algumas atividades biológicas como a antitumoral (PATI *et al*., 2005; WINTER *et al*., 2010; TAMIELLO *et al*., 2013).

Sabendo-se a) do papel central das mitocôndrias na provisão de energia celular e no mecanismo de indução de morte celular por apoptose por via intrínseca; b) da necessidade de busca por novas moléculas anticarcinogênicas que sejam mais efetivas, e com maior seletividade contra células tumorais; e, c) os escassos relatos na literatura sobre os efeitos de chalconas sobre o metabolismo mitocondrial, o presente estudo tem como objetivo principal verificar os efeitos da chalcona, da 3-nitrochalcona e da 4-nitrochalcona sobre mitocôndrias isoladas de tecido normal (fígado de rato) com a finalidade de verificar os efeitos destes compostos sobre parâmetros respiratórios mitocondriais e sobre a geração de espécies reativas de oxigênio, além da possibilidade de realizar um estudo comparativo para verificar a importância do grupamento nitro para estes efeitos.

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 MITOCÔNDRIAS – ASPECTOS FUNCIONAIS E METABOLISMO DE XENOBIÓTICOS

As mitocôndrias são as principais organelas responsáveis pela produção de energia celular, por gerarem de 80% a 90% do ATP necessário para a sobrevivência celular durante a fosforilação oxidativa (SORGATO *et al*., 2007).

A membrana interna da mitocôndria contém enzimas do processo de fosforilação oxidativa (Figura 1), e é impermeável a pequenas moléculas e íons incluindo o $H^+$. No espaço intermembranar encontram-se proteínas especializadas, como o citocromo c, e na matriz, encontram-se enzimas que participam de diferentes vias metabólicas, como: ciclo de Krebs e β-oxidação de ácidos graxos (WINTER *et al*., 2010).

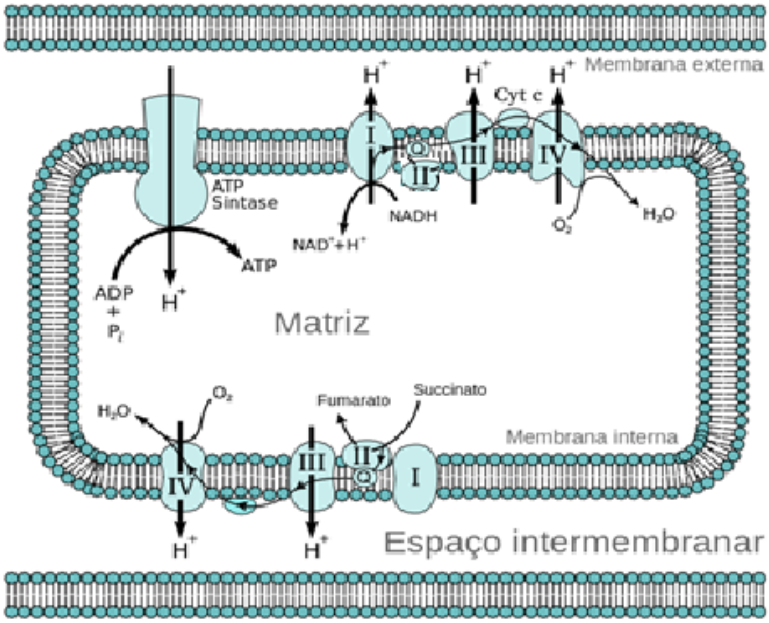



Figura 1 – Componentes da cadeia respiratória mitocondrial
Fonte: Adaptado de Vasconcellos (2007)

A energia resultante da transferência de elétrons é utilizada para o bombeamento de prótons da matriz em direção ao espaço intermembranas, gerando um gradiente eletroquímico de prótons ($\Delta\mu_{H+}$), composto pela variação de pH (ΔpH) e do potencial de membrana mitocondrial ($\Delta\Psi_m$) (NICHOLLS; FERGUSON, 2002; WILLEMSA, 2009). Os prótons retornam à matriz através da ATP sintase, tornando possível a liberação do ATP sintetizado a partir de ADP e fosfato inorgânico. Quando o vazamento de prótons pela membrana mitocondrial interna é mínimo, obtém-se o máximo acoplamento entre as

bombas de prótons da cadeia respiratória e a fosforilação do ADP (NICHOLLS; FERGUSON, 2002).

As mitocôndrias são alvos primários para uma série de xenobióticos indutores de “colapso bioenergético” devido as suas características estruturais e funcionais. Compostos químicos podem afetar o funcionamento da mitocôndria interferindo na formação do gradiente eletroquímico de prótons ($\Delta\mu_{H+}$), através da inibição de um ou mais complexos da cadeia respiratória e do desacoplamento promovido por alterações nas propriedades da membrana mitocondrial (WALLACE & STARKOV, 2000).

Existem muitos compostos que manifestam sua toxicidade por interferirem na bioenergética mitocondrial, sendo importante o esclarecimento destes efeitos para o entendimento de seus mecanismos de ação (WALLACE & STARKOV, 2000). Entre eles encontram-se alguns flavonoides, como a hispidulina (DABAGHI-BARBOSA *et al*., 2003), a quercetina e a galangina (DORTA *et al*., 2008), a eupafolina (HERRERIAS *et al*., 2008) e o núcleo básico da flavona (VALDAMERI *et al*., 2010).

## 2.2 FLAVONOIDES

Flavonoides são compostos polifenólicos biossintetizados em plantas a partir da via dos fenilpropanóides e do acetato, precursores de vários grupos de substâncias como aminoácidos alifáticos, terpenóides, ácidos graxos e outros (DORNAS *et al*., 2007).

Os flavonoides são classificados segundo seus substituintes e subdivididos em classes principalmente em: flavonas, flavonóis, chalconas, auronas, flavanonas, flavanas, antocianidinas, leucoantocianidinas, proantocianidinas, isoflavonas e neoflavonóides (TSAO, 2010).

Cerca de 9.000 flavonoides já foram identificados (HARBORNE E BAXTER, 1999). A estrutura básica de um flavonoide com anel C (Figura 2A) consiste em um núcleo fundamental, constituído de quinze átomos de carbono, contendo dois anéis fenólicos substituídos (A e B) e um pirano (cadeia heterocíclica C) acoplado ao anel A (DORNAS *et al*., 2007). O anel C pode estar presente, em flavonóides pertencentes a classe das flavonas, ou ausente (Figura 2B), como nas chalconas.

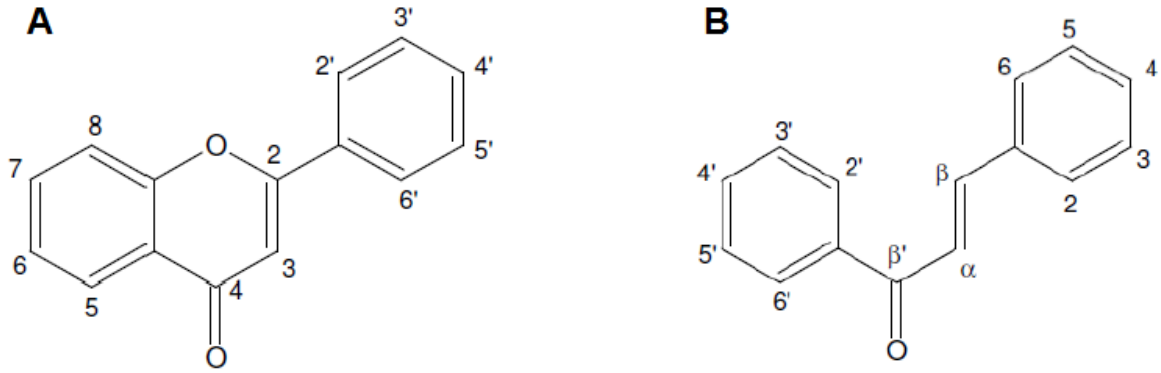



Figura 2 – A) Estrutura básica dos flavonoides com anel C. B) Estrutura básica dos flavonoides sem anel C.
Fonte: O autor (2014)

O primeiro flavonoide formado é uma chalcona (Figura 3) e todos os outros flavonoides são obtidos a partir de reações de oxidação e de redução deste intermediário (MARCANO & MASAHISA, 1991).

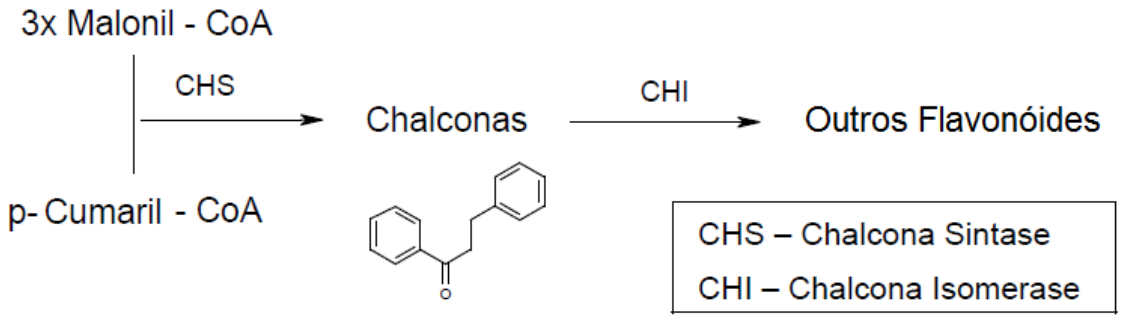



Figura 3 – Esquema da síntese das chalconas
Fonte: Adaptado de Martens e Mithofer (2005)

Durante a síntese, grupamentos hidroxil são adicionados, podendo também ser metilados, sulfatados, e/ou glicosilados (HEIN *et al*., 2002). Os flavonoides podem ser diferenciados também em dois tipos, aqueles que não possuem grupamento de açúcar ou com resíduos de açúcares ligados (PETERSON; DWYNER, 1998).

### 2.2.1 Estrutura versus Atividade Biológica

Entre as várias atividades biológicas dos flavonoides pode-se citar: antioxidante, antialergênica, anti-inflamatória, antiviral, anticarcinogênica, vasoprotetora, antimicrobiana

e protetora da função hepática (DUTHIE *et al*., 2000; MIDDLETON *et al*., 2000; DI CARLO *et al*., 1999; AFANAS'EV *et al*., 1989).

A maioria dos efeitos biológicos protetores dos flavonoides pode ser atribuída as suas habilidades seqüestradoras de radicais livres e a sua capacidade de transferir elétrons para radicais livres através da formação de radicais fenoxil de baixa reatividade (PROCHÁZKOVÁ *et al.*, 2011; CAMMERER, 2012). Além disso, alguns flavonoides podem agir como potentes quelantes de metais (DULIC *et al*., 2012), como a quercetina e o kaempferol (MACÁKOVÁ *et al*., 2012). Essas e outras habilidades dos flavonoides estão correlacionadas com a sua estrutura química.

A presença de diferentes grupamentos ocasiona também diferentes potenciais antioxidantes, provavelmente devido a uma alteração na distribuição e estabilização do *spin* no radical fenoxil do flavonoide (ARORA *et al*., 1998). Hidroxilas que podem doar elétrons e promover a deslocalização em torno do sistema aromático também influenciam na atuação dos flavonoides (DORNAS *et al*., 2007).

Contudo, os flavonoides são conhecidos também por seus efeitos pró-oxidantes importantes para algumas de suas atividades biológicas, entre elas a atividade antitumoral. Herrerias (2005) demonstrou que a eupafolina (5, 7, 3', 4'- tetrahidroxi - 6 - metoxiflavona), na dose de 100 $\mu mol.L^{-1}$ e no tempo de 24 horas, diminui em 40% a viabilidade de células HeLa (carcinoma uterino humano). Em mitocôndrias isoladas de fígado de rato tratadas com doses de 25 - 200 $\mu mol.L^{-1}$ de eupafolina verificou-se redução da atividade das enzimas entre os complexos I e III da cadeia respiratória.

Dabaghi-Barbosa (2003) observou inibição de atividade das enzimas da cadeia respiratória entre os complexos I e III quando mitocôndrias isoladas de fígado de rato foram tratadas com hispidulina (5,7,4'-tri-hidroxi-6-metoxiflavona) nas doses de 75 a 200 $\mu mol.L^{-1}$, sugerindo ação inibidora do transporte de elétrons deste composto.

Além disso, Valdameri *et al*. (2010), verificaram que o núcleo básico estrutural das flavonas é importante para variações de parâmetros do metabolismo mitocondrial, como: diminuição na velocidade respiratória no estado 3 e do potencial elétrico transmembrana.

### 2.2.2 Flavonoides na Alimentação

Os flavonoides são amplamente distribuídos em todo o reino vegetal (Tabela 1) e representam os antioxidantes mais abundantes da dieta (KHLEBNIKOV *et al*., 2007). O consumo total estimado de flavonoides varia entre 26 mg e 1 g/dia, de acordo com o

consumo de fontes específicas como vinho tinto, chá preto, cerveja, frutas (maçã, uva, morango), vegetais (cebola, couve, vagem, brócolis), grãos, nozes, sementes e especiarias (BELING *et al*., 2004). Estudos demonstraram que o consumo em longo prazo de uma dieta rica em verduras, frutas e legumes reduz o risco de doenças crônicas, especialmente câncer (BATRA *et al*., 2013). Manach *et al*. (2004) afirmam que pessoas que têm alimentação balanceada, comem várias porções de frutas e vegetais, ingerem aproximadamente 1 g/dia de flavonoides, quantidade que estaria dentro dos padrões preconizados de consumo.

Tabela 1- Fontes dos Flavonoides

| **Grupo - Flavonoides** | **Exemplos** | **Alimentos Fonte** |
|---|---|---|
| Antocianinas | Cianidina | Amora, mirtilo, groselha-preta |
| Flavanonas | Naringina, Hesperidina | Laranja, limão, toranja |
| Flavonas | Apigenina, Luteolina | Salsa, aipo, pimenta |
| Flavonóis | Quercetina, Miricetina | Cebola, maçã, alho-poró |
| Isoflavonas | Genisteína, Glicisteína | Soja |

Fonte: Adaptado de Egert *et al*. (2011)

## 2.3 CHALCONAS

As chalconas, cuja estrutura básica (1,3-difenil 2-propenona) está representada nas Figuras 2B e 4, constituem uma das maiores classes de produtos naturais, sendo encontradas em plantas rasteiras e superiores, frutos, folhas e raízes (TRISTÃO, 2008). Segundo Tsao (2010) dihidroxichalconas podem ser encontradas na maçã e prenilchalconas podem ser encontradas no lúpulo e na cerveja.

As chalconas naturais ocorrem principalmente como pigmento amarelo nas pétalas, justificando o nome (derivado do grego chalcos = bronze), que passam à cor vermelha em meio alcalino, mas também têm sido encontradas em diferentes estruturas vegetais, como em caules, raízes, folhas, frutos e sementes, podendo estar sob a forma livre ou ligada a açúcares e proteínas (OSÓRIO *et al*., 2012).

As chalconas são flavonóides de cadeia aberta, em que os dois anéis aromáticos são unidos por um sistema de três carbonos, constituindo cetonas α, β insaturadas, onde tanto a carbonila (C=O) quanto à porção olefínica (-C=C-) estão ligadas a grupamentos aromáticos (RAO & TZENG, 2004). Essas características estruturais das chalconas são importantes para vários efeitos biológicos que serão descritos a seguir.

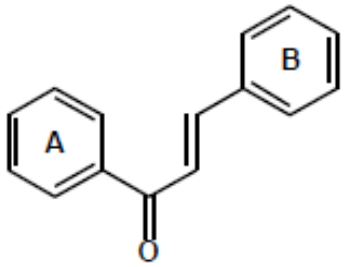



Figura 4 - Estrutura básica das chalconas
Fonte: Adaptado de Tsao (2010)

### 2.3.1 Efeitos biológicos das chalconas

As chalconas têm demonstrado atividade hipocolesterolêmica, antinociceptiva, antioxidante e antitumoral, imunomoduladora, anti-inflamatória, antibacteriana, antifúngica e antiviral (CAMPOS *et al.*, 2003; LIMING *et al*., 2004; BHAT *et al*., 2005; NOWAKAWSKA, 2007) entre outras.

Szliszka *et al*. (2010) avaliaram os efeitos da estrutura básica da chalcona (1,3-difenil 2-propenona) combinada, ou não, com o fator de necrose tumoral relacionado com indução de apoptose (TRAIL) em células tumorais de próstata (LNCaP). Quando o tratamento foi realizado apenas com a chalcona ou com TRAIL o efeito citotóxico foi menor do que quando comparado ao tratamento combinado (chalcona e TRAIL). A partir do método do MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio), foi observada uma redução de 60% da viabilidade celular após o tratamento combinado da chalcona (50 $\mu mol.L^{-1}$) com TRAIL (100 $ng.mL^{-1}$) no tempo de 48 horas.

Shen *et al*. (2007) também avaliaram os efeitos da estrutura básica da chalcona em células tumorais de bexiga (T24 e HT-1376) e os resultados demonstraram que houve inibição da proliferação pela indução da apoptose, identificada através da liberação de citocromo c e ativação das caspases 3 e 9, no tempo de 12 horas e parada do ciclo celular na fase G2/M após tratamento de 6 horas, ambas na dose de 6 $\mu mol.L^{-1}$ .

Resultados similares foram descritos por Hsu *et al*. (2005) que analisaram a ação da chalcona em células tumorais de coração (MCF-7 e MDA-MB-231) e observaram indução da apoptose por via intrínseca, por aumento da expressão de Bax e Bak e

subsequente ativação da caspase-9, no tempo de 24 horas. Também observaram parada do ciclo celular em G2/M por aumentar a expressão de p21 e p27 e reduzir níveis de ciclinas após tratamento por 12 horas, ambas na dose de 10 $\mu mol.L^{-1}$ .

Navarini *et al*. (2009) observaram que chalconas hidroxiladas nas posições 5 ou 6 do anel-A na dose de 100 $\mu mol.L^{-1}$ apresentam maior efeito citotóxico em células de melanoma B16-F10 que chalconas metoxiladas e hidroxiladas em outras posições. Os autores atribuem este efeito à indução da morte celular por apoptose, demonstrada através do aumento da fragmentação do DNA.

Rao *et al*. (2010) observaram que 2'-hidroxi-2,3,4',6'-tetrametoxichalcona (HTMC) apresenta atividade citotóxica sobre células de adenocarcinoma de pulmão (A549), diminuindo a viabilidade celular em 59% na dose de 50 $\mu mol.\ L^{-1}$ e induzindo a parada do ciclo celular na fase G1 na dose de 12,5 $\mu mol.L^{-1}$, ambas no tempo de 24 horas.

Winter *et al*. (2010) verificaram que as chalconas derivadas de 2-nafitilacetofenona (100 $\mu mol.L^{-1}$ – 24 horas) induzem a morte de células leucêmicas (L1210), por um mecanismo de ação que pode estar relacionado com a indução da morte celular pela ativação da via das caspases, danos diretos a mitocôndria ou através da indução do estresse oxidativo.

Neves *et al*. (2012) avaliaram os efeitos da 2'-hidroxichalconas sobre células de adenocarcinoma de coração (MCF-7) e constataram que este grupo de chalconas aumenta a quantidade de células em sub-G1 e de células em apoptose, verificada por marcação com Anexina, de 11,1% até 23,9% nas doses 4,1 – 10,5 $\mu mol.L^{-1}$, respectivamente.

Quanto aos efeitos sobre o metabolismo mitocondrial, Martineau (2012) verificou que a chalcona (50 $\mu mol.L^{-1}$) reduz a velocidade respiratória no estado 3 (32%) utilizando succinato como substrato oxidável. Entretanto, o autor analisou apenas velocidade respiratória durante o estado 3 e 4, somente nas doses de 25, 50 e 100 $\mu mol.L^{-1}$, e não verificou os demais parâmetros respiratórios. O autor sugere que a redução da velocidade respiratória promovida pela chalcona poderia ser consequência de uma possível inibição das enzimas da cadeia respiratória entre o complexo II e IV, porém essa hipótese não foi analisada.

O mesmo autor avaliou os efeitos das chalconas homobuteína (2',4,4'-triidroxi-3-metoxichalcona), 4'-hidroxichalcona e isoliquiritigenina (2',4',4-trihidroxichalcona) em mitocôndrias isoladas e observou que a primeira e a segunda reduziram a capacidade funcional residual ($CCR_{controle}/CCR_{tratado}$) em 80% ambas na dose de 25 $\mu mol.L^{-1}$. Já a

isoliquiritigenina apresentou atividade desacopladora, aumentou a taxa de consumo basal de oxigênio (estado 4 da respiração), em 108% na dose de 100 µmol.$L^{-1}$.

### 2.3.2 Efeitos biológicos das chalconas nitradas

A respeito das chalconas nitradas, classe a que pertencem dois flavonóides foco deste estudo, foram descritos poucos efeitos biológicos. Chiaradia *et al*. (2008) verificaram redução da produção de nitrito em macrófagos murinos da linhagem RAW 264.7 (estimulados com LPS) promovido por chalconas metoxiladas nas posições 2, 4 e 6 do anel A, tendo como substituintes do anel B um grupo nitro nas posições 3 e/ou 4, na dose de 10 µmol.$L^{-1}$ após 48 horas de tratamento. Os autores não avaliaram a ação de chalconas contendo apenas o grupamento nitro na sua estrutura, mas sugeriram que as chalconas nitradas e metoxiladas estudadas podem ser potencialmente ativas em processos inflamatórios.

Alberton *et al*. (2008) verificaram que a presença de grupamentos nitro adicionados às posições 4´ e 3´ de análogos de chalconas (adição do grupo metilenodioxila no anel B) na dose de 10 mg/Kg (administradas por gavagem) conferiram efeito hipoglicemiante em ratos hiperglicêmicos, através da redução dos níveis séricos de glicose.

Estes resultados corroboram aos encontrados por Damazio *et al*. (2009) que observaram que quando administradas chalconas com grupamentos nitro nas posições 3´ e 4´ juntamente com glucose a ratos hiperglicêmicos, a secreção de insulina aumentava (325% e 265%) em relação aos controles que recebiam somente glucose. Os autores descrevem que o efeito das chalconas nitradas em termos de indução da secreção de insulina foi 25% maior do que o da glipizida (fármaco indutor da secreção de insulina) após 30 minutos.

A 3-nitrochalcona (figura 5), que foi um dos alvos deste estudo, é um derivado sintético das chalconas, possui fórmula química $C_{15}H_{11}NO_3$ e peso molecular 235,25 g/mol. Sob temperatura ambiente é encontrada em pó com coloração amarela.

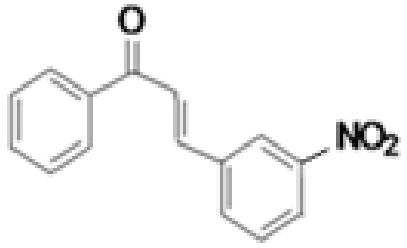



Figura 5 - Estrutura da 3-nitrochalcona
Fonte: O autor (2014)

Recentemente, Tamiello et al. (2013) verificaram que a 3-nitrochalcona aumenta a produção de espécies reativas de oxigênio (ERO), diminui os níveis de expressão de mRNA e a atividade da catalase, modifica o ciclo celular e diminui a viabilidade de células HepG2 em 56% na dose de 20 $\mu mol.L^{-1}$ no tempo de 48 horas. Porém, não são conhecidos seus efeitos sobre mitocôndrias isoladas ou mesmo sobre hepatócitos normais.

O terceiro composto utilizado neste estudo foi a 4-nitrochalcona (Figura 6), sobre a qual já foram descritas algumas atividades antitumorais. Dimmock *et al*. (2002) verificaram que após o tratamento por 24 horas com 4-nitrochalcona as células leucêmicas L1210 o $IC_{50}$ da foi de 59 $\mu mol.L^{-1}$, enquanto que, para CEM T-linfócitos o $IC_{50}$ foi de 13,8 $\mu mol.L^{-1}$.

Dalla Via *et al*. (2009) verificaram que a 4-nitrochalcona reduz a viabilidade de células de melanoma JR8 tendo sido obtido o valor de 6 $\mu mol.L^{-1}$ de $IC_{50}$ após 72 horas de tratamento. Ilango *et al*. (2010) observaram que a 4-nitrochalcona com tratamento por 24 horas diminui a viabilidade de células tumorais de coração MCF-7 e T47D tendo como $IC_{50}$ 55 e 52 $\mu mol.L^{-1}$, respectivamente.

No laboratório de Oxidações Biológicas e Cultivo Celular da UFPR verificou-se que a 4-nitrochalcona reduz aproximadamente 50% da viabilidade de células HepG2 na dose de 20 $\mu mol.L^{-1}$ no tempo de 24 horas, já em células de flibroblastos L929 não houve alteração da viabilidade nesta mesma dose e tempo de tratamento, sugerindo um efeito seletivo sobre células tumorais.

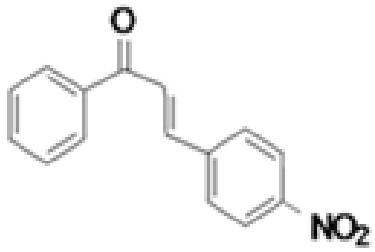



Figura 6 - Estrutura da 4-nitrochalcona
Fonte: O autor (2014)

# 3 JUSTIVICATIVA E OBJETIVOS

## 3.1 JUSTIFICATIVA

As justificativas deste trabalho estão pautadas nas seguintes considerações: a) a presença do grupo nitro nas chalconas aumenta e/ou altera algumas atividades biológicas de alguns flavonoides, b) existem relatos na literatura da indução de morte por apoptose por via intrínseca promovida pela chalcona em algumas linhagens celulares, c) há poucos estudos (chalcona) ou ausência (nitrochalconas) no modelo de mitocôndrias isoladas de fígado e d) a necessidade de conhecer os efeitos e o mecanismo de ação das chalconas em mitocôndrias de tecidos normais. Desta forma, os objetivos deste trabalho foram:

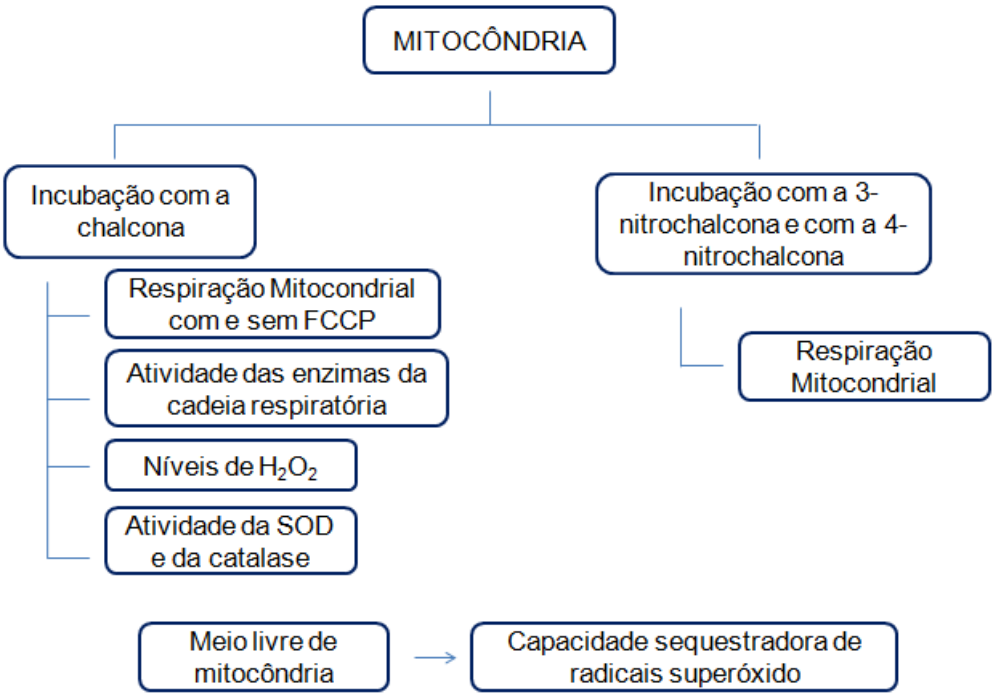
## 3.2 OBJETIVO GERAL

Avaliar e comparar os efeitos da chalcona, da 3-nitrochalcona e da 4-nitrochalcona sobre mitocôndrias isoladas de fígado de rato.

## 3.3 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos da chalcona sobre parâmetros mitocondriais de consumo de oxigênio na ausência e presença de FCCP;
- Mensurar a atividade das enzimas da cadeia respiratória e das enzimas antioxidantes superóxido dismutase e catalase na presença da chalcona;
- Quantificar os níveis de peróxido de hidrogênio na presença da chalcona;
- Avaliar os efeitos da 3-nitrochalcona e da 4-nitrochalcona sobre o consumo de oxigênio em mitocôndrias isoladas;
- Em sistemas livres de mitocôndrias avaliar a capacidade sequestradora de radicais superóxido da chalcona;
- Comparar os efeitos obtidos com a chalcona com aqueles obtidos com a 3-nitrochalcona, e 4-nitrochalcona e com os descritos na literatura para outras chalconas substituídas.

## 4 ESTRATÉGIA EXPERIMENTAL



## 5 ARTIGO CIENTÍFICO

# Effects of chalcones on the oxidative properties of isolated rat liver mitochondria

Stephane Janaina de Moura Escobar[a], Sílvia Maria Suter Correia Cadena[a], Alfredo Ricardo Marques de Oliveira[b], Maria Eliane Merlin Rocha[a]

[a]Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, PR, Brazil
[b]Department of Chemistry, Federal University of Paraná, Curitiba, PR, Brazil

ABSTRACT

Chalcones have many biological activities including anti-inflammatory, antioxidative and anticancer properties. The mechanism of action for death induction promoted by chalcones in some tumor cells involves apoptose by intrinsic pathway. Chalcone and nitrochalcones are capable to reduce cell viability in HepG2 cells, however studies are scarce about effects of these compounds on mitochondrial respiratory chain in normal cells. It is important that anticancer drugs have selectivity for tumor cells in order to reduce side effects of the treatment. The objective of this study was to investigate the effect of chalcone, 4-nitrochalcone and 3-nitrochalcone on the mitochondrial respiration parameters and its redox properties in rat liver mitochondria. The chalcone (20 – 200 µM) promoted a reduction in respiratory rate in state 3 (~20 - 30%), and RCC (~30 - 40%) when glutamate/malate were used as oxidizable substrates. Chalcone (20 – 200 µM) inhibited activities of the NADH oxidase (~20 - 90%) and succinate oxidase (~30 – 70%). The other enzymes activities of respiratory chain were not affected by chalcone. Catalase activity was reduced around ~10% in all doses tested of the chalcone. Chalcone demonstrated scavenger activity of superoxide *in vitro* (20 – 35%). Nonetheless, the addition of the nitro group in position 3 or 4 of chalcone structure reduces its effects on mitochondrial respiratory chain to ~10% in respiratory rate in state 3 with 3-nitrochalcone (20 µM) and ~15% (state 3 and RCC) with 4-nitrochalcone (20 µM). The data suggest that redox properties of the chalcone probably affect electron flux through the respiratory chain components, promoting decrease in mitochondrial respiration modifying significantly the oxygen reactive species levels.

Keywords: chalcones, nitrochalcones, mitochondria

Abbreviations: ADP, adenosine 5’ diphosphate; ATP, adenosine 5’ triphosphate; BSA, bovine serum albumin; Cat, catalase; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N’ ,N’-tetraacetic acid; FCCP, p-trifluoromethoxy-carbonyl cyanide phenyl hydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; PMS,

phenazine methosulfate; RCC, respiratory control coefficient; ROS, reactive oxygen species; SOD, superoxide dismutase; TRIS, tri(hydroxymethyl)-aminomethane.

## 5.1. Introduction

Flavonoids are widely distributed throughout the plant kingdom and represent the most abundant dietary antioxidants [1]. Chalcones are intermediate compounds in the flavonoids biosynthesis in plants that have many biological activities including anti-inflammatory [2], antioxidative [3] and anticancer [4,5] properties.

The chalcone core (1,3-diphenyl-2-propen-1-ones) (Fig. 1A) is implicated in intrinsic apoptotic pathway in bladder cancer cells (T24 e HT-1376) [6] and in extrinsic pathway in prostate tumor cells (LNCaP) [5]. Martineau [7] evaluated the effects of chalcone on oxygen consumption, although the author did not hold detailed analyzes of the mechanism of action of this compound. Despite these effects, the mechanism of action of this chalcone in mitochondrial metabolism is not well known.

Structural modification of compounds can generate pharmacologically active structures or optimize their action, and the addition of nitro group was effective in several cases [2,8,9], including as an antitumor agent [10,11,12]. However, effects of these nitrochalcones on mitochondrial metabolism are not known.

Moreover, mitochondria is an important model to be the organelle responsible for the synthesis of almost all the ATP required for cellular function, being involved in the process of cell death by apoptosis (intrinsic pathway), and also in the generation of reactive oxygen species, parameters that may be modified by chalcones [6]. Furthermore, modification of mitochondrial functions by xenobiotics may contribute to their cytotoxic and antineoplastic activities [13].

Given the central role of mitochondria in supplying cellular energy and the induction of cell death by apoptosis (intrinsic pathway), the search for new anticarcinogenic molecules that show more effective mechanism with increased selectivity against tumor cells are needed. Rare reports in the literature describe the effects of chalcones on mitochondrial metabolism, the aim of our study is to investigate the effect of chalcones (chalcone, 4-nitrochalcone and 3-nitrochalcone) (Fig. 1) on the mitochondrial metabolism and oxidative stress in rat liver mitochondria.

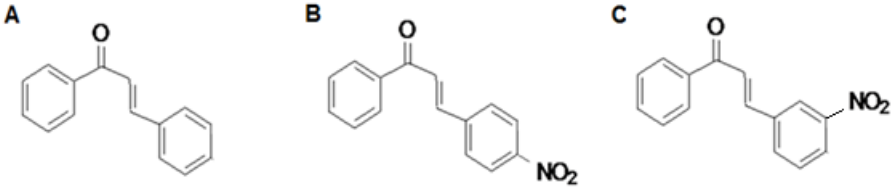



**Fig. 1** – Chemicals structures of chalcones. (A) chalcone; (B) 4-nitrochalcone; (C) 3-nitrochalcone.

## 5.2. Materials and methods

### *5.2.1. Chemicals*

Glutamic acid, succinic acid, NADH, ATP, ADP, EGTA, EDTA, FCCP, DPPH, rotenone, d-mannitol, sucrose, HEPES, BSA, phosphoenolpyruvate (PEP), pyruvate kinase, oligomycin, cytochrome c and Tris were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate,ammonium heptamolybdate and ferrous sulfate were purchased from Merck (Brazil). Other reagents were of analytical grade. Solutions were prepared with Millipore Milli-Q deionized water.

### *5.2.2. Animals*

The animals, male albino rats (Wistar strain; 220–300 g), were fed a standard laboratory diet (Nuvilab®) and water *ad libitum*. All the animals were starved for 12 h prior to being sacrificed, and none of them showed any pathological lesion. The recommendations of the Brazilian Law (No. 6.638.05, November 1979) for the scientific management of animals were followed.

### *5.2.3. Preparation of chalcones solutions*

Chalcone and 3-nitrochalcone were synthesized by Dr. Alfredo Ricardo Marques de Oliveira Department of Chemistry, UFPR, and kindly provided for this study. The 4-nitrochalcone was purchased from Alfa Aeser. All chalcones were dissolved in DMSO and then further diluted with the assay medium. Solvent controls with DMSO were carried out in each assay. Chalcones stock solution was stored at −20°C and warmed to 25°C before use.

*5.2.4. Preparation of rat liver mitochondria*

Rat liver mitochondria were isolated by differential centrifugation, following the procedure described by Voss et al. [14]. The extraction medium contained D-mannitol (210 mM), sucrose (70 mM), Hepes buffer (10mM) (pH 7.4), EGTA (0.5 mM), and BSA (0.05 g%). Rat liver was homogenized in extraction medium in a Potter–Elvehjem homogenizer and the suspension centrifuged at 2500×g for 5 min. The supernatant solution was then centrifuged at 12,500×g for 10 min, after which the sediment was washed twice with the same medium at 10,000×g for 10 min. The final suspension was prepared by adding the original medium to the sediment to obtain a final protein concentration of 20–100 mg/mL. To evaluate the enzyme complexes of the respiratory chain, mitochondrial suspensions were frozen in liquid $N_2$, and immediately prior to their use, each aliquot was disrupted by a freeze/thaw treatment (×3) and maintained at 4°C during the enzymatic tests.

*5.2.5. Mitochondrial respiration*

Highly sensitive Oroboros Oxygraphy-2K (Oroboros, Innsbruck, Austria) was used to determine mitochondrial respiration at 28°C. A reaction medium containing D-mannitol (125 mM), KCl (65 mM), HEPES–KOH buffer (pH 7.2) (10 mM), EGTA (0.1 mM), and BSA (0.1 g%) was supplemented with Pi (0.8 mM), ADP (0.16mM) and mitochondrial protein (1 mg/mL) when sodium glutamate (5mM) and malate (0,5mM) were used as substrate. Chalcone in varying concentrations (20–200μM) was added to each system. The respiration rate is expressed as pmol of oxygen consumed per second per milligram of mitochondrial protein. The ADP:O ratio was calculated according to Chance and Williams [15]. RCC values were obtained as the ratio of the rate of mitochondrial oxygen consumed in state 3 to that consumed in state 4. To induce the mitochondrial uncoupling was utilized FCCP 1 μM. In the other experiments were used various substrates and inhibitors for the mitochondrial ETC complexes. Glutamate (10 mM) + malate (5 mM), succinate (10 mM), or ascorbate (5 mM) + TMPD (0.5 mM) were used as substrates for Complex I, II/III, or IV, respectively. Rotenone (1 μM), antimycin A (1 μg/ml), or potassium cyanide (0.25 mM) was used as inhibitor for Complex I, III, or IV, respectively.

*5.2.6. Measurement of respiratory chain enzyme activities*

NADH oxidase and NADH dehydrogenase (NADH:ubiquinone oxidoreductase) were assayed spectrophotometrically, as described by Singer [16]. NADH cytochrome c reductase (NADH: cytochrome c oxidoreductase) activity was measured by reduction of cytochrome c at 550 nm, as described by Somlo [17], while cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase) activity was measured at 550 nm, according to Mason et al. [18]. NADH, succinate and cytochrome c oxidases were assayed polarographically in Oxygraphy Oroboros. ATPase activity in disrupted mitochondria was evaluated as described by Pullman et al. [19], but with modifications. The ATPase activity in disrupted mitochondria was assayed at 28°C, using a medium consisting of sucrose (250 mM), Tris–HCl buffer (50mM)(pH 8.4), magnesium sulfate (3.0 mM), PEP (2.5 mM), pyruvate kinase (10 U), ATP (4mM) and mitochondrial protein (100µg). The results are expressed as nmol of Pi released per min per mg.

*5.2.7. Superoxide dismutase (SOD)*

SOD activity was evaluated by the decrease in absorbance at 480 nm as proposed by Misra and Fridovish [20]. The reaction system consisted of buffer 50 mM sodium carbonate (pH 10.2), 0,1 mM EDTA, 1 mM epinephrine and protein at 30°C. Results were expressed as U of SOD, where 1U corresponds to the amount required to inhibit 50% of the autoxidation of epinephrine.

*5.2.8. Catalase (Cat)*

Catalase activity was evaluated by the decrease in absorbance at 240 nm as proposed by Aebi [21]. The reaction system consisted of buffer 50 mM phosphate (pH 7.0), 10 mM $H_2O_2$ and 500 mg protein.$mL^{-1}$ at 25°C. Results were expressed as U of catalase, where 1U corresponds to 1 µM of $H_2O_2$ decomposed (mg protein per min) considering the molar extinction coefficient $H_2O_2$ 0.394 $mM^{-1}.cm^{-1}$).

*5.2.9. Mitocondrial $H_2O_2$ release*

Mitochondrial $H_2O_2$ release was measured with Amplex®Red probe as proposed by Tahara et al. [22]. The reaction system consisted in 0.125 mg protein/ml mitochondrial suspensions in experimental buffer 125 mM sucrose and 65 mM KCl, 10 mM Hepes, 2 mM inorganic phosphate, 2 mM $MgCl_2$, and 0.01% bovine serum albumin, adjusted to pH 7.2 with KOH, at 28°C, with continuous stirring.

*5.2.10. Superoxide anion scavenging activity*

The scavenging ability of superoxide radical was assessed by the method described by Nishimiki et al. [23], with some modifications. The reaction mixture consisted of 10mM Tris–HCl buffer pH 8.0, 338 µM NADH, 72 µM NBT and 30 µM PMS. The reaction was monitored at 560 nm and the scavenging ability of superoxide radicals was calculated using the following equation 1 [24]:

$$\text{scavenging effect}\,(\%) = 1 - \left(\frac{\text{Abs sample}}{\text{Abs control}}\right) \times 100 \quad (1)$$

*5.2.11. Protein determination*

Mitochondrial protein was assayed by the method of Lowry et al. [25] calibrated with bovine serum albumin.

*5.2.12. Statistical analysis*

Data are presented as means ± SD. Statistical analysis of the data was carried out as analysis of variance, linear regression and Tukey test for average comparison. Results were considered significant when $p < 0.05$.

## 5.3. Results

### *5.3.1. Effects of chalcone on mitochondrial respiration*

The rates of oxygen consumption, after an addition of ADP (state 3) and after its exhaustion (state 4) in the presence of chalcone (20–200 µM), and the respiratory control coefficient (RCC) as well as the ADP/O ratio were measured. The effects of chalcone on rat liver mitochondria respiration are shown in Table 1. The addition of chalcone caused a decrease in respiration in presence of ADP (state 3) of ~26, ~21, ~34 and ~75% at doses of 20, 50, 100 and 200 µM, respectively. Oxygen consumption rate after ADP exhaustion (state 4) and ADP/O ratio have not been altered with the addition of chalcone. In presence of 200 µM, mitochondrial respiration did not return to state 4 after ADP phosphorylation (data not shown). As a result of the inhibitory effect of chalcone on state 3, the respiratory control coefficient (RCC) values decreased similarly. These data suggest inhibitory effect of chalcone on the respiratory chain.

**Table 1**
Effects of chalcone on oxygen consumption using the glutamate/malate as oxidizable substrate

| Chalcone ($\mu mol.L^{-1}$) | State 3 (%) | State 4 (%) | RCC (%) | ADP/O (%) |
|---|---|---|---|---|
| 0 | 100 ± 10.65 | 100 ± 9.78 | 100 ± 2.43 | 100 ± 4.6 |
| 20 | 73.9 ± 15.58* | 82.9 ± 16.72 | 85.9 ± 1.62* | 108 ± 4.5 |
| 50 | 79.6 ± 16.08* | 78.6 ± 21.24 | 76.7 ± 7.39* | 107.6 ± 9.3 |
| 100 | 66.6 ± 15.01* | 97.2 ± 16.5 | 63.9 ± 9.68* | 100.9 ± 7.7 |
| 200 | 24.7 ± 6.3* | - | - | - |

Experimental conditions are described in Section 5.2. DMSO control (100%) values obtained were: 291.4 ± 19 pmols $O_2$ consumed.$min.^{-1}mg^{-1}$ protein in state 3; 115.1 ± 9 pmols $O_2$ consumed.$min.^{-1}mg^{-1}$ protein in state 4; 2.9 ± 0.2 (nmols ADP/nanoatoms $O_2$ consumed) ADP/O ratio and RCC 2.8 ± 0.2. Each value represents the means of three different experiments was expressed as percentage of control.
* Significantly different from the control (100%), $p < 0.05$.

### *5.3.2. Effects of nitrochalcones on mitochondrial respiration*

The effects of nitrochalcones on rat liver mitochondria respiration are shown in Table 2. The RCC was reduced of ~9% in the presence of 20 µM of 3-nitrochalcone. However, the respiratory rate in state 3, state 4 and ADP/O ratio have not been altered. The respiratory rate in state 3 and RCC were decreased of ~14% in the presence of 20 µM of 4-nitrochalcone. Oxygen consumption rate in state 4 and ADP/O ratio have not been

altered with the addition of 4-nitrochalcone. These data suggest that the addition of the nitro group in chalcone structure reduces the inhibitory effect of the mitochondrial respiration.

**Table 2**
Effects of nitrochalcones on oxygen comsuption using the glutamate/malate as oxidizable substrate

| Nitrochalcones (µmol.L$^{-1}$) | | State 3 (%) | State 4 (%) | RCC (%) | ADP/O (%) |
|---|---|---|---|---|---|
| | 0 | 100 ± 7.5 | 100 ± 5.3 | 100 ± 2.7 | 100 ± 3.1 |
| 3 - nitrochalcone | 20 | 96.7 ± 8.2 | 98 ± 4.6 | 91.2 ± 1.4* | 100.7 ± 4.6 |
| 4 - nitrochalcone | 20 | 85.8 ± 5.6* | 92.3 ± 4 | 89.2 ± 1.5* | 99.6 ± 4.4 |

Experimental conditions are described in Section 5.2. DMSO control (100%) values obtained were: 427.5 ± 30 pmols $O_2$ consumed.min.$^{-1}$mg$^{-1}$ protein in state 3; 136.5 ± 7.3 pmols $O_2$ consumed.min.$^{-1}$mg$^{-1}$ protein in state 4; 2.8 ± 0.1 (nmols ADP/nanoatoms $O_2$ consumed) ADP/O ratio and RCC 3.3 ± 0.1. Each value represents the means of three different experiments was expressed as percentage of control.
* Significantly different from the control (100%), $p < 0.05$.

### *5.3.3. Chalcone effects on enzyme activities*

The inhibition promoted by chalcone in mitochondrial respiration motivates us to study its effects in enzyme activities of the complexes in the respiratory chain. The effects of chalcone on respiratory chain enzyme activities are shown in Table 3. It promoted inhibition in a dose-dependent manner of NADH oxidase. At the lowest concentration (20 µM), the inhibition was 23% and at the highest concentration (200 µM) the inhibition was 84%. However, NADH- dehydrogenase, NADH- cytochrome *c* redutase and Cytochrome *c* oxidase activities have not been changed. To confirm the inhibition of oxidases, assays were performed in oxygraphy (Table 4). NADH oxidase was inhibited of ~25, ~56, ~78 and ~89% at doses of 20, 50, 100 and 200 µM, respectively. Succinate oxidase was inhibited of ~30, ~47, ~57 and ~64% at doses of 20, 50, 100 and 200 µM, respectively. The results of NADH oxidase and cytochome c oxidase activity were similar in both methodologies.

**Table 3**
Effects of chalcone on respiratory chain enzyme activities

| Chalcone ($\mu mol.L^{-1}$) | NADH oxidase | NADH- dehydrogenase | NADH- cytochrome *c* reductase | Cytochrome *c* oxidase |
|---|---|---|---|---|
| 0 | 100 ± 5.4 | 100 ± 2.9 | 100 ± 8.4 | 100 ± 4.3 |
| 20 | 76.8 ± 6.3* | 98.2 ± 5.1 | 98.1 ± 9.8 | 98.2 ± 8.6 |
| 50 | 52.6 ± 3.5* | 98.4 ± 5.2 | 96.3 ± 8 | 99.2 ± 7.6 |
| 100 | 33.5 ± 1.5* | 100.8 ± 2.5 | 102.7 ± 8.5 | 100.6 ± 12 |
| 200 | 16.6 ± 3.7* | 100.2 ± 5.2 | 98.1 ± 6.3 | 95.2 ± 8.6 |

Experimental conditions are described in Section 5.2. DMSO control (100%) values obtained were: NADH oxidase: 44.6 ± 7.9 nmol of NADH oxidized $min^{-1}$ $mg^{-1}$ of mitochondrial protein; NADH citochrome c reductase: 401.1 ± 19 nmol of cytochrome c reduced. $min^{-1}$ $mg^{-1}$ of mitochondrial protein; NADHdehydrogenase: 1.6 ± 0.5 µmol of ferricyanide reduced $min^{-1}$ $mg^{-1}$ of mitochondrial protein; cytochrome c oxidase: 348.5 ± 48 nmol cytochrome c oxidized $min^{-1}$ $mg^{-1}$ of mitochondrial protein. Results (means of three independent experiments in triplicate) are expressed as percentage of control activities.
* Significantly different from the control (100%), $p < 0.05$.

**Table 4**
Effects of chalcone on respiratory chain enzyme verified polarographically

| Chalcone ($\mu mol.L^{-1}$) | NADH Oxidase | Succinato Oxidase | Cytochrome c oxidase |
|---|---|---|---|
| 0 | 100 ± 2.3 | 100 ± 7.7 | 100 ± 4.8 |
| 20 | 76.4 ± 3.7* | 69.6 ± 7.0* | 98.2 ± 3.4 |
| 50 | 43.8 ± 1.6* | 52.6 ± 5.1* | 97.6 ± 4.6 |
| 100 | 21.5 ± 1.0* | 42.3 ± 3.4* | 97 ± 3.8 |
| 200 | 10.6 ± 0.7* | 35.4 ± 4.8* | 98.8 ± 7.3 |

Experimental conditions are described in Section 5.2. DMSO control (100%) values obtained were: NADH oxidase: 17.1 ± 1.2 nmol of $O_2$ consumed $min^{-1}$ $mg^{-1}$ of mitochondrial protein; succinate oxidase: 29.7 ± 4.2 nmol of $O_2$ consumed $min^{-1}$ $mg^{-1}$ of mitochondrial protein; cytochome c oxidase: 31.4 ± 10 nmol of $O_2$ consumed $min^{-1}$ $mg^{-1}$ of mitochondrial protein. Results (means of three independent experiments in triplicate) are expressed as percentage of control activities.
* Significantly different from the control (100%), $p < 0.05$.

In order to confirm the spectrophotometric results in enzymes activities, the effects of chalcone on respiratory chain were evaluated using polarografic analyses with additions of complex specific substrates and inhibitors in the same experiment. In Fig. 2A is possible to verify the decline of oxygen concentration (blue curves) and oxygen consumption in response to substrates for Complex I (glutamate + malate), Complexes II and III (succinate), or Complex IV (ascorbate + TMPD) (red curves). Oxygen consumption in various mitochondrial enzymatic complexes was evaluated by the addition of complex-specific inhibitors (rotenone for Complex I, antimycin A for Complex III, or potassium

cyanide for Complex IV). As shown in Fig.2 A and B, chalcone did not affect significantly oxygen consumption in mitochondrial enzymatic complexes when compared to control (with DMSO). These results are in concordance with the data of the Table 3 and suggest that only when NADH or succinate oxidases (total respiratory chain enzymatic activities) are added it is possible to verify reduction in oxygen consumption.

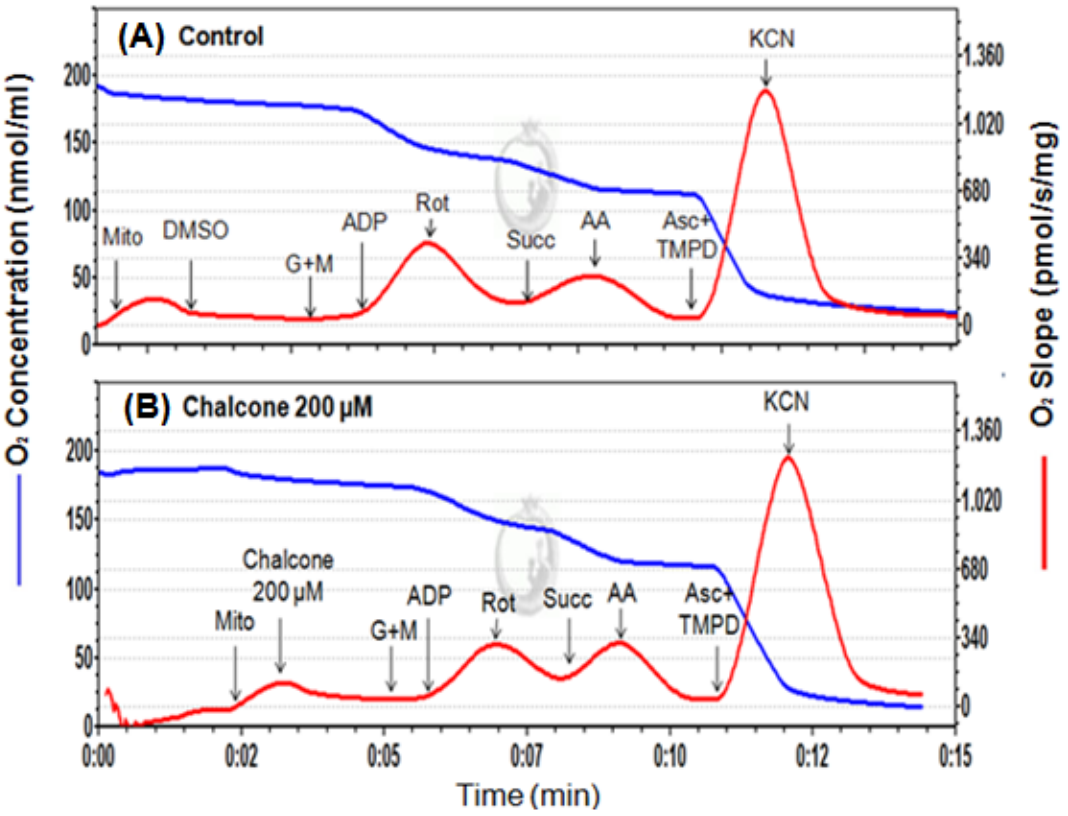



**Fig. 2**. Representative profiles of oxygen consumption in mitochondria treated with vehicle or chalcone. Oxygen consumption was assessed in mitochondria treated with vehicle DMSO (control) (A) or chalcone 200 µM (B) using oxygraphy as described in Section 5.2. Mito, mitochondria; G, glutamate; M, malate; ADP, adenosine diphosphate; Rot, rotenone; Succ, succinate; AA, antimycin A; Asc, ascorbate; TMPD, N.N.N′.N′-tetramethyl-p-phenylenediamine dihydrochloride; KCN, potassium cyanide. Blue line is the decline of oxygen concentration in chamber of oxygraphy. Red line is the oxygen consumption in response to substrates for Complexes I–IV.

In order to confirm the inhibitory effects of chalcone (200 µM) in state 3, it was added during that state of the respiration (Fig. 3B). It was possible to observe that oxygen consumption decreases (Fig. 3B) instantly. It may suggest, at first, a possible effect on ATPase activity. The other minor doses of chalcone did not show effect (data not shown).

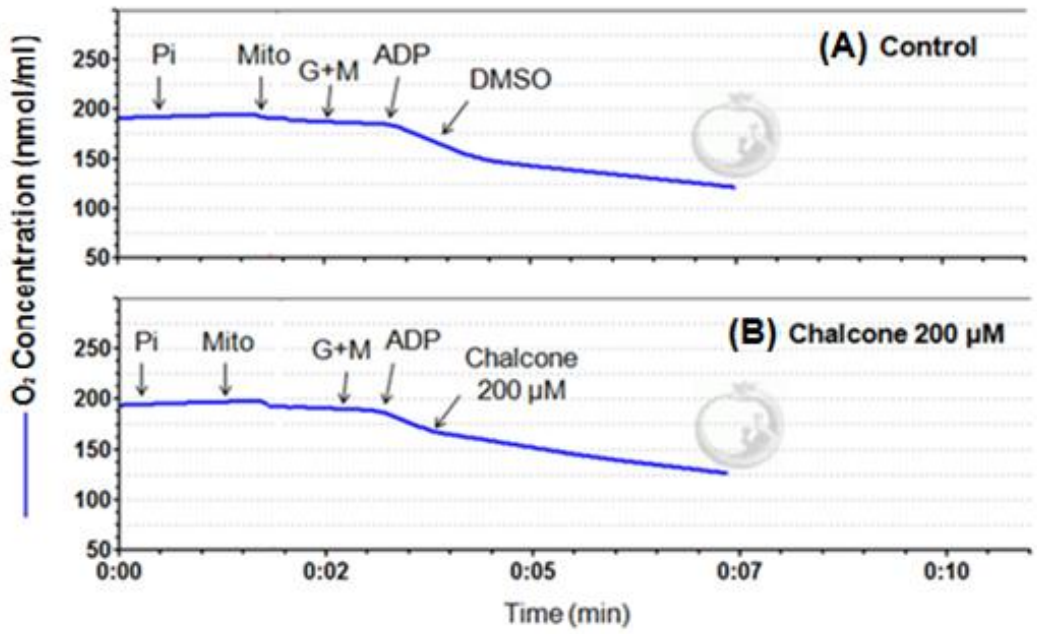



**Fig. 3**. Representative profiles of oxygen consumption in mitochondria treated with vehicle (A), or chalcone (B). Oxygen consumption was assessed in mitochondria treated with (control) vehicle or (chalcone) chalcone 200 µM using oxygraphy as described in Section 5.2. Pi, phosphate; Mito, mitochondria; G, glutamate; M, malate; ADP, adenosine diphosphate. Blue line, decline of oxygen concentration in chamber of oxygraphy.

This hypothesis was analyzed, but the ATPase activity using disrupted mitochondria was not affected by chalcone (Table 5). ATPase activity was measured by two methodologies (Pi liberation and NADH oxidation). When ATPase was verified by NADH oxidation system, the results were similar (date not shown) at Table 5. These data suggest that chalcone reduces oxygen consumption quickly but it was not possible to verify in what complex in respiratory chain occurs the inhibition by the used methodologies.

**Table 5**
Effects of chalcone on ATPase activity in disrupted mitochondria

| Chalcone ($\mu mol.L^{-1}$) | ATPase activity |
|---|---|
| 0 | 100 ± 4.3 |
| 20 | 96.8 ± 12 |
| 50 | 94.8 ± 3.8 |
| 100 | 93.7 ± 2.5 |
| 200 | 94.8 ± 3.9 |

Experimental conditions are described in Section 5.2. DMSO control (100%) value obtained was 31.8 ± 4.7 µmol of Pi liberated. Results (means of three independent experiments in triplicate) are expressed as percentage of control activities.
* Significantly different from the control (100%), $p < 0.05$.

*5.3.4. Effects of chalcone on mitochondrial respiration with FCCP*

To confirm the chalcone effects on the respiratory chain, the measure oxygen consumption was performed in the presence of uncoupling FCCP. Oxygen consumption decreased (~30%) only at a dose of 200 µM of chalcone. These results suggest that the mechanism of action of the chalcone is involved with the reduction of electron transport in the respiratory chain and this effect is more intense in presence at 200 µM of chalcone.

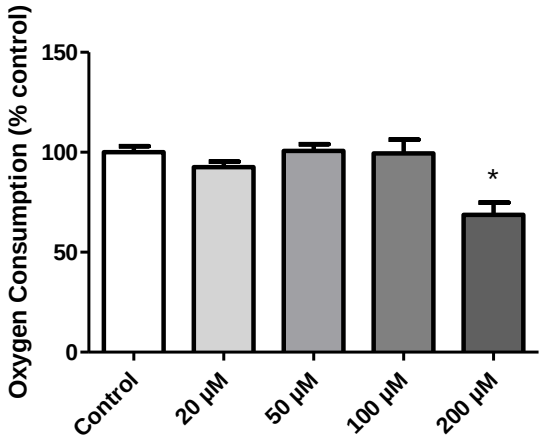



**Fig. 4** – Mitochondrial respiratory with FCCP. Experimental conditions are described in Section 5.2. DMSO control (100%) value obtained was 305.6 ± 72 pmols $O_2$ consumed.min.$^{-1}$mg$^{-1}$ protein. Each value represents the means of three different experiments was expressed as percentage of control.
* Significantly different from the control (100%), $p < 0.05$.

*5.3.5. Effects of chalcone on $H_2O_2$ release, SOD and catalase activity*

According to Guzy et al. [13] the toxic effects of chalcones might be due to their pro-oxidant activities. To test this possibility, we investigated the effects of chalcone on activities of the mitochondrial antioxidant enzymes, SOD and catalase and ROS generation using Amplex®Red probe. SOD activity (Table 6) and $H_2O_2$ concentration during the state 3 and 4 respiration were not affected (Fig. 5). State 3 respiration was induced by adding ADP and state 4 by the addition of oligomycin. These analyses were important to evaluate whether chalcone modified the levels of $H_2O_2$ during oxidative phosphorylation (state 3), which could mean inhibition of enzymes during the basal activity of respiratory chain (state 4), which could mean uncoupling. Catalase activity was inhibited ~10% in all the doses of chalcone (Table 6).

**Table 6**
Effects of chalcone on antioxidant enzymes activity

| Chalcone ($\mu mol.L^{-1}$) | SOD | Catalase |
|---|---|---|
| 0 | 100 ± 9.1 | 100 ± 2.7 |
| 20 | 123.5 ± 10 | 93.6 ± 6* |
| 50 | 99.8 ± 15 | 87.5 ± 6.8* |
| 100 | 105.7 ± 19 | 90.5 ± 4.2* |
| 200 | 90.5 ± 20 | 88.7 ± 3.6* |

Experimental conditions are described in Section 5.2. DMSO controls (100%) values obtained were: catalase 2.97 ± 0.5 unid catalase.$min^{-1}$.$mg^{-1}$ mitochondrial protein; SOD 11 ± 2 unid SOD.$min^{-1}$.$mg^{-1}$ mitochondrial protein. Each value represents the means of three different experiments was expressed as percentage of control.
* Significantly different from the control (100%), $p < 0.05$.

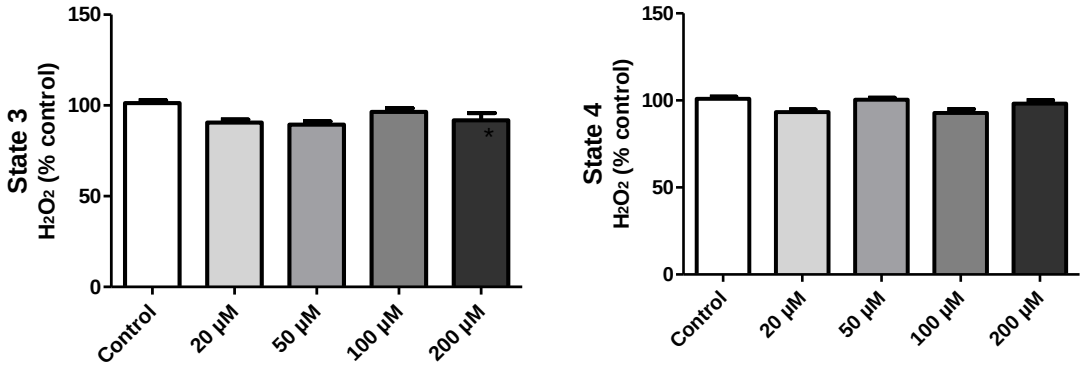



**Fig. 5** – Effects of chalcone in state-dependent $H_2O_2$ release. Experimental conditions are described in Section 5.2. DMSO controls (100%) values obtained were: 60 ± 6 µM $H_2O_2$ consumed.$min^{-1}$.$mg^{-1}$ mitochondrial protein in state 3; 54 ± 7 µM $H_2O_2$ consumed.$min^{-1}$.$mg^{-1}$ mitochondrial protein in state 4. Each value represents the means of three different experiments was expressed as percentage of control.

To evaluate if the chalcone has *in vitro* scavenging ability, specifically in the case of the superoxide radical, assays were performed in a system where PMS/NADH reduces NBT in the presence of this radical [23]. Compounds that are able to scavenge superoxide radicals decreased NBT reduction and the results are shown in Fig. 6. The ability of chalcone to scavenge $O_2^{\bullet -}$ was to ~20% at 20 and 50 µM and to ~35% at 100 and 200 µM.

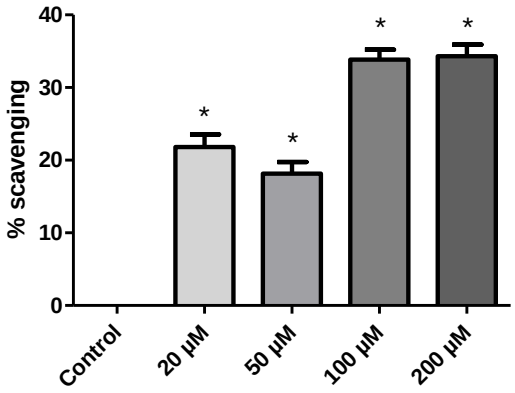



**Fig. 6.** Scavenging effect of chalcone on superoxide radical production. Experimental conditions are described in Section 5.2. The results are expressed as percentage of trapped superoxide radical in relation to the control (absence of derivatives). Each value represents the mean ± SD (n = 9).
* Significantly different from the control (100%), $p < 0.05$.

## 4. Discussion

Chalcones are flavonoids that have many biological properties including anticancer activity. Previous results of our laboratory verified that chalcone and nitrochalcones are capable to reduce cell viability in HepG2 cells with different magnitude that observed in L929 cells. The selectivity for tumor cells is important to reduce the side effects in cancer treatment. The objective of this study was to investigate the effect of chalcone and two nitrochalcones (4-nitrochalcone and 3-nitrochalcone) on the parameters related to oxygen consumption and redox properties in rat liver mitochondria.

It was verified that chalcone promoted inhibition in the rate of oxygen consumption at state 3, using glutamate as oxidizable substrate, and decreased RCC (Table 1). These results agree with the obtained by Martineau [7] that used the chalcone (50 µM) and verified inhibition of 32% in state 3, when succinate was used as a substrate, we observed a reduction of 21% at the same dose with glutamate/malate as substrates. In addition, other authors verified that other class of flavonoids (eg.: flavones) promoted inhibition of state 3 of respiration [26,27,28]. The decrease in oxygen consumption at state 3 may be an indicative of inhibition of mitochondrial electron transfer in respiratory chain [13].

The addition of the nitro group in chalcone reduced the activity of the compounds in mitochondrial respiration (Table 2), probably because this group modifies the polarity of the molecule and hampers its passage through the mitochondrial membrane. Another point is that the α,β - unsaturated bond confers electrophilic capacity to chalcone [29] what is important to some biological activities of this flavonoid. It is possible that the addition of

the nitro group in chalcone core structure could reduce this capacity. These results differ from results obtained by other authors with tumor cells, where the addition of the nitro group increased the antimumor activity of flavonoids [30,31], what may suggest that the mechanism of action of these compounds is not by mitochondrial pathway. Nitrochalcones promotes greater decrease of HepG2 cells viability when compared to chalcone in same conditions. This fact may be favorable for selectivity of the compound by tumor cells and consequently, reduction of side effects.

The chalcone did not change the activity of the segments NADH desidrogenase, cytochrome c oxidase, NADH cytochrome c reductase of the mitochondrial respiratory chain, when analyzed spectrofotometrically. However, enzymes activities of NADH and succinate oxidases were reduced (Table 3 and 4). The activities of the enzymes NADH oxidase and cytochrome c oxidase were similarly affected in both analysis types, polarografically and spectrophotometrically, and these results discard the hypothesis that the differences are related with the methodology sensitivity. Results exhibit in Fig. 2 confirm that chalcone does not reduce the oxygen consumption of the mitochondrial respiratory chain when inhibitors are used to analyze different complexes. The decrease of respiration promoted by chalcone (200 µM) obtained in presence of FCCP (Fig. 4) suggests that this effect is due to an inhibition in electron transport.

ATPase inhibition could be suspected because several flavonoids are inhibitors of ATPase [32,33]. Moreover, Fig. 3 shows that the chalcone at 200 µM promotes similar oligomycin effect, inducing state 4 when added during state 3. However, the chalcone did not modify the ATPase activity (Table 5) in disrupted mitochondria in all doses tested.

Respiratory chain inhibition activity of several flavonoids has been frequently associated to the oxidation potentials of these compounds [26]. In the literature was described that chalcone suffers oxidation and reduction when submitted to an electrochemical process occurring at the glassy carbon electrode (working electrode) using platinum wire as counter electrode and Ag/AgCl as a reference electrode [34]. These analyses by cyclic voltammetry, demonstrated that chalcone was oxidized between pH 3.0 and 11.2 in phosphate buffer, producing two well-defined oxidation peaks and one reduction peak [34]. It was observed two anodic peaks at about 0.541 V and 1.478 V and cathodic peak at about -0.689 V in pH 3.0 phosphate buffer using Ag/AgCl as a reference electrode. The authors proposed mechanisms of electrooxidation of chalcone. Now our hypothesis is that the reduction of oxygen consumption observed in this work could be related to the redox properties of chalcone. This hypothesis is based in fact of

electrochemical analyses demonstrated that chalcone undergoes oxidation in +0.514 V, if this value were converted to standard hydrogen electrod, the value is +0.316 V (SHE). These data suggest a possible direct interference in transport of electrons in respiratory chain, what could explain why enzymatic activities inhibition in mitochondrial respiratory chain was not observed except when NADH or succinate oxidases were analyzed.

Other hypothesis is that chalcone effects are consequence of the interaction of this flavonoid with the mitochondrial membrane. This fact may contribute to alterations in membrane properties as fluidity and consequently interferes in electron flow and with the formation of the electrochemical proton gradient ($\Delta\mu_{H+}$). Further studies are necessary to clarify the causes of this chalcone effects in mitochondria function, as well as to demonstrate if the sequestering and/or donating electrons occurs directly to component of the complexes in respiratory chain.

Despite the inhibition in oxygen consumption rate at state 3 the hydrogen peroxide levels did not increase and this result may be related to the scavenger activity ($O_2^{\bullet-}$) of chalcone observed *in vitro* (Fig. 5). Some flavonoids, due to their structural aspects, appear to be efficient radical scavengers [35]. Another factor to be considered is that, in mitochondria isolated from rat liver had lower variations in the rates of reactive oxygen species during state 3 when traditional inhibitors of chain were added [22].

The catalase activity was reduced (Table 6) however, the presence of catalase in mitochondria is controversial. It is not clear if this enzyme is an intrinsic component of the mitochondrial antioxidant system or if its presence results from a contamination by peroxisomes [36].

In conclusion, the results suggest that the chalcone is able to decrease oxygen consumption probably because this flavonoid reduces the electron transport, and may act directly in components of the respiratory chain, promoting its oxidation and/or reduction. It was also observed that the addition of the nitro group in position 3 or 4 of chalcone structure reduces the action of this flavonoid in mitochondrial respiration.

### 5.5. Acknowledgments

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## 6 CONCLUSÕES

- A chalcona promove redução da velocidade respiratória durante o estado 3 e do coeficiente de controle respiratório (CCR) nas doses de 20 - 200 µmol.$L^{-1}$, quando se utilizou glutamato/malato como substrato oxidável, porém, não altera a velocidade respiratória durante o estado 4 e a razão ADP/O;
- Os resultados com as chalconas nitradas (20 - 50 µmol.$L^{-1}$) sugerem que a adição do grupamento nitro nas posições 3 ou 4 da estrutura da chalcona diminui a ação do flavonoide sobre a cadeia respiratória mitochondrial;
- A chalcona inibe a atividade da NADH e succinato oxidase de maneira dependente da dose, porém, não altera as demais atividades das enzimas da cadeia respiratória mitocondrial;
- A chalcona inibe discretamente a atividade da catalase, não modifica a atividade da superóxido dismutase e não altera os níveis de $H_2O_2$ durante o estado 3 e 4 da respiração mitocondrial, contudo, possui atividade scavenger de radical superóxido em sistema *in vitro*;
- Os resultados observados sugerem que a chalcona, por suas propriedades redox, é capaz de reduzir a respiração mitocondrial, possivelmente por doar e/ou receber elétrons, interferindo assim diretamente no fluxo destes através dos componentes da cadeia respiratória sem, porém, afetar significativamente os níveis de ERO.

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