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ATIVIDADE HEPATOPROTETORA DO GAMA ORIZANOL EM MODELOS DE HEPATITE FULMINANTE AGUDA EM CAMUNDONGOS

DISSERTAÇÃO DE MESTRADO

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Itaqui, RS, Brasil.
2015

ATIVIDADE HEPATOPROTETORA DO GAMA ORIZANOL EM MODELOS DE HEPATITE FULMINANTE AGUDA EM CAMUNDONGOS

por

Marcelo Gomes de Gomes

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ATIVIDADE HEPATOPROTETORA DO GAMA ORIZANOL EM MODELOS DE HEPATITE FULMINANTE AGUDA EM CAMUNDONGOS

elaborada por
Marcelo Gomes de Gomes

Como requisito parcial para obtenção do grau de
Mestre em Bioquímica

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DEDICO

Aos meus familiares, tios e avós, mas
em especial aos meu pais
pelo amor e apoio incondicional
sempre quando eu precisei
nas ocasiões apresentadas
pela vida.

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RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa

ATIVIDADE HEPATOPROTETORA DO GAMA ORIZANOL EM MODELOS DE HEPATITE FULMINANTE AGUDA EM CAMUNDONGOS

Autor: Marcelo Gomes de Gomes

Orientador: Cristiano Ricardo Jesse

Local e data da defesa: Itaqui-RS, 02 de março de 2015.

O lipopolissacarídeo (LPS) associado com a D-galactosamina (D-GalN), tetracloreto de carbono (CCl_4) e o paracetamol (PCM), são modelos animais de hepatite fulminante aguda amplamente utilizados. O gama orizanol (γ-ORY) é uma mistura de 10 ésteres de ácido trans ferúlico e é encontrado no óleo do farelo de arroz. O objetivo deste estudo foi avaliar o efeito hepatoprotetor do γ-ORY nos diferentes modelos animais de hepatite fulminante aguda em camundongos. O experimento foi realizado através do tratamento com γ-ORY (50 mg/kg, por via oral, p.o.) durante uma semana, após uma hora do último tratamento foi administrado por via intraperitoneal o LPS (50 mg/kg) e D-GalN (500 mg/kg), foi utilizado silimarina como controle positivo. O γ-ORY foi administrado oralmente (50 mg/kg) durante uma semana, após 1 hora da última administração o dano foi induzido por uma única dose de CCl_4 (1 mg/kg i.p.), a silimarina foi utilizada como controle positivo. Os animais receberam γ-ORY durante dois dias em duas doses (10 mg e 50 mg/kg p.o.), no terceiro dia o PCM (2 g/kg, p.o.). Após 24 horas os animais foram submetidos à eutanásia. Foram feitas as seguintes determinações bioquímicas: marcadores de estresse oxidativo (TBARS, NPSH, 4-HNE), defesas antioxidantes enzimáticas (CAT, SOD, GPx), marcadores de dano hepático (AST, ALT, GGT), marcadores de funcionalidade hepática (bilirrubina), marcadores inflamatórios (TNF-α, IL-1β), marcadores de apoptoses (Caspase 3 e 9) e análise histológica. O γ-ORY foi capaz de proteger significantemente em todas as determinações analisadas, podendo ser comparado ao um fármaco utilizado para o tratamento da hepatite fulminante aguda que é a silimarina. Portanto, sugerimos que o γ-ORY pode ser uma alternativa para o tratamento da hepatite fulminante aguda, pois ficou comprovado seu efeito hepatoprotetor está associado com sua capacidade antioxidante, anti-inflamatória e anti-apoptótica.

Palavras-chave: doenças hepáticas, hepatoprotetor, antioxidante, antiinflamatório.

ABSTRACT

Dissertation of Master

Program of Post-Graduation in Biochemistry

Federal University of Pampa

HEPATOPROTECTIVE ACTIVITY OF GAMMA ORYZANOL IN MODELS OF ACUTE FULMINANT HEPATITIS IN MICE

Author: Marcelo Gomes de Gomes

Advisor: Cristiano Ricardo Jesse

Site and Date of defence: Itaqui-RS, March 02, 2015.

The lipopolysaccharide (LPS) associated with D-galactosamine (D-GalN), carbon tetrachloride (CCl_4) and paracetamol (PCM) are acute fulminant hepatitis widely used animal models. The gamma oryzanol (γ-ORY) is a mixture of 10 esters of trans-ferulic acid and is found in rice bran oil. The objective was to assess the potential hepatoprotective effect of γ-ORY on mechanisms involved in different animal models of acute fulminant hepatitis in mice. The animals were divided into different groups depending on the experiment. γ-ORY treatment (50 mg/kg, per oral, p.o.) for a week after an hour of the last treatment before the administration by intraperitoneal of LPS (50 mg/kg) and D-GalN (500 mg/kg). γ-ORY was administered orally (50 mg/kg) for one week, after 1 hour of the last administration damage was induced by a single dose of CCl4 (1 mg /kg i.p.). The animals received γ-ORY for two days in two doses (10mg and 50mg /kg p.o.), on the third day PCM (2 g/ kg, p.o.). After 24 hours the animals were euthanized. The following biochemical determinations were made: non-enzymatic antioxidant defenses, defenses enzymatic antioxidants, liver damage markers, liver function markers, inflammatory markers, apoptosis markers and histological analysis. The γ-ORY was able to significantly protect in all analyzed determinations, and can be compared to a drug used for acute fulminant hepatitis. Therefore, we suggest that the γ-ORY can be an alternative for the treatment of acute fulminant hepatitis, as was proved its hepatoprotective effect is associated with its antioxidant capacity, anti-inflammatory and anti-apoptotic effect.

Keywords: Liver diseases, hepatoprotective, antioxidant, anti-inflammatory.

LISTA DE FIGURAS

Parte I

LISTA DE ABREVIATURAS

AA = Ácido ascórbico

ALP = Fosfatase alcalina

ALT = Alanina aminotransferase

AST = Aspartato aminotransferase

CAT = Catalase

CCl_4 = Tetracloreto de carbono

D-GalN = D-Galactosamina

EROS = Espécies reativas ao oxigênio

GGT = Gama glutamil transferase

GPx = Glutationa Peroxidase

GR = Glutationa Redutase

GSH = Glutationa reduzida

H_2O = Água

HOCL = Ácido hipocloroso

IFN-γ = Interferon gama

IL-1β = Interleucina 1-beta

IL-6 = interleucina 6

LDH = Desidrogenase láctica

LPS = Lipopolissacarídeo

MCP-1 = Proteína quimiotática de monócitos 1

MPO = Mieloperoxidase

NO = Óxido nitríco

NPSH = Tióis não protéicos

O_2 = Oxigênio

P.i. = Por via intraperitoneal

P.o. = Por via oral

PCM = Paracetamol

SOD = Superóxido dismutase

TBARS = Espécies reativas ao ácido tiobarbitúrico

TGF-β1 = Fator de transformação de crescimento 1-beta

TNF-α = Fator de necrose tumoral alfa

γ-ORY = Gama Orizanol

δ-ALA-D = Delta aminolevulenato desidratase

1. INTRODUÇÃO

1.1 Doenças hepáticas

O fígado é um dos órgãos mais importantes dos seres vertebrados e desempenha um papel importante no metabolismo de proteínas, carboidratos e lipídios. As doenças hepáticas já foram confirmadas como um dos mais graves problemas de saúde no mundo (Williams, 2006). Cerca de 25 mil mortes a cada ano, tem como causa distúrbios hepáticos (Sharma e Sharma, 2010). Geralmente a síntese da patologia está em uma infecção viral, esteatose não alcoólica ou outro agente hepatotóxico e são altamente associados com inflamação aguda ou crônica (Tseng et al., 2014). A lesão hepática aguda faz com que o desenvolvimento de doença no fígado evolua para insuficiência terminal do órgão, sendo o fator inicial e uma via comum para numerosas doenças hepáticas. Sabe-se que a lesão hepática muitas vezes conduz a fibrose hepática, cirrose, e ainda câncer de fígado, e que estas doenças têm um sério impacto sobre o corpo (Liu et al., 2014).

Como síntese das patologias hepáticas já é conhecido o papel do estresse oxidativo, o qual gera uma depleção das defesas antioxidantes, conduzindo ao dano celular, causando alterações de macromoléculas como lipídeos da membrana celular, proteínas e ácidos nucléicos (Chen et al., 2013). Tais alterações prejudicam a função celular, podendo progredir à apoptose celular e conduzir à necrose tecidual (Majhi et al., 2011). Quando persistente o estresse oxidativo, conduz a ativação de vias de sinalização e aumento da expressão de genes reguladores do processo inflamatório (Esposito et al, 2002; Evans et al., 2002). O estresse oxidativo ativando vias pró-inflamatórias é um mecanismo complexo os quais são interdependentes os quais geram um dano grave no tecido hepático (Rutter, 2000).

1.2 Modelo de dano hepático com Lipopolissacarídeo (LPS) e D-Galactosamina (D-GalN)

O LPS é um componente tóxico das paredes celulares de bactérias gram-negativas e está amplamente presente no trato digestivo dos seres humanos e animais (Jacob et al., 1977). Nos seres humanos, nanogramas de LPS injetados na corrente sanguínea pode resultar em todas as manifestações fisiológicas de choque séptico (van Deventer et al., 1990). A D-GalN é um amino açúcar seletivamente metabolizado pelos hepatócitos, induzindo um esgotamento do pool de uridina trifosfato e assim, uma inibição das macromoléculas (RNA, proteínas e glicogênio) sintetizadas no fígado (Decker e Keppler, 1974). Uma dose baixa de LPS em combinação com D-GalN foi mostrado que é capaz de induzir lesão hepática experimental, a qual é semelhante a falência hepática aguda nos casos clínicos (Galanos et al., 1979), como foi ilustrado pela figura 1. Estudos iniciais sobre o mecanismo de hepatotoxicidade, com LPS e D-GalN sugeriram que a lesão resultou nos hepatócitos à partir do excesso de produção de várias citocinas e mediadores inflamatórios tais como fator de necrose tumoral-α (TNF-α), interferon-γ (IFN-γ) e óxido nítrico (NO) (Eipel et al., 2007).

Tem sido sugerido que as espécies reativas de oxigênio (EROS), produzidos pelos macrófagos ativados nos hepáticos pode ser a causa primária dos danos no fígado induzido por D-GalN (Shiratori et al., 1988). Como demonstrado por Sun et al. (2003), a necrose induzida por D-GalN foi o resultado de extensas reações de geração de radicais livres ou de estresse oxidativo. É também sabido que os radicais livres estão envolvidos na fisiopatologia direta ou indiretamente da sepsia ou endotoxemia (Abe et al., 1988). Além disso, a D-GalN em combinação com uma pequena quantidade de LPS conduz a uma maior geração de EROS (Neihorster et al., 1992). As EROS estimulam os macrófagos do tecido para produzir citocinas pró-inflamatórias, as quais, posteriormente, atraem as células inflamatórias no fígado (Mayer et al., 1993). Assim, os radicais livres são tóxicos para as várias células, incluindo hepatócitos e iniciam a cascata mediada pelos EROS causando a morte celular de hepatócitos, conduzindo a hepatite aguda.

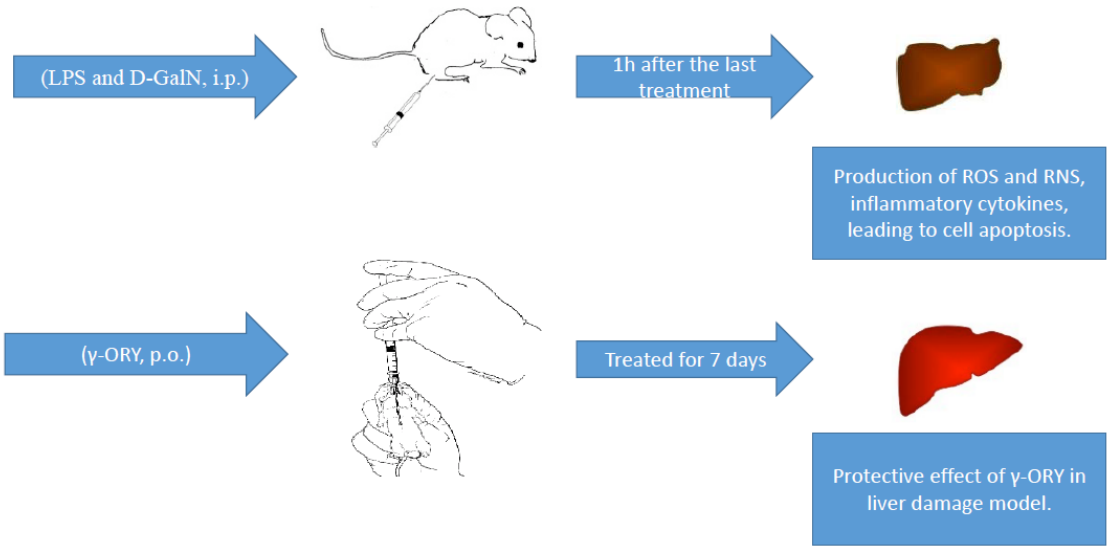

Figura 1: Esquema do modelo de dano hepático com LPS/D-GalN

1.3 Modelo de dano hepático com Tetracloreto de Carbono (CCl_4)

O CCl_4 é um agente hepatotóxico clássico que causa danos ao fígado com rápida progressão desde esteatose à necrose centrolobular (Sebastiani et al., 2011; Nogueira et al., 2009). O CCl_4 é metabolizado no citocromo P450, sofrendo bioativação pela fase I, no fígado para formar seus metabólitos reativos, o radical triclorometil (CCl_3·), e o radical peroxil triclorometil (·OOCCl3) (Hung et al., 2006). Estes radicais livres se ligam com ácidos graxos poliinsaturados para produzir radicais livres alcoxila (R·) e peroxil (ROO·) (Hung et al., 2006). Estes radicais livres reagem com grupamentos sulfidrilos tais como a glutationa (GSH) e os tióis proteicos (Filho et al., 2013). A ligação covalente de radicais livres triclorometil com as proteínas celulares é considerado o passo inicial de uma cadeia de acontecimentos que conduzem, eventualmente, a peroxidação lipídica da membrana e finalmente, a necrose celular (Brattin et al., 1985), ilustrado pela figura 2. Consequentemente, o CCl_4 é conhecido por induzir a formação de EROS, esgotar GSH (importante para enzimas de fase I), e reduzir enzimas antioxidantes e substratos para induzir o estresse oxidativo, que é um fator importante em lesões hepáticas agudas e crônicas (Brattin et al., 1985).

É importante notar que o estresse oxidativo causado por CCl_4 é indutor de apoptose, a qual está envolvida nos efeitos deletérios, tais como fibrose e cirrose hepática. Sabe-se que a ativação subsequente de células de Kupffer, juntamente com o aumento da produção de citocinas pró-inflamatórias, tais como TNF-α, IL-6 e IL-8 é também um fator que contribui para o desenvolvimento de lesão hepática aguda induzida pelo CCl_4 (Lin et al., 2012). Sabe-se também que um dos mecanismos de ação do CCl_4 é a produção de citocinas inflamatórias e o recrutamento de células inflamatórias, conduzindo a danos no fígado e disfunção celulares (Weber et al., 2003; Ebaid et al., 2013; Hamdy e El-Demerdash, 2012). Assim, a indução de lesão hepática em camundongos por exposição CCl_4 representa um modelo animal promissor para elucidar o mecanismo de disfunção clínica e para avaliar a eficácia de agentes hepatoprotetores.

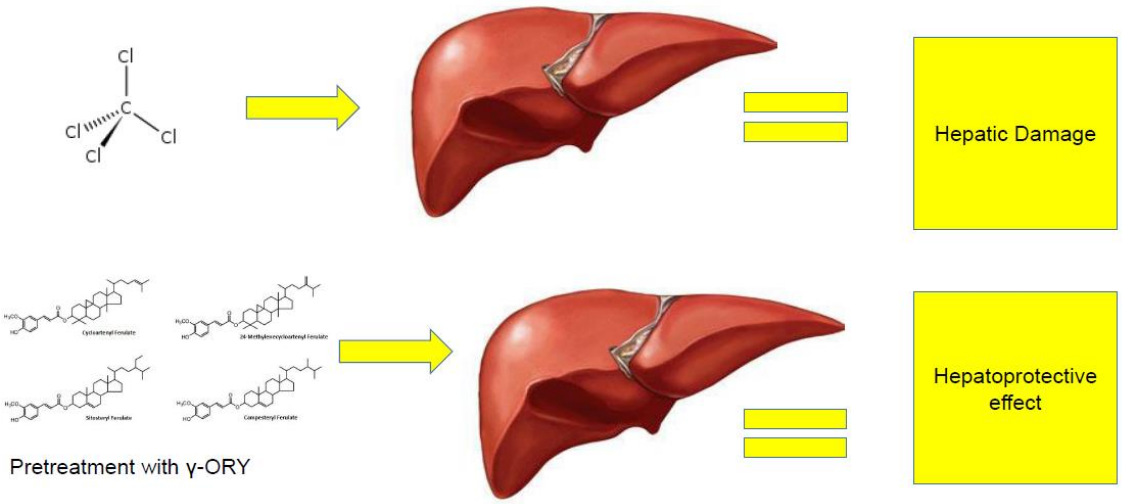

Figura 2: Esquema do modelo de dano hepático com CCl_4

1.4 Modelo de dano hepático com Paracetamol (PCM)

O PCM é um analgésico e antipirético amplamente utilizado, que é empregue de forma segura no uso terapêutico no homem em animais (Dargan e Jones, 2002). Seu uso em overdose (suicida ou acidental) ou com abuso crônico de álcool provoca insuficiência hepática fulminante e contribui de forma significativa para os casos intensivos de internações unidades hospitalares (Prescott, 2000), conforme ilustrado na figura 3. O metabolito tóxico produzido

em doses terapêuticas é detoxificado pela GSH, cisteína e o ácido mercaptúrico para ser subsequentemente excretado do corpo. No entanto, PCM tomado em overdose ou em longo prazo por uso terapêutico pode conduzir à saturação da via conjugação que conduz à depleção de GSH e o aumento da formação de metabolitos reativos tóxicos (Ethel et al., 2009). A hepatotoxicidade do PCM é o resultado da formação de um metabolito tóxico, N-acetil-p-benzoquinona imina (NAPQUI) pelo citocromo P-450 2E1 que pode induzir uma dose dependente da depleção de GSH intracelular e perturbação de do homeostase cálcio (Chen et al., 1999). Esse metabólito tóxico está relacionado ao estresse oxidativo excessivo.

O estresse oxidativo provocado por altas doses de NAPQUI também tem sido demonstrado afetar o sistema de defesas antioxidante (Bessems e Vermeulen, 2001). Acredita-se que a formação excessiva de EROS pode abalar as membranas celulares através de peroxidação lipídica e modificar ou danificar biomoléculas, ou seja, proteínas, lipídios, carboidratos e de DNA *in vitro* e *in vivo* (Halliwell, 1996). O dano celular significativo ocorre quando a quantidade de radicais livres produzidos excede a capacidade de uma produção endógena sistema de defesa antioxidante celular. De acordo com a teoria de radicais livres, bloqueando ou retardando a reação de oxidação em cadeia é uma das estratégias praticáveis para a prevenção da hepatotoxicidade induzida por estresse oxidativo (Jesse et al.,2009). Os dados da literatura também sugerem que a inflamação desempenha um papel na toxicidade de uma variedade de produtos químicos, incluindo hepatotoxicidade causada por PCM (Luster et al., 2001).

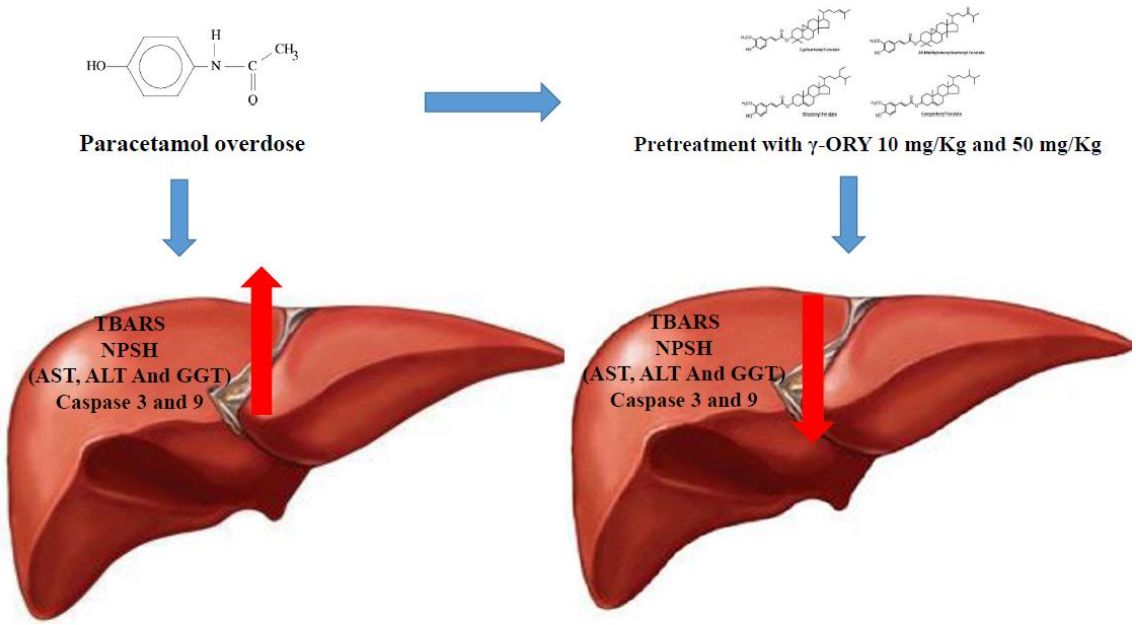

Figura 3: Esquema do modelo de dano hepático com PCM

1.5 γ-ORY

O arroz é uma cultivar muito popular no Brasil, a produção anual atingindo cercade 11,661 milhões de toneladas, o estado do Rio Grande do Sul é responsável por cerca de 62,8% dessa produção (Conab – Companhia Nacional de Abastecimento, 2011). O farelo de arroz, um subproduto do arroz processado, representa cerca de 8-11% do grão, em peso, e contém 16-22% de lipídios, assim sendo comumente utilizado para extração do óleo do farelo de arroz (Silva et al., 2006; Pestana et al., 2009). O refino é decisivo para melhorar a estabilidade, a qualidade (sabor e cor) e funcionalidade (composição de ácidos graxos, vitaminas e antioxidantes) do óleo do farelo de arroz (Pestana et al., 2012). O processo de refino químico do óleo do farelo de arroz inclui degomagem, neutralização, branqueamento e desodorização e deceragem (Pestana et al., 2008).

Assim, o gama orizanol (γ-ORY) é um importante composto encontrado no óleo do farelo de arroz. O γ-ORY é uma mistura de ésteres de ácido trans-ferúlico. Óleo de farelo de arroz é uma fonte rica de γ-ORY, o qual contém uma série de ferulatos e fitoesteróis como o 24-metileno cicloartenol (24 MCAF), cicloartenol ferulato (CAF), campestrol ferulato, β- sitosterol - (β-SF), e

ciclobranol ferulato (Xu et al., 1999), ilustrado pela figura 4. Sendo, o γ-ORY um dos principais compostos bioativos presentes no óleo do farelo de arroz, e tem sido sugerido possuir efeitos de redução nos níveis de colesterol no soro (Rong et al., 1997; Wilson et al., 2007), efeitos anti-inflamatórios (Akihisa et al., 2000), anticancerígenas (Yasukawa et al 1998) e também como antioxidante (Isram et al 2009; Xu et al 2001). A atividade antioxidante do γ-ORY torna estes compostos candidatos para formulações cosméticas, drogas farmacêuticas, e de alimentos funcionais (Juliano et al., 2005).

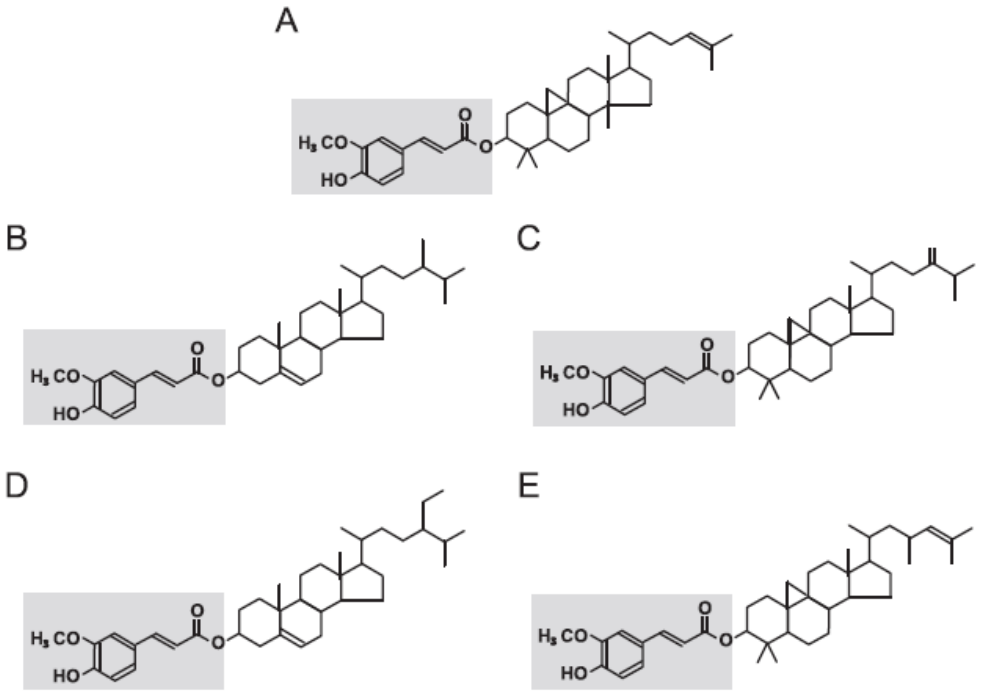

Figura 4: Estéres formadores do γ-ORY

2.Objetivos

2.1 Objetivo Geral

Avaliar o efeito hepatoprotetor do γ-ORY em diferentes modelos animais de hepatite fulminante aguda em camundongos.

2.2 Objetivos específicos

2.3 No modelo animal com LPS e D-GalN

Avaliar o efeito hepatoprotetor do γ-ORY no modelo induzido por LPS e D-GalN em camundongos, através da medida dos seguintes marcadores para o estudo dos mecanismos envolvidos:

Determinar os níveis de TBARS e NPSH hepáticos.

Avaliar a atividade das enzimas marcadoras de dano hepático no plasma: AST, ALT, ALP, GGT e LDH.

Determinar os níveis de marcadores de funcionalidade hepática no plasma: Albumina e Bilirrubina.

Avaliar a atividade enzimas sensíveis ao estresse oxidativo no tecido hepático: CAT, SOD, GPx, GR, GST e δ-ALA-D.

Determinar os níveis de citocinas pró-inflamatórias no plasma: TNF-α e IL-1β.

Análise histológica através de microscopia ótica para verificar as lesões causadas ao tecido hepático.

2.4 No modelo animal com CCl_4

Avaliar o efeito hepatoprotetor do γ-ORY no modelo induzido por CCl_4 em camundongos, através da medida dos seguintes marcadores para o estudo dos mecanismos envolvidos:

Determinar os níveis de TBARS, NPSH e AA hepáticos.

Avaliar a atividade das enzimas marcadoras de dano hepático no plasma: AST, ALT, ALP, GGT e LDH.

Determinar o nível do marcador de funcionalidade hepática no plasma: Bilirrubina.

Determinar os níveis de citocinas pró-inflamatórias no tecido hepático: TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 e IFN-γ.

Determinar os marcadores da resposta inflamatórias aguda no tecido hepático: MPO e NO.

Avaliar a atividade das caspases pró-apoptóticas no tecido hepático: Caspase 3 e Caspase 9.

Análise histológica através de microscopia ótica para verificar as lesões causadas ao tecido hepático.

2.5 No modelo animal com PCM

Avaliar o efeito hepatoprotetor do γ-ORY no modelo induzido por PCM em camundongos, através da medida dos seguintes marcadores para o estudo dos mecanismos envolvidos:

Determinar os níveis de TBARS, NPSH, AA e 4-HNE hepáticos, após o pré- tratamento com γ-ORY.

Avaliar a atividade enzimas sensíveis ao estresse oxidativo no tecido hepático: CAT, SOD, GPx, GR, GST e δ-ALA-D.

Avaliar a atividade das caspases pró-apoptóticas no tecido hepático: Caspase 3 e Caspase 9.

Análise histológica através de microscopia ótica para verificar as lesões causadas ao tecido hepático.

PARTE II

Hepatoprotective effect of γ-Oryzanol in inflammatory and oxidative stress markers induced by lipopolysaccharide and D-galactosamine in mice

Marcelo Gomes de Gomes, Franciele Donato, Leandro Cattelan Souza, André Rossito Goes, Carlos Borges Filho, Lucian Del Fabbro, Elza Eliza Tenorio Altvater, Francielli W.Santos, Silvana P.Boeira, Cristiano R.Jesse

Submetido à Food and Chemical Toxicology.

Hepatoprotective effect of γ-Oryzanol in inflammatory and oxidative stress markers induced by lipopolysaccharide and D-galactosamine in mice

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Abstract

Lipopolysaccharide (LPS) and D-Galactosamine (D-GalN), is a model widely used in hepatic damage. γ-Oryzanol (γ-ORY) is a mixture of esters 10 trans-ferulic acid and it is found in rice bran oil. The purpose of the present study was to investigate the possible hepatoprotective and anti-inflammatory effects of γ-ORY In mice in a model induced by LPS and D-GalN. γ-ORY treatment (50 mg/kg, per oral, p.o.) for a week after an hour of the last treatment before the administration by intraperitoneal of LPS (50 mg/kg) and D-GalN (500 mg/kg). Treatment with γ-ORY (50 mg/kg) protected against increases in thiobarbituric reactive species (TBARS), and decreasing non-protein thiols (NPSH), levels in liver of mice. γ-ORY protected against aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities increases induced by LPS and D-GalN plasma exposure. Treatment with γ-ORY protected against increases in albumin and bilirubin levels in serum of mice. Superoxide dismutase (SOD) and δ-Aminolevulic acid dehydratase (δ-ALA-D) activities was inhibited by LPS and D-GalN and remained unaltered even after treatment with γ-ORY. Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) activities inhibition caused by LPS and D-GalN were protected against by treatment with γ-ORY. Exposure of animals to LPS and D-GalN increase levels of tumor necrosis factor alfa TNF-α and interleukin-1β (IL-1β), were protected against by treatment with γ-ORY. Sections of liver from LPS and D-GalN-exposed mice presented an intense infiltration of inflammatory cells, and loss of the cellular architecture. γ-ORY attenuated LPS and D-GalN-induced hepatic histological alterations. γ-ORY was able to protect against oxidative stress and inflammation induced by LPS and D-GalN. Altogether, our results indicate that the gamma was able to protect against damage induced by LPS and

D-GalN. **Keywords:** Disease hepatic, Hepatic damage, Hepatoprotective, Inflammation, Antioxidant.

1. Introduction

Fulminant hepatic failure is a life-threatening clinical syndrome that results from severe impairment of liver function. Lipopolysaccharide (LPS) and D-Galactosamine (D-GalN) induced liver failure is a widely used model that resembles human liver failure (Nakama et al., 2001). And they caused hepatic failure in rodents (Galanos et al., 1979). LPS, a major component of the outer membrane of Gram-negative bacteria, is an endotoxin that is thought to contribute significantly to hepatic failure (Bohlinger et al., 1996). Upon stimulation with LPS/GalN activates Kupffer cells and produces various cytokines, e.g., interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which lead to hepatic necrosis, the decreasing of antioxidant enzyme activities and so on (Yang et al., 2014). D-GalN is a specific hepatotoxic agent that depletes the uridine triphosphate pool and thereby inhibits macromolecule synthesis (Decker and Keppler., 1974). As a transcriptional inhibitor, D-GalN potentiates the toxic effects of LPS in liver (Xiong et al., 1999).

Oxidative stress is an increase in intracellular levels of reactive oxygen species (ROS), such that cellular antioxidant defenses are insufficient to secure them below a toxic threshold level. (Hung et al., 2006). The harmful effects of LPS are, in part, suspected to its ability to induce an oxidative stress status characterized by depletion of endogenous antioxidant enzyme activities such as superoxide dismutase, catalase and glutathione peroxidase. The pro-oxidant action of the endotoxin is due to its ability to induce excessive ROS and reactive nitrogen species (RNS) accumulation leading to

cellular injury by impairment of vital macromolecules as protein and lipid (Mallis et al., 2001) resulting in altered membrane fluidity and mitochondrial function (Cadenas and Cadenas., 2002). Excess production of ROS and or defective cellular antioxidant systems are involved in many pathological conditions, including chronic liver injury and fibrogenesis (Bataller and Brenner, 2005; Friedman, 2000; Lotersztajn et al., 2005). LPS activates inflammatory cells and subsequently amplifies the inflammatory response by releasing various cytokines, such as TNF-α and IL-1β (Sompamit et al., 2010).

Rice bran oil derived from rice bran has been found to possess promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, and heart disease (Jariwalla RJ., 2001). Rice bran oil is a rich source of γ-Oryzanol (γ-ORY), which contains a number of phytosteryl ferulates such as 24-methylenecycloartanyl ferulate (24-mCAF), cycloartenyl ferulate (CAF), campesteryl ferulate, β-sitosteryl ferulate (β-SF), and campestanyl ferulate; and the mixture of these is called as γ-ORY (Xu et al., 1999). It is of particular importance that γ-ORY exhibits antioxidant properties *in vitro* systems (Kim et al., 1995). γ-ORY also have some physiological activities such as inhibition of tumor promotion (Yasukawa et al., 1998), reduction of serum cholesterol levels (Guardiola et al., 1996) and antioxidant properties in several models (Xu et al., 2001; Nystrom et al., 2005). The antioxidant potency of γ-ORY makes it a good candidate for pharmaceutical drugs, cosmetic formulations, and health food (Juliano et al., 2005).

The purpose of the present study was to investigate the possible hepatoprotective and anti-inflammatory effects of γ-ORY in a model in mice induced by LPS and D-GalN. Through the evaluation of enzymatic and non-enzymatic markers of oxidative stress, histological analysis, marker enzymes of liver damage, markers of liver function and inflammatory markers. Silymarin was also used in our study as a well-known

hepatoprotectant reference drug extracted from the seeds of *Silybum marinum* plant and it was used as a positive control (Pradhan SC, Girish C., 2006).

2. Materials and methods

2.1. Experimental animals

Male adult Swiss mice (25–35 g) from our own breeding colony were used. Animals were kept in separate animal rooms, on a 12 h light/dark cycle, at a temperature of 22±2 ◦C, food and water *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil. This study was approved with number of protocol 021/2014.

2.2 Reagents

γ-ORY was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). LPS, D-GalN, glutathione reductase from baker's yeast, b-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and oxidised glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co. (USA).

2.3 Exposure

The animals were divided into six groups:

Group 1: Canola oil (10 mg/kg) + saline (10 mg/kg);

Group 2: γ-ORY (50 mg/kg) + saline (10 mg/kg);

Group 3: Silymarin (200 mg/kg) + saline (10 mg/kg);

Group 4: Canola oil (10 mg/kg) + LPS and D-GalN (50 and 500 mg/kg);

Group5: γ-ORY (50 mg/kg) + LPS and D-GalN (50 and 500 mg/kg);

Group 6: Silymarin (200 mg/kg) and LPS and D-GalN (50 and 500 mg/Kg).

The animals received γ-ORY orally (50 mg/kg) once a day for 7 days. After one hour of the last treatment was administered γ-ORY-inducing agents of liver damage intraperitoneal LPS (50 mg / kg) and D-GalN (500 mg / kg). After 24 hours the animals were anesthetized and blood collected by cardiac puncture. After this procedure, mice were their euthanized using sodium pentobarbital (100 mg/kg, i.p.) and livers were removed, dissected and kept on ice until the assay of team. The liver samples were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10 w /v), and centrifuged at 2400g for 15 min. The low-speed supernatants were used for biochemical analyzes. For histological a small sample was collected and kept in liver formaldehyde to retain its structure.

2.4 Histopathological analysis

Small pieces of liver tissues from individual mice were fixed in 10% formalin. For optical microscopy examination, tissues were embedded in paraffin, sectioned at 4μm, and stained with haematoxylin and eosin. Liver sections were numerically graded to assess the histological features for degree of acute hepatic injury. Centrilobular necrosis, or zonal necrosis (damage of several liver cells around the central vein), vascular congestion, megalocytosis, cell infiltration, eosinophilic cells and tumefaction of hepatocytes of liver cells were characterized (Wills and Asha., 2006).

2.5 Markers of oxidative stress

2.5.1 Thiobarbituric acid reactive species (TBARS)

An aliquot of S1 (200 μl) from mice belonging to the experimental groups was added to the reaction mixture containing 500 μl thiobarbituric acid (0.8%), 200 μl SDS (sodium dodecyl sulfate, 8.1%) and 500 μl acetic acid (pH 3.4), and was incubated for 2 hours at 95°C. Thiobarbituric reactive species (TBARS) levels were determined as

described by Ohkawa, et al., 1979. Malondialdehyde (MDA), which formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a colored product that can be measured optically at 532 nm. The lipid peroxidation was expressed as nmol MDA equivalents/g tissue.

2.5.2 Non-protein thiols (NPSH)

NPSH levels were determined by the method of Ellman (1959). To determine NPSH, a sample aliquot was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free–SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The colour reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue.

2.6 Markers of hepatic damage

2.6.1 Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities

The enzymes activities was measured in serum using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil). The enzymatic activity were expressed as U/L.

2.7 Markers of hepatic functionality

2.7.1 Albumin and Bilirubin levels

Levels were measured in serum using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

2.8 Marker enzymes of oxidative stress

2.8.1 Catalase (CAT) activity

CAT is an enzymatic antioxidant defense that is involved in protecting against the injurious effects of RS. Catalase activity was assayed spectrophotometrically by the Aebi method (1984), which involves monitoring the disappearance of H_2O_2 in the presence of S_1 at 240 nm. Enzymatic reaction was initiated by adding an aliquot of S_1 20 µl, and the substrate (H_2O_2) at a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units of (1 U decomposes 1 µmol of H_2O_2 per minute at pH 7 at 25°C).

2.8.2 Superoxide dismutase (SOD) activity

SOD is an enzymatic antioxidant defense that protects against active free radicals by scavenging excess superoxide. SOD activity in liver homogenate was assayed spectrophotometrically as described by Misra and Fridovich (1972). The method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 37 ◦C.

2.8.3 Glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity is measured in a system containing reduced glutathione (GSH) by Wendel (1981), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR). GPx acts oxidizing GSH into glutathione disulphide (GSSG). To complete the cycle, GR reduces GSSG back to GSH at the expenses of NADPH. The decline in the concentration of NADPH can be monitored at 340 nm. The activity of GPx is given by the consumption of NADPH in nmol/min/mg of protein.

2.8.4 Glutathione Reductase (GR) Activity

GR activity was determined spectrophotometrically as described by Calberg and Mannervik (1985). In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which is followed at 340 nm. GR activity is proportional to NADPH decay. An aliquot of 50llofS1was added in the system containing 0.15 M potassium phosphate buffer, pH7.0, 1.5 mM EDTA, 0.15 mM NADPH. After the basal reading, 50ll of 20 mM GSSG (substrate) was added. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.8.5 Glutathione S-transferase (GST) activity

GST is an enzymatic antioxidant defense that protects against RS-induced injury. GST activity was assayed through the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by Habig et al. (1974). An aliquot, (100 µl of 0.1 M potassium phosphate buffer (pH 7.4), with CDNB as the substrate) was added to 50 mM GSH. The enzymatic activity was expressed in µmol/min mg protein.

2.9 Sensitive to oxidative stress marker

2.9.1 δ-Aminolevulic acid dehydratase activity

δ-Aminolevulic acid dehydratase (δ-ALA-D) activity was assessed by measuring the formation of porphobilinogen (PBG), according to Sassa (1982) method, except that 45mM sodium phosphate buffer and 2.2mM ALA were used. Samples were homogenized in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2400 × g for 15 min. An aliquot of 50 µL of homogenized tissue was incubated for 2 h at 37 ◦C. PBG formation was detected with the addition of modified Erlich's reagent at 555 nm.

3. Inflammation markers

3.1 TNF-α and IL-1β Levels

Levels of TNF-α and IL-1β were determined in blood using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg.

4. Protein Determination

Protein content was measured colorimetrically according to the method of Bradford (1976) and bovine serum albumin (1 mg/ml) was used as standard.

5. Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a Two-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. The level of significance was set at $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

6.RESULTS

6.1 Histopathological analysis

The liver tissue from mice exposed to LPS and D-GalN when compared with the control group (Figure A) revealed extensive injuries, characterized by vascular congestion, megalocytosis, cell infiltration, eosinophilic cells and tumefaction (Figure D). γ-ORY significantly reduced the liver damage and markedly ameliorated the degree of liver damage (Figure E). It was observed that γ-ORY administration at the dose of 50 mg/kg p.o. did not cause changes to the liver histopathological analysis in this study (Figure B). Silymarin caused no changes in the histopathological analysis of mouse liver (Figure C). Silymarin significantly reduced the liver damage and markedly ameliorated the degree of liver damage (Figure F).

6.2 TBARS levels

A two-way ANOVA of TBARS levels revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 33.67$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to decrease the levels of TBARS in the liver of mice with liver injury induced by LPS and D-GalN (Table 1). Two-way ANOVA of TBARS levels revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 43.21$; $p<0.001$). Post hoc comparisons showed that the silymarin was able to decrease the levels of TBARS in the liver of mice with liver injury induced by LPS and D-GalN (Table 1).

6.3 NPSH levels

A two-way ANOVA of NPSH levels revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 11.50$; $p<0.003$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to increase the levels of NPSH in the liver of mice with liver injury induced by LPS and D-GalN (Table 1). Two-way ANOVA of NPSH levels revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 5.75$; $p<0.02$). Post hoc comparisons showed that the silymarin was able to increase the levels of NPSH in the liver of mice with liver injury induced by LPS and D-GalN (Table 1).

6.4 Plasmatic AST, ALT, ALP, GGT and LDH activities

Two-way ANOVA of plasma AST activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 102.13$; $p<0.001$). The post-hoc comparison revealed that the γ-ORY reversed the increase in the ALT activity induced by LPS and D-GalN administration(Table 2). Two-way ANOVA of plasma ALT activity revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 106.17$; $p<0.001$). It has

been demonstrated by post hoc comparisons that treatment with silymarin reversed liver damage caused by LPS and D-GalN (Table 2).

Two-way ANOVA of plasma ALT activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 100.24$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY reversed the increase in the ALT activity induced by LPS and D-GalN administration (Table 2). Two-way ANOVA of plasma ALT activity revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 98.80$; $p<0.001$). The post-hoc comparison revealed that the silymarin reversed the ALT activity caused by administration of LPS and D-GalN (Table 2).

A two-way ANOVA of plasma ALP activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 53.21$; $p<0.001$). The post-hoc comparison revealed that the γ-ORY reversed the increase in the ALP activity induced by LPS and D-GalN administration (Table 2). Two-way ANOVA of plasma ALP activity revealed a significant Silymarin x LPS + D-GalN interaction ($F(1,36) = 69.19$; $p<0.001$). The post-hoc comparison revealed that the silymarin reversed the increase in the ALT activity induced by LPS and D-GalN administration (Table 2).

A two-way ANOVA of plasma GGT activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 80.81$; $p<0.001$). The post-hoc comparison revealed that the γ-ORY reversed the increase in the GGT activity induced by LPS and D-GalN administration (Table 2). Two-way ANOVA of plasma GGT activity revealed a significant silymarin x LPS and D-GalN interaction ($F(1,36) = 99.73$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with Silymarin reversed the increase in the GGT activity induced by LPS and D-GalN administration (Table 2).

A two-way ANOVA of plasma LDH activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 34.89$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY reversed the increase in the LDH activity induced by LPS and D-GalN administration (Table 2). Two-way ANOVA of plasma LDH activity revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 56.05$; $p<0.001$). The post-hoc comparison revealed that the silymarin reversed the LDH activity caused by administration of LPS and D-GalN (Table 2).

6.5 Albumin and Bilirubin levels

A two-way ANOVA of liver Albumin levels yielded a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 4.59$; $p<0.02$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to increase the levels of albumin in the group where the damage was induced by hepatic LPS and D-GalN (Table 3). Two-way ANOVA of blood Albumin levels yielded a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 6.02$; $p<0.001$). The Post-hoc comparison showed that Silymarin was able to increase the levels of albumin in the group where the damage was induced by hepatic LPS and D-GalN (Table 3).

A two-way ANOVA of blood Bilirubin levels yielded a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 123.71$; $p<0.001$). The Post-hoc comparison showed that γ-ORY was able to increase the levels of bilirubin in the group where the damage was induced by hepatic LPS and D-GalN (Table 3). Two-way ANOVA of blood Bilirubin levels yielded a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 146.32$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with Silymarin was able to increase the levels of bilirubin in the group where the damage was induced by hepatic LPS and D-GalN (Table 3).

6.7 CAT activity

Two-way ANOVA of CAT activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 6.62$; $p<0.02$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to enhance the enzymatic activity of the group exposed to LPS and D-GalN (Table 4). A two-way ANOVA of CAT activity revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 17.21$; $p<0.008$). Post hoc comparisons showed that the Silymarin was able to enhance the enzymatic activity of the group exposed to LPS and D-GalN (Table 4).

6.8 SOD activity

A significant main effect of LPS and GalN ($F(1,36) = 63.34$; $p<0.001$) on SOD activity in mice livers was observed (Table 4). Results demonstrated that LPS and D-GalN exposure caused a decrease (40%) in SOD activity, and treatment with γ-ORY did not modify this inhibition.

A significant main effect of LPS and GalN ($F(1,36) = 7.01$; $p<0.01$) and silymarin ($F(1,16) = 16.91$; $P < 0.008$) on SOD activity in mice livers was observed (Table 4). Treatment with Silymarin significantly increased the SOD activity inhibition induced by LPS and GalN. SOD activity remained unaltered in the livers of mice which received γ-ORY and Silymarin (Table 4).

6.9 δ-ALA-D activity

A significant main effect of LPS and GalN ($F(1,36) = 25.96$; $p<0.001$) and γ-ORY ($F(1,36) = 6.06$; $p<0.02$) on δ-ALA-D activity in mice livers was observed (Table 4). Results demonstrated that LPS and GalN exposure caused a decrease (38%) in δ-ALA-D activity, and treatment with γ-ORY prevented this inhibition.

Two-way ANOVA of δ-ALA-D activity revealed a significant γ-ORY x LPS and GalN interaction ($F(1,36) = 7.16$; $p<0.001$). Post hoc comparisons demonstrated

that LPS exposure inhibited the δ-ALA-D activity, and Silymarin at the dose of 200 mg/kg protected against the inhibition of δ-ALA-D activity induced by LPS and GalN exposure (Table 4). γ-ORY at the dose tested (50 mg/kg, p.o., for seven days) and silymarin did not alter δ-ALA-D activity in the mice livers.

7 GPx activity

Two-way ANOVA of GPx activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 6.94$; $p<0.01$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to enhance the enzymatic activity of the group exposed to LPS and D-GalN (Table 5). A two-way ANOVA of GPx activity revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 10.38$; $p<0.005$). Post hoc comparisons showed that the Silymarin was able to enhance the enzymatic activity of the group exposed to LPS and D-GalN (Table 5).

7.1 GR activity

A two-way ANOVA of GR activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 14.16$; $p<0.001$). Post hoc comparisons showed that the γ-ORY was able to reduce the enzymatic activity of the group exposed to LPS and D-GalN (Table 5). Two-way ANOVA of GR activity revealed a significant Silymarin x LPS + D-GalN interaction ($F(1,36) = 14.85$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with Silymarin was able to reduce the enzymatic activity of the group exposed to LPS and D-GalN (Table 5).

7.2 GST activity

A two-way ANOVA of GST activity revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 15.30$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to reduce the enzymatic activity of the group exposed to LPS and D-GalN (Table 5). Two-way ANOVA of GST activity

revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) = 17.21$; $p<0,008$). Post hoc comparisons showed that the Silymarin was able to reduce the enzymatic activity of the group exposed to LPS and D-GalN (Table 5).

7.3 TNF-α levels

A two-way ANOVA of TNF-α levels revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 22.74$; $p<0.003$). Post hoc comparisons showed that the γ-ORY was able to decrease the plasmatic levels of TNF-α in injury induced by LPS and D-GalN (Table 6). Two-way ANOVA of TNF-α levels revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) =44.89$; $p<0.02$). It has been demonstrated by post hoc comparisons that treatment with Silymarin was able to decrease the plasmatic levels of TNF-α in injury induced by LPS and D-GalN (Table 6).

7.4 IL-1β levels

Two-way ANOVA of IL-1β levels revealed a significant γ-ORY x LPS and D-GalN interaction ($F(1,36) = 63.21$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to decrease the plasmatic levels of IL-1β in injury induced by LPS and D-GalN (Table 6). A two-way ANOVA of IL-1β levels revealed a significant Silymarin x LPS and D-GalN interaction ($F(1,36) =65.18$; $p<0.001$). Post hoc comparisons showed that the Silymarin was able to decrease the plasmatic levels of IL-1β in induced by LPS and D-GalN (Table 6).

8. Discussion

As expected, a single oral dose of LPS and D-GalN showed significant hepatotoxicity, evidenced by a dramatic elevation in AST, ALT, ALP, GGT and LDH plasma activities, and an increase of histopathological lesions in the mice livers. In addition, LPS and D-GalN exposure caused oxidative damage evidenced by a significant increase in hepatic lipid peroxidation levels, decrease NPSH levels.

Inhibition of CAT, GPx, GR and glutathione S-transferase GST activities. Also increases in albumin and bilirubin levels in sérum, TNF-α and IL-1β in blood. The γ-ORY was able to protect against damage induced by LPS and D-GalN.

Oxidative stress plays a prominent causative role in many diseases, including liver damage (Pereira et al., 2012). Free radicals are capable of binding to proteins and lipids, abstracting hydrogen atoms from unsaturated lipids, and initiating both lipid peroxidation and liver damage, they play a significant role in the pathogenesis of disease (Knockaert et al., 2012) Lipid peroxidation, usually measured through its catabolites such as MDA, is accepted as one of the principal causes of LPS and D-GalN- induced liver injury. γ-ORY has been suggested to possess effects of anti-inflammatory (Akihisa et al., 2000) and antioxidant (Isram et al., 2009; Xu et al., 2001).

Serum aminotransferase activities have long been considered as sensitive indicators of hepatic injury (Sclafani et al., 1986). Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells (Molander et al., 1955). Therefore, the marked release of AST and ALT into the circulation indicates severe damage to hepatic tissue membranes during LPS and D-GalN intoxication. In the present study, the single oral dose of LPS/D-GalN caused a dramatic elevation in serum AST, ALT, ALP, GGT and LDH activities, indicating an acute hepatotoxicity induced by administration of LPS and D-GalN. The acute hepatotoxic effects induced by LPS and D-GalN exposure were confirmed histopathologically, revealing extensive hepatocellular degeneration and necrosis, inflammatory cell infiltration, congestion, and sinusoidal dilatation (Goto et al., 2006). Pretreatment with γ-ORY dose of 50 mg/kg efficiently prevented the LPS and D-GalN-induced elevation of serum AST, ALT, ALP, GGT and LDH activities, indicating the hepatoprotective activity of γ-ORY against the acute intoxication of LPS and D-GalN.

This phenomenon was also confirmed by the results of histopathological examination, as evidenced by a decrease in the incidence and severity of histopathological hepatic lesions.

The mechanism of action of inducing agents generate oxidative stress damage. Antioxidant therapies could provide a potential means to treat conditions in which the formation of reactive oxygen species exceeds the capability of natural protective mechanisms. Several studies have suggested that several oxygen-generating radicals, which remove GSH from tissue (Dupont et al., 2000; Masalkar and Abhang, 2005), inhibit glutathione synthesis (Videla et al., 1991; Oh et al., 1997), and impair the antioxidant defense system (Nordmann et al., 1992). The NPSH constitutes (mainly GSH) are the first line of defense against free radicals. In our study showed that with the depletion caused in LPS and D-GalN and increased levels of NPSH in the group treated with γ-ORY group. The hepatic lipid peroxidation has been associated with liver damage inducing agents as an indicator of oxidative stress. The results presented showed significant decrease in the levels of MDA in liver tissue of mice treated with γ-ORY, a result similar to the animals treated with silymarin, hepatoprotective agent used as positive control in several studies, as an example of Xing et al., 2014.

The oxidative stress can alter the physiology and therefore cellular functionality. Thus, there are marker enzymes of liver damage. LPS and D-GalN was able to increase the activity of AST, ALT, GGT, ALP and LDH enzymes recognized in several studies the marker hepatic damage. γ-ORY was able to drastically reduce the activity of marker enzymes of hepatic damage, so we can infer that it has a hepatoprotective effect. High ALP and bilirubin indicated dysfunction of hepatocytes and increased biliary pressure (Breikaa et al., 2013). The inducing agents caused an increase in activity ALP and bilirubin levels. But γ-ORY was able to decrease the ALP activity and bilirubin levels,

and it is believed that the hepatocytes may have regained their functionality, similar results were found for the positive control silymarin. On the levels of serum albumin the treated mice had a significant increase in the levels, these results indicate that γ-ORY could promote functional recovery of the liver. It is believed that these results are due to the antioxidant and anti-inflammatory effect that has the γ-ORY.

The body has defense mechanisms to prevent and neutralize free-radical-induced damage. CAT, an antioxidant enzyme, is one of the most important enzymes to ameliorate the effects of oxygen metabolism (Linares et al., 2006; Pande et al., 2002). CAT catalyzes the breakage of hydrogen peroxide produced in the cell into O_2 and H_2O (Linares et al., 2006). γ-ORY treatment caused a protective effect on CAT activity, minimizing the deleterious effects caused by LPS and D-GalN, which also happened to silymarin. This is due to the antioxidant activity of gamma. SOD This enzyme keeps the concentration of superoxide radicals at low levels and therefore plays an important role in the defence against oxidative stress (Fridovich., 1997). The γ-ORY was not able to restore the activity of SOD significantly. However had a main effect which increased the activity of the enzyme inhibited by LPS and D-GalN. The same did not happen with silymarin, then we can infer that the range has no mediation of the action of this enzyme. It is believed that the gamma is acting on the reactive species so mudula the activity of antioxidant enzymes. Taking into account δ-ALA-D is a thiol enzyme (Spiazzi et al., 2013). She is sensitive to oxidative stress, also serving as a marker of functionality because it is synthesized in the liver. Our results show inhibition of δ-ALA-D activity induced by LPS and D-GalN, demonstrating a toxic effect in hepatic tissue. The γ-ORY has a protective effect, because it was able to increase the activity of δ-ALA-D because it is a thiol enzyme. This is due to its antioxidant capacity, results similar results to those obtained in the positive control.

GSH is the dominant NPSH in mammalian cells; as such it is essential in maintaining the intracellular redox balance and the essential thiol status of proteins (Lu, 1999; Schulz et al., 1996). GSH is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism. GSH is synthesized and degraded in most cell types by a series of well characterized enzymatic reactions. It exists in both GSH and GSSG form, the former by far the largest fraction. GSH and GSSG are interconvertible by the action of two enzymes, GPX and GR. GSH strongly modulates the redox state ratio of oxidizing to reducing equivalents of the cell, a role which is critical for cell survival (Salvemini et al., 1999. GPx and Gr met with inhibited due to stressful action of LPS and D-GalN activities. The γ-ORY has a protective effect reestablishing the activities of enzymes The same results were found for silymarin. Supporting our findings, it was suggested that the depletion of antioxidants induced by LPS and D-GalN could be also one of the reasons for increased oxidative stress in liver. Parameter changes as the levels of TBARS increased by example. The damage shown in histological slides, demonstrate that there are changes in enzymatic defenses. GST is a cytosolic enzyme involved in the detoxification of a range of xenobiotic compounds by conjugation to GSH which is essential in the maintenance of normal physiological processes (Dagget et al., 1998). LPS and D-GalN were able to dramatically decrease GST enzyme activity but the animals treated with γ-ORY had enzyme activity maintained is equivalent to control and positive control. This confirms the results found in this study, demonstrating the efficiency of the γ-ORY as an antioxidant.

Many studies also demonstratedthat IL-1β and TNF-α play a key role in the development and maintenance of inflammatory and those cytokines elevationis associated with many liver diseases (Shin et al., 2013; Weber et al., 2003; Shim et al.,

2010). LPS and D-GalN activates inflammatory cells and subsequently amplifies the inflammatory response by releasing various cytokines, such as TNF-α and IL-1β (Sompamit et al., 2009) .TNF-α can induce apoptosis of hepatocytes at an early stage in LPS/D-GalN-induced liver injury, and neutrophil transmigration can represent a critical step leading to necrosis of hepatocytes at a later stage (Tiegs, 1994; Chosay et al., 1997). IL-1β is one of interleukins is directly responsible for the inflammatory cascade. We can say that the γ-ORY attenuated the inflammatory process that is precisely mechanism action of the causative agents of hepatic damage. Because it was able to decrease the levels of c, silymarin showed similar results. This is due to its anti-inflammatory capabilities also found in the study of Akihisa et al., 2000.

In conclusion, the results indicate that LPS and D-GalN acute liver injury caused. γ-ORY but had a protective effect for its antioxidant and anti-inflammatory. These results indicate that the beneficial effects of treatment with the γ-ORY reestablishment of liver function are mediated by its antioxidant action (TBARS, NPSH, CAT, SOD, GPx, Gr GST) and anti-inflammatory (TNF-α, IL-1β). Also restored the enzymatic activity was inhibited enzyme markers of liver injury (AST, ALT, GGT, LDH, ALP), a result also demonstrated by histology. Moreover, the γ-ORY appears to act on the antioxidant and anti-inflammatory mechanisms against liver injury induced by LPS and D-GalN. Therefore, the γ-ORY may powerhouse considered a hepatoprotective agent against liver injury induced by LPS and D-GalN.

Conflict of interest

The author has declared that there is no conflict of interest.

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Legends

Figure 1- Photomicrography of segment of the hepatic lobe (A) of an animal control. (B) of an animal treated with γ-Oryzanol. (C) of an animal treated with silymarin. (D) of an animal treated with LPS and D-GalN showed necrosis. (E) of an animal treated with γ-Oryzanol + LPS and D-GalN . (F) of an animal treated with silymarin + LPS and D-GalN. Note the hepatocyte strings (arrow), the centrilobular vein (VC) and sinusoid capillaries (*) with normal aspect in figures 1A, 1B and 1C. Observe around the centrilobular vein (VC) some hepatocytes with vacuolation and acidophilic cytoplasm (arrows). H.E. 100X.

Table 1- γ-ORY (50 mg/kg, p.o.) was administered for 7 days, 1 hour after the last treatment, the animals were exposed to agents that cause liver damage LPS (50 mg/kg, i.p.) and D-GalN (500 mg/kg ,i.p.) after 24 hours analyzes were performed. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p <0.001$ as compared to the LPS and D-GalN.

Table 2- γ-ORY (50 mg/kg, p.o.) was administered for 7 days, 1 hour after the last treatment, the animals were exposed to agents that cause liver damage LPS (50 mg/kg, i.p.) and D-GalN (500 mg/kg, i.p.) after 24 hours analyzes were performed. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the LPS and D-GalN.

Table 3- γ-ORY (50 mg/kg, p.o.) was administered for 7 days. 1 hour after the last treatment, animals were exposed to LPS (50 mg/kg i.p.) and D-GalN (500 mg/kg i.p.). Biochemical analysis were performed after 24 hours. Data are expressed as means ±

S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the LPS and D-GaIN.

Table 4- γ-ORY (50 mg/kg, p.o.) was administered for 7 days, 1 hour after the last treatment, the animals were exposed to agents that cause liver damage LPS (50 mg/kg i.p.) and D-GalN (500 mg/kg i.p.) after 24 hours analyzes were performed. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p <0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the LPS and D-GaIN.

Table 5- γ-ORY (50 mg kg, p.o.) was administered for 7 days, 1 hour after the last treatment, the animals were exposed to agents that cause liver damage LPS (50 mg/kg i.p.) and D-GalN (500 mg/kg i.p.) after 24 hours analyzes were performed. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p < 0.001$ as compared to the LPS and D-GaIN.

Table 6- γ-ORY (50 mg/kg, p.o.) was administered for 7 days, 1 hour after the last treatment, the animals were exposed to agents that cause liver damage LPS (50 mg/kg i.p.) and D-GalN (500 mg/kg i.p.) after 24 hours analyzes were performed. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the LPS and D-GalN.

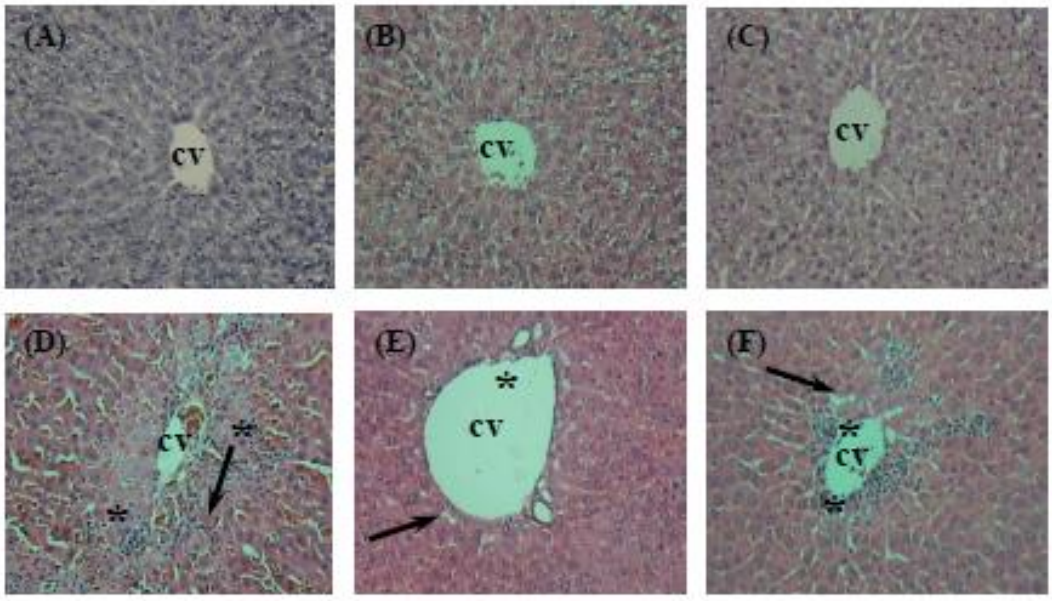

Fig 1.

Table 1. Effect of γ-ORY administration on TBARS and NPSH levels in liver of mice exposed to LPS and D-GalN

Groups	TBARS (nmol MDA equivalents/g tissue)	NPSH (µmol/g tissue)
Control	20.03 ± 1.55	0.97 ± 0.03
γ-ORY	19.83 ± 1.49	1.04 ± 0.02
Silymarin	16.61 ± 1.29	1.16 ± 0.05
LPS/D-GalN	56.12 ± 4.09[a]	0.57 ± 0.04[a]
γ-ORY + LPS/D-GalN	26.52 ± 2.06[b]	0.89 ± 0.04[b]
Silymarin + LPS/D-GalN	21.22 ± 1.44[b]	0.95 ± 0.02[b]

Table 2. Effect of γ-ORY administration on ALT, AST, FAL, GGT and LDH activities in serum of mices exposed to LPS and D-GalN

Groups	**AST** (U/L)	**ALT** (U/L)	**ALP** (U/L)	**GGT**(U/L)	**LDH** (U/L)
Control	37.60 ± 2.24	29.40 ± 3.26	178.2 ± 11,76	6.12 ± 0.54	321.2 ± 37.61
γ-ORY	39.80 ± 2.13	33.60 ± 3.93	162.0 ± 15,72	5.27 ± 0.33	344.0 ± 31.86
Silymarin	38.40 ± 4.42	32.80 ± 1.74	148.4 ± 14,82	5.15 ± 0.40	312.6 ± 35.90
LPS/D-GaIN	243.2 ± 15.52[a]	214.8 ± 13.68[a]	547.2 ± 25,33[a]	55.10 ± 3.96[a]	1052 ± 79.86 [a]
γ-ORY + LPS/D-GaIN	76.60 ± 5.35[ab]	63.60 ± 5.25[ab]	231.2 ± 25,73[b]	14.28 ± 1.9[ab]	489.4 ± 31.66[b]
Silymarin + LPS/D-GaIN	64.20 ± 6.25[b]	62.00 ± 6.78[ab]	189.6 ± 23.53[b]	12.82 ± 0.97[ab]	328.0 ± 33.67[b]

Table 3. Effect of γ-ORY administration on Albumin and Bilirubin levels in plasma of mice exposed to LPS and D-GalN

Groups	**Albumin** **(g/dL)**	**Bilirubin** **(mg/dL)**
Control	35.89 ± 2.02	0.58 ± 0.03
γ-ORY	35.62 ± 1.76	0.66 ± 0.03
Silymarin	34.25 ± 1.9	0.61 ± 0.02
LPS/D-GalN	20.24 ± 1.12[a]	3.46 ± 0.19[a]
γ-ORY + LPS/D-GalN	27.59 ± 2.0[ab]	1.03 ± 0.10[ab]
Silymarin + LPS/D-GalN	26.66 ± 1.25[ab]	0.94 ± 0.07[b]

Table 4. Effect of γ-ORY administration on CAT, SOD and δ-ALA-D activities in liver of mice exposed to LPS and D-GalN

Groups	CAT (U/mg protein)	SOD (U/mg protein)	δ-ALA-D (nmol PBG/mg protein/h)
Control	34.61 ± 1.64	9.37 ± 0.51	10.26 ± 0.52
γ-ORY	33.10 ± 1.59	10.14 ± 0.74	10.58 ± 0.49
Silymarin	30.87 ± 1.32	9.74 ± 0.61	10.51 ± 0.82
LPS/D-GalN	20.89 ± 1.08[a]	5.60 ± 0.38[a]	6.26 ± 0.45[a]
γ-ORY + LPS/D-GalN	26.56 ± 1.17[ab]	5.59 ± 0.34[a]	8.75± 0.75[b]
Silymarin + LPS/D-GalN	29.22 ± 1.68[b]	10.27 ± 0.84[b]	9.93 ± 0.68[b]

Table 5. Effect of γ-ORY administration on GPx, GR and GST activities in liver of mice exposed to LPS + D-GalN

Groups	GPx (mmol NADPH/min/mg protein)	GR (mmol NADPH/min/mg protein)	GST (µmol/min mg protein)
Control	185.4 ± 9.49	12.20 ± 0.47	68.92 ± 2.72
γ-ORY	184.8 ± 9.01	11.42 ± 0.70	72.23 ± 2.71
Silymarin	188.6 ± 8.99	11.64 ± 0.83	69.80 ± 2.40
LPS/D-GalN	123.8 ± 6.67[a]	20.51 ± 0.53[a]	115.6 ± 6.21[a]
γ-ORY + LPS/D-GalN	177.8 ± 14.62[b]	14.89 ± 0.80[b]	86.74 ± 3.80[ab]
Silymarin + LPS/D-GalN	184.6 ± 10.20[b]	13.79 ± 1.15[b]	82.26 ± 4.49[b]

Table 6. Effect of γ-ORY administration on TNF-α and IL-1β plasmatic levels of mice exposed to LPS and D-GalN

Groups	**TNF-α** (pg/ mg)	**IL-1β** (pg/ mg)
Control	38.60 ± 3.44	23.80 ± 2.57
γ-ORY	36.60 ± 2.15	35.20 ± 2.85
Silymarin	37.40 ± 2.80	16.60 ± 1.72
LPS/D-GalN	307.8 ± 24.02[a]	250.6 ± 21.05[a]
γ-ORY + LPS/D-GalN	131.8 ± 27.17[ab]	76.40 ± 9.34[ab]
Silymarin + LPS/D-GalN	111.4 ± 15.8[ab]	57.00 ± 8.97[b]

PARTE III

Evaluation of the protective effect of gamma oryzanol on inflammatory markers in liver injury induced by carbon tetrachloride in mice

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Evaluation of the protective effect of gamma oryzanol on inflammatory markers in liver injury induced by carbon tetrachloride in mice

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Abstract

Objective: The aim of this study was to evaluate the possible hepatoprotective effect, anti-inflammatory and anti apoptotic of γ-ORY in acute liver injury induced by CCl_4 in mice.

Methods: γ-ORY was administered orally (50 mg/kg) for one week, after 1 hour of the last administration damage was induced by a single dose of CCl4 (1 mg / kg i.p.). Also the following biochemical tests: Lipid peroxidation (TBARS), Determination of non-protein thiols (NPSH), Ascorbic Acid (AA), levels of bilirubin. Markers of hepatic damage: Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH). Inflammatory markers: TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 and IFN-γ. Apoptotic markers: Caspase 3 and 9.

Results: As the histological analysis the γ-ORY was able to attenuate liver injury caused by the model. γ-ORY was able to decrease the lipid peroxidation caused by CCl_4, and restore the activity of the enzymes marker of liver damage, all enhanced by the model similar to one reference drug silymarin. Was effectively modulating the inflammatory markers, proving its anti-inflammatory wont. Was also able to modulate the caspase decreasing apoptosis.

Conclusion: This study demonstrated a protective effect of γ-ORY in acute liver injury induced by CCl_4 in mice, indicating that it could be useful as a therapy for the treatment of acute hepatic damage.

Keywords: Anti-inflammatory, Hepatoprotective, Apoptotic modulator, Antioxidant, Rice bran oil

Introduction:

Liver disease has been confirmed as one of the most serious health problems in the world [1]. The condition is usually caused by viral infection, non-alcoholic steatohepatitis or other hepatotoxic agent that are highly associated with acute or chronic inflammation [2]. The hepatic inflammation is mediated by the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2) [3]. It is well known that the hepatotoxicity induced by carbon tetrachloride (CCl_4) via trichloromethyl-free radicals (CCl_3 or CCl_3OO). During the course of liver injury, CCl_4 is metabolized by cytochrome P450 to form reactive intermediates such as trichloromethyl free radicals (CCl_3) and peroxyl radical ($OOCCl_3$), which then initiate lipid peroxidation associated with cellular damage [4].

Of note, the subsequente activation of Kupffer cells along with increased production of proinflammatory cytokines such as TNF-α, IL-6 and IL-8 is also a contributing factor to the development of CCl4-induced acute liver injury [4]. Is the well characterized animal model for evaluating the therapeutic potential of dietary antioxidants [5]. It is also known that CCl4, mechanism of action is the production of inflammatory cytokines and recruitment of inflammatory cells, leading to liver damage and dysfunction architectural [6,7,8]. Importantly, the oxidative stress caused by CCl4 which induces apoptosis and is involved in harmful effects such as cirrhosis and fibrosis in the liver [9]. Nowadays, many hepatoprotective medicines have been widely used, and however, some of them have potential adverse effects [10]. Like this, there is much evidence indicating that natural substances from edible and medicinal plants show highly antioxidant capacity which may antagonize hepatic toxicity caused by CCl_4 [11].

Gamma Oryzanol (γ-ORY) is one of major bioactive components in rice bran, and has been suggested to possess effects, anti-inflammatory [12], and to function as an antioxidant [13,14] (Isram et al. 2009; Xu et al. 2001). γ-ORY is thought to be the main antioxidant componente of rice bran oil [14]. Comparing other components of rice bran oil as vitamin E (α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol), the gamma is the major constituent in rice bran oil. It was demonstrated that the last had higher activities, being 24-methylenecycloartenyl ferulate the one with the highest [15]. The antioxidant potency of γ-ORY makes it a good candidate for pharmaceutical drugs, cosmetic formulations, and health food [16].

In this context, the aim of this study is to evaluate the possible hepatoprotective effect, anti-inflammatory and anti apoptosis of γ-ORY in acute liver injury induced by CCl_4 in mice. Silymarin was also used in our study as a well-known hepatoprotective reference drug extracted from the seeds of Silybum marinum plant and it was used as a positive control [17].

Materials and methods:

Experimental animals

Male adult Swiss mice (25–35 g) from our own breeding colony were used. Animals were kept in separate animal rooms, on a 12 h light/dark cycle, at a temperature of 22±2 ◦C, food and water ad libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil. This study was approved with number of protocol 021/2014.

Reagents

γ-ORY was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). LPS, D-GalN, glutathione reductase from baker's yeast, b-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and oxidised glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co. (USA).

Exposure

The animals were divided into six groups:

Group 1: Olive oil (10 mg/kg, gavage),

Group 2: γ-ORY (50 mg/kg, Per oral, p.o.) + olive oil (10 mg/kg, gavage),

Group 3: Silymarin (200 mg/kg, p.o.) + olive oil (10 mg/kg, gavage),

Group 4: olive oil (10 mg/kg, gavage) + CCl_4 (1 mg/kg body weight, intraperitoneal, i.p.;1:1 in canola oil),

Group5: γ-ORY (50 mg / kg, p.o) + CCl_4 (1 mg/kg, i.p.),

Group 6: Silymarin (200 mg / kg, p.o.) + CCl_4 (1 mg/kg, i.p.).

It was made γ-ORY treatment with once daily orally (50 mg/kg,) for a week after an hour of the last treatment was administered by intraperitoneal CCl_4 (1 mg/kg i.p.). Was expected 24 hours the animals were anesthetized and blood collected by cardiac puncture. After this procedure, mice were their euthanized using sodium pentobarbital (100 mg/kg, i.p.) and livers were removed, dissected and kept on ice until the assay of team. The liver samples were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10 w /v), and centrifuged at 2400g for 15 min. The low-speed supernatants were used for biochemical

analyzes. For histological a small sample was collected and kept in liver formaldehyde to retain its structure.

Histopathological analysis

Small pieces of liver tissues from individual mice were fixed in 10% formalin. For optical microscopy examination, tissues were embedded in paraffin, sectioned at 4µm, and stained with haematoxylin and eosin. Liver sections were numerically graded to assess the histological features for degree of acute hepatic injury. Centrilobular necrosis, or zonal necrosis (damage of several liver cells around the central vein), vascular congestion, megalocytosis, cell infiltration, eosinophilic cells and tumefaction of hepatocytes of liver cells were characterized [18].

Markers of oxidative stress

Thiobarbituric acid reactive species (TBARS)

Lipid peroxidation was estimated by measuring TBARS assay, by a method previously proposed [19]. The low-speed supernatant (S1) of liver, were incubated with 0.8% thiobarbituric acid (TBA), acetic acid buffer pH 3.4 and 8.1% sodium dodecyl sulphate at 95 °C for 2 h. The amount of TBARS produced was measured at 532 nm, using MDA as a biomarker of lipid peroxidation. TBARS levels are expressed as nmol of MDA/mg protein.

Non-protein thiols (NPSH)

NPSH levels were determined by a method previously proposed [20]. To determine NPSH, a sample aliquot was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free –SH groups were determined in

the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The colour reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue.

Ascorbic acid determination (AA)

Ascorbic acid (AA) levels are non-enzymatic antioxidant defenses that are involved in protecting against the injurious effects of RS. AA levels were determined a by a method previously proposed [21] with some modifications. Briefly, S1 was precipitated in 10 % trichloroacetic acid solution. An aliquot of S1 (300 ml) at a final volume of 575 ml of solution was incubated for 3 h at 38°C, then 500 ml H2SO4 65% (v/v) was added to the medium. The reaction product was determined using a colour reagent containing 4.5 mg/ml dinitrophenyl hydrazine and CuSO4 (0.075 mg/ml) at 520 nm. The AA contente is related to the amount of tissue (mmol AA/g tissue).

Markers of hepatic damage

Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities

The enzymes activities was measured using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil). The enzymatic activities were expressed as U/L.

Marker of hepatic functionality

Bilirubin levels

Levels were measured using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil).

Inflammation markers

Interleukins: tumor necrosis factor-alfa (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), growth factor beta-1 (TGF- β1), monocyte chemotactic protein-1 (MCP-1) and interferon-gamma (IFN-γ) Levels

Levels of TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 and IFN-γ were determined in plasma, using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

Markers of the acute inflammatory response

Measurement of hepatic myeloperoxidase (MPO) activity

The extent of neutrophil accumulation in the liver was measured by assaying (MPO) activity by a method previously proposed [22] with a slight modification. After processing the supernatant of liver homogenate, a part of the corresponding pellet (50 mg) was weighed, homogenized in 1 ml of the buffer (0.1 M NaCl, 0.02 M NaH2PO4, 0.015 M EDTA, pH 4.7) and centrifuged at 6000g for 20 min at 4°C. The pellets were then resuspended in 0.5 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% (w/v) hexadecyltrimethylammonium bromide. The suspensions were freeze–thawed three times, heated for 2 h at 60°C to increase myeloperoxidase recovery, and finally centrifuged at 6000g for 20 min at 4°C to separate the supernatants for MPO assay. The reaction was started by mixing 0.2 ml of 1.6 mM 3,30,5,50-tetramethylbenzidine in dimethylsulfoxide with 0.8 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.006% (v/v) H2O2 and 0.2 ml of the 6000g supernatant from the liver tissue sample. MPO activity was assayed by measuring the change in optical density (OD) for 5 min at 650 nm. Results were expressed as change in OD per g of wet tissue.

Nitric oxide (NO) levels

The liver was dissected and homogenized with ZnSO4 (200 mM) and acetonitrile (96 %), centrifuged at 16,0009g at 4 °C for 30 min and the supernatant was collected. The supernatant was used to determine the nitrate and nitrite content, an indicator of nitric oxide (NO) production by a method previously proposed [23]. Nitrate/nitrite content was estimated in a medium containing 300 ml of 2 % VCl3 (in 5 % HCl), 200 ml of 0.1 % N-(1-naphthyl) ethylene-diamine dihydrochloride and 200 ml of 2 % sulfanilamide (in 5 % HCl). After incubating at 37 °C for 60 min, nitrite levels were determined spectrophotometrically at 540 nm, based on the reduction of nitrate to nitrite by VCl3. Tissue nitrate/nitrite levels were expressed as nmol of NOx/g of tissue.

Apoptotic markers

Caspase 3 and 9 activities assay

Caspase 3 and 9 activities in liver were measured using a Caspase-Glo assay kit (Promega, Madison, USA) according to the manufacturer's instructions.The activities was calculated as pmol/min/mg protein.

Statistical Analysis:

The results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a Two-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. The level of significance was set at $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results:

Histopathological analysis

The liver tissue from mice exposed to CCl_4 when compared with the control group (Figure A) revealed extensive injuries, characterized by vascular congestion, megalocytosis, cell infiltration, eosinophilic cells and tumefaction (Figure D). γ-ORY significantly reduced the liver damage and markedly ameliorated the degree of liver damage (Figure E). It was observed that γ-ORY administration at the dose of 50 mg/kg p.o. did not cause changes to the liver histopathological analysis in this study (Figure B). Silymarin caused no changes in the histopathological analysis of mouse liver (Figure C). Silymarin significantly reduced the liver damage and markedly ameliorated the degree of liver damage (Figure F).

Measurement of hepatic myeloperoxidase (MPO) activity

Two-way ANOVA of plasma MPO activity revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 13.88$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY reversed the increase in the MPO activity induced by CCl_4 administration (Table 1). Two-way ANOVA of plasma MPO activity revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 13.59$; $p<0.002$). The post-hoc comparison revealed that the silymarin reversed the MPO activity caused by administration of CCl_4 (Table 1).

Nitric oxide (NO) levels

A significant main effect of CCl4 ($F(1,16) = 16.40$; $p<0.001$) on NO levels in mice livers was observed (Table 1). Results demonstrated that CCl4 exposure caused a decrease in NO levels, and treatment with γ-ORY did not modify this levels.

A significant main effect of CCl4 ($F(1,16) = 17.90$; $p<0.01$) and silymarin ($F(1,16) = 8.96$; $p< 0.001$) on NO levels in mice livers was observed (Table 1). Treatment with

silymarin significantly increased the NO levels induced by CCl4. NO levels remained unaltered in the livers of mice which received γ-ORY + vehicle and silymarin + vehicle (Table 1).

Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities

Two-way ANOVA of plasma AST activity revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 22.63$; $p<0.001$). The post-hoc comparison revealed that the γ-ORY prevented significantly AST activity increased by CCl_4 (Table 2). Two-way ANOVA of plasma AST activity revealed a significant silymarin x CCl_4 ($F(1,16) = 14.39$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with silymarin prevented significantly AST activity increased by CCl_4 (Table 2).

Two-way ANOVA of plasma ALT activity revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 17.09$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY reversed the increase in the ALT activity induced by CCl_4 administration (Table 2). Two-way ANOVA of plasma ALT activity revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 14.79$; $p<0.001$). Post-hoc comparison revealed that the silymarin reversed the ALT activity caused by administration of CCl_4 (Table 2).

Two-way ANOVA of plasma ALP activity revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 22.01$; $p<0.001$). Post-hoc comparison revealed that the γ-ORY reversed liver damage caused by CCl_4 (Table 2). Two-way ANOVA of plasma ALP activity revealed a significant silymarin x CCl_4 interaction ($F(1,16) =23.36$; $p<0.001$). Post-hoc comparison reversed the ALP activity caused by administration of CCl_4 (Table 2).

Two-way ANOVA of plasma GGT activity revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 17.74$; $p<0.001$). Post-hoc comparison revealed that the γ-ORY reversed liver damage caused by CCl_4 (Table 2). A two-way ANOVA of plasma GGT activity revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 16.22$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with silymarin reversed liver damage caused by CCl_4 (Table 2).

Two-way ANOVA of plasma LDH activity revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 14.96$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY prevented significantly LDH activity increased by CCl_4 (Table 2). Two-way ANOVA of plasma LDH activity revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 12.23$; $p<0.003$). The post-hoc comparison revealed that the silymarin reversed the LDH activity caused by administration of CCl_4 (Table 2).

Thiobarbituric acid reactive species (TBARS)

A significant and principal effect of CCl_4 ($F1,16= 37.65$; $p<0.001$) on lipid peroxidation levels in mice livers was observed (Table 3). Results demonstrated that CCl_4 exposure caused an increase in lipid peroxidation. Treatment with γ-ORY and sylimarin significantly decreased the lipid peroxidation levels induced by CCl_4. Lipid peroxidation levels remained unaltered in the mice livers which received only γ-ORY (Table 3).

NPSH levels

A significant main effect of CCl_4 ($F(1,16) = 47.36$; $p<0.001$) on NPSH levels in mice livers was observed (Table 3). Results demonstrated that CCl_4 exposure caused a decrease in NPSH levels, and treatment with γ-ORY significantly increased this levels.

A significant main effect of CCl_4 ($F(1,16) = 47.41$; $p<0.01$) and silymarin ($F(1,16) = 26.14$; $p< 0.001$) on NPSH levels in mice liver was observed (Table 3). Treatment with silymarin significantly increased the NPSH levels induced by CCl_4.

Determination (AA)

A significant main effect of CCl_4 ($F(1,16) = 58.27$; $p<0.001$) on AA levels in mice livers was observed (Table 3). Results demonstrated that CCl_4 exposure caused a decrease (52.92%) in AA levels, and treatment with γ-ORY significantly increased this levels.

A significant main effect of CCl4 ($F(1,16) = 45.62$; $p<0.01$) and silymarin on AA levels in mice livers was observed (Table 3). Treatment with silymarin significantly increased the AA levels induced by CCl4.

Bilirubin levels

Two-way ANOVA of liver Bilirubin levels yielded a significant γ-ORY x CCl_4 interaction ($F(1,16) = 19.31$; $p<0.001$). Post-hoc comparison showed that γ-ORY was able to decrease the levels of bilirubin in the group where the damage was induced by hepatic CCl_4 (Table 3). Two-way ANOVA of liver Bilirubin levels yielded a significant silymarin x CCl_4 interaction ($F(1,16) = 17.47$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with silymarin was able to decrease the levels of bilirubin in the group where the damage was induced by hepatic CCl_4 (Table 3).

Interleukins: TNF-α, IL-1β, IL-6, TGF- β1, MCP-1 and IFN-γ levels

Two-way ANOVA of TNF-α levels revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 12.72$; $p<0.002$). Post hoc comparisons showed that the γ-ORY was able to decrease the levels of TNF-α in plasma of mice with liver injury induced by CCl_4 (Figura 1). Two-way ANOVA of TNF-α levels revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 8.45$; $p<0.01$). It has been demonstrated by post hoc comparisons

that treatment with silymarin was able to decrease the levels of TNF-α in plasma of mice with liver injury induced by CCl_4 (Figura 1).

Two-way ANOVA of IL-1β levels revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 8.38$; $p<0.01$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to decrease the levels of IL-1β in plasma of mice with liver injury induced by CCl_4 (Figura 1). Two-way ANOVA of IL-1β levels revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 4.80$; $p<0.04$). Post hoc comparisons showed that the silymarin was able to decrease the levels of IL-1β in plasma of mice with liver injury induced by CCl_4 (Figura 1).

Two-way ANOVA of IL-6 levels revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 25.83$; $p<0.001$). Post hoc comparisons showed that the γ-ORY was able to decrease the levels of IL-6 in plasma of mice with liver injury induced by CCl_4 (Figura 1). Two-way ANOVA of IL-6 levels revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 15.71$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with silymarin was able to decrease the levels of IL-6 in plasma of mice with liver injury induced by CCl_4 (Figura 1).

Two-way ANOVA of TGF-β1 levels revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 38.01$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to decrease the levels of TGF-β1 in plasma of mice with liver injury induced by CCl_4 (Figura 1). Two-way ANOVA of TGF-β1 levels revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 26.68$; $p<0.001$). Post hoc comparisons showed that the silymarin was able to decrease the levels of TGF-β 1 in plasma of mice with liver injury induced by CCl_4 (Figura 1).

A significant main effect of CCl_4 ($F(1,16) = 106.92$; $p<0.001$) on MCP-1 levels in mice livers was observed (Figura 1). Results demonstrated that CCl_4 exposure caused a decrease in MCP-1 levels, and treatment with γ-ORY did not modify this levels. Two-way ANOVA of MCP-1 levels revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 15.65$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with silymarin was able to decrease the levels of MCP-1 in plasma of mice with liver injury induced by CCl_4 (Figura 1).

Two-way ANOVA of IFN-γ levels revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 17.94$; $p<0.001$). It has been demonstrated by post hoc comparisons that treatment with γ-ORY was able to decrease the levels of IFN-γ in plasma of mice with liver injury induced by CCl_4 (Figura 1). A two-way ANOVA of IFN-γ levels revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 26.85$; $p<0.001$). Post hoc comparisons showed that the silymarin was able to decrease the levels of IFN-γ in plasma of mice with liver injury induced by CCl_4 (Figura 1).

Caspase 3 and 9 activities assay

Two-way ANOVA of activity of caspase 3 revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 10.69$; $p<0.004$). Post hoc comparisons showed that the γ-ORY was able to decrease the activity of caspase 3 in the liver of mice with liver injury induced by CCl_4 (Figure 2). Two-way ANOVA of activity of caspase 3 revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 6.17$; $p<0.02$). It has been demonstrated by post hoc comparisons that treatment with silymarin was able to decrease the activity of caspase 3 in the liver of mice with liver injury induced by CCl_4 (Figure 2).

Two-way ANOVA of activity of caspase 9 levels revealed a significant γ-ORY x CCl_4 interaction ($F(1,16) = 11.01$; $p<0.004$). It has been demonstrated by post hoc

comparisons that treatment with γ-ORY was able to decrease the activity of caspase 9 in the liver of mice with injury induced by CCl_4 (Figure 2). Two-way ANOVA of activity of caspase 9 revealed a significant silymarin x CCl_4 interaction ($F(1,16) = 10.63$; $p<0.004$). Post hoc comparisons showed that the silymarin was able to decrease the activity of caspase 9 in the liver of mice with injury induced by CCl_4 (Figure 2).

Discussion:

In the present study, CCl_4 administration to mice induced severe hepatic damage, which was attenuated by γ-ORY treatment. Carbon tetrachloride (CCl_4) is a toxic substance used to induce liver damage in rats [24]. The hepatoprotective effects of γ-ORY in the liver injury were evidenced by the suppressed elevation of biochemical markers of hepatic injury in serum (AST, ALT, ALP, GGT, LDH and bilirubin) and alleviated histopathological lesions. CCl_4 is metabolized by cytochrome P450 in the liver, leading to hepatotoxic metabolites, both are free radicals, peroxyl trichloromethyl and trichloromethyl. The free radicals stimulate tissue macrophages to produce proinflammatory cytokines, which subsequently attract inflammatory cells into the liver [25]. Thus, the protective effect of γ-ORY were associated with inflammatory injury because the levels of plasmatic pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 and IFN-γ) decreased significantly. The increase in inflammatory cytokines can induce activation of apoptotic mediators (Caspase 3 and 9), which was γ-ORY capable of modulating the activity. In addition, the CCl_4-induced elevation of the hepatic contents of MDA was also suppressed by γ-ORY. Furthermore, γ-ORY showed a remarkable hepatoprotective effect similar to that of silymarin, a positive control, CCl_4–induced hepatic damage.

Excessive production of ROS attack the biological membrane, which can lead to the oxidative destruction of the membrane polyunsaturated fatty acids through lipid

peroxidation [25]. TBARS in our study, were significantly elevated due to the administration of treatment with CCl_4 and γ-ORY was able to significantly reduce the levels of TBARS similar silymarin used as positive control. Confirming that the γ-ORY was capable of reducing the lipid peroxidation induced by CCl_4. The NPSH constitutes (mainly GSH) are the first line of defense against free radicals. The CCl_4 caused a depletion of NPSH levels and γ-ORY did not exert a protective unlike that silymarin increased levels of NPSH effect, keeping antioxantes defenses. Ascorbic acid is considered a marker of oxidative stress and the reduction of its content may indicate an increase in oxidative stress [21]. In our study, administration of CCl_4 decreased levels of AA and γ-ORY was not able to reverse this levels. Already, silymarin was able to increase the levels of AA. Thus, increased lipid peroxidation and depletion of non-enzymatic antioxidant defenses, contributes pro increased synthesis of pro-inflammatory cytokines.

The loss of membrane integrity caused by lipid peroxidation leads to the release of the cytosolic contents from ruptured hepatocytes such as ALT and AST [26]. The administration of CCl_4 showed an acute hepatotoxicity, because it elevated the activity of marker enzymes of liver damage AST, ALT, ALT, GGT and LDH. The γ-ORY was able to enhance the enzymatic activity of all enzymes in the same manner as silymarin. Thus confirming its hepatoprotective effect. MPO is an important enzyme abundantly present in neutrophils and involved in the catalysis of H2O2 to hypochlorous acid (HOCl) [27]. HOCl contributes to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates [28]. The CCl_4 to be a causative agent of damage, eventually increasing the enzyme MPO activity, the γ-ORY was able to restore the enzymatic activity of MPO, the same happened with silymarin. Another marker that evaluates the liver function is bilirubin. Accumulating evidences have

showed that bilirubin is an efficient scavenger of ROS and nitric oxide [29,30], decrease the level of pro-inflammatory cytokines and inhibited the migration of immune cell [31,32]. Levels of bilirubin in our study were high with CCl_4 administration for the γ-ORY was able to decrease the levels almost control similar silymarin. Inferring so had a positive mechanism on a marker of liver function. It can also be the mechanism by which γ-ORY has the anti-inflammatory effect because studies show that normal levels of bilirubin cause a decrease in pro-inflammatory cytokines.

In liver injury, the injured cells release a number of cytokines and stimulate the kupffer cells to release more inflammatory mediators and various free radicals [33]. Some studies have demonstrated that NO regulates pro-inflammatory cytokine expression during inflammatory processes [34,35]. Already in the levels of NO were increased by CCl_4 and the γ-ORY or silymarin were able to decrease these levels. An early rise of TNF-α level induces pro-inflammatory genes [36]. The IL-1β and IL-6 are the cytokines with multiple and diverse regulatory roles. They are pro-inflammatory molecules (TNF-α, IL-1β, IL-6, TGF- β1, MCP-1 and IFN-γ) which induce cytotoxic effects, such as the activation of synthesis and secretion of acute phase proteins [37,38], and oxidative cell damage [39]. The CCl_4 was able to increase the levels of inflammatory mediators such as TNF-α, IL-1β and IL-6, since the γ-ORY was able to restore the levels of these pro-inflammatory cytokines, the same happened with silymarin which is a hepatoproteor drug. Thus it is evident that the γ-ORY is able to mediate pro-inflammatory cytokines. TGF-β1 is an anti-inflammatory cytokine which plays an important role in the inflammatory process. It has become evident that TGF-β1 not only inhibits IL-1β production [40] but also blocks IL-1β-stimulated IFN production [41]. IFN-γ is a potent cytokine produced by cells activation of cells in the inflammatory process. During liver injury, IFN-γ is produced as a self-defensive mechanism [42]. MCP-1 is a key pro-

inflammatory cytokine inflammatory process, because it activates signaling pathways for numerous other inflammatory cytokines. The administration of CCl_4 caused an increase in synthesis of interleukins TGF-β1, IFN-γ and MCP-1, the γ-ORY was able to prevent the increase reducing the levels of interleukins, the same effect was silymarin. These results indicated that γ-ORY might suppress the inflammatory response via the inhibition of pro-inflammatory cytokines supporting our biochemical and histological analysis.

Several studies have shown that liver damage induced by CCl_4 is partly involved in the apoptosis pathway in vivo and in vitro [43,44]. CCl_4 damages liver mitochondria by inhibiting cytochrome oxidase and by enhancing oxidative stress [45]. Mitochondrial initiated apoptosis triggered by ROS plays an important role in CCl_4-induced hepatotoxicity, and antioxidants effectively reversed liver damage caused by CCl4 [44]. Apoptosis or programmed cell death is an important process involved in normal development and cell maintenance. The apoptotic process can cause serious damage to the liver. Usually the apoptotic process is activated by caspases. The main intrinsic pathway is characterized by mitochondrial dysfunction, with the release of cytochrome c, activation caspase 9, and subsequently of caspase 3 [46]. In our study CCl_4 highly activities of caspases 3 and 9, increasing the apoptotic process in cells of the liver, the γ-ORY was able to normalized activities of caspases and demonstrated its anti-apoptotic effect, the same happened with silymarin. These results appear to be mediated by anti-inflammatory and antioxidant effect of both.

Conclusion:

Thus, the results of our present study found that γ-ORY significantly attenuated CCl_4–induced liver injury in mice largely due to its anti-inflammatory effect by associating with its antioxidant effect. These results indicated that the beneficial effects of treatment

with the γ-ORY reestablishment of liver function is mediated by anti-inflammatory action. Was also effective against pro-inflammatory cytokines, thereby reducing inflammation caused by CCl_4. It was also able to modulate the activity of caspases, thus decreasing apoptosis activated by pro-inflammatory cytokines. It is therefore suggested that the γ-ORY may represent a new type of protective agent against hepatic damage caused by CCl_4 and it may provide a potent hepatoprotective effect for clinical use.

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Legends

Fig A,B,C,D,E,F. Photomicrography of segment of the hepatic lobe (A) of an animal control. (B) of an animal treated with γ-Oryzanol. (C) of an animal treated with silymarin. (D) of an animal treated with CCl4 showed necrosis. (E) of an animal treated with γ-Oryzanol + CCl4. (F) of an animal treated with silymarin + CCl4. Note the hepatocyte strings (arrow), the centrilobular vein (VC) and sinusoid capillaries (*) with normal aspect in figures 1A, 1B and 1C. Observe around the centrilobular vein (VC) some hepatocytes with vacuolation and acidophilic cytoplasm (arrows). H.E. 100X.

Fig 1-A. Effect of administration of γ-ORY in the levels of interleukin TNF-α in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the CCl_4.

Fig 1-B. Effect of administration of γ-ORY in the levels of interleukin IL-1β in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the CCl_4.

Fig 1-C. Effect of administration of γ-ORY in the levels of interleukin IL-6 in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the CCl_4.

Fig 1-D. Effect of administration of γ-ORY in the levels of interleukin TGF-β1 in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the CCl_4.

Fig 1-E. Effect of administration of γ-ORY in the levels of interleukin MCP-1 in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the CCl_4.

Fig 1-F. Effect of administration of γ-ORY in the levels of interleukin IFN-γ in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the CCl_4.

Fig 2-A. Effect of administration of γ-ORY activity (%) of caspase 3 in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p< 0.001$ as compared to the CCl_4.

Fig 2-B. Effect of administration of γ-ORY activity (%) of caspase 9 in the liver of mice exposed to CCl_4. Values are mean±S.E.M. (n=6 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p< 0.001$ as compared to the CCl_4.

Table 1. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted p<0.001 as compared to the control group. b Denoted p<0.001 as compared to the CCl4.

Table 2. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted p<0.001 as compared to the control group. b Denoted p<0.001 as compared to the CCl4.

Table 3. Data are expressed as means ± S.E.M of 6 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted p<0.001 as compared to the control group. b Denoted p<0.001 as compared to the CCl4.

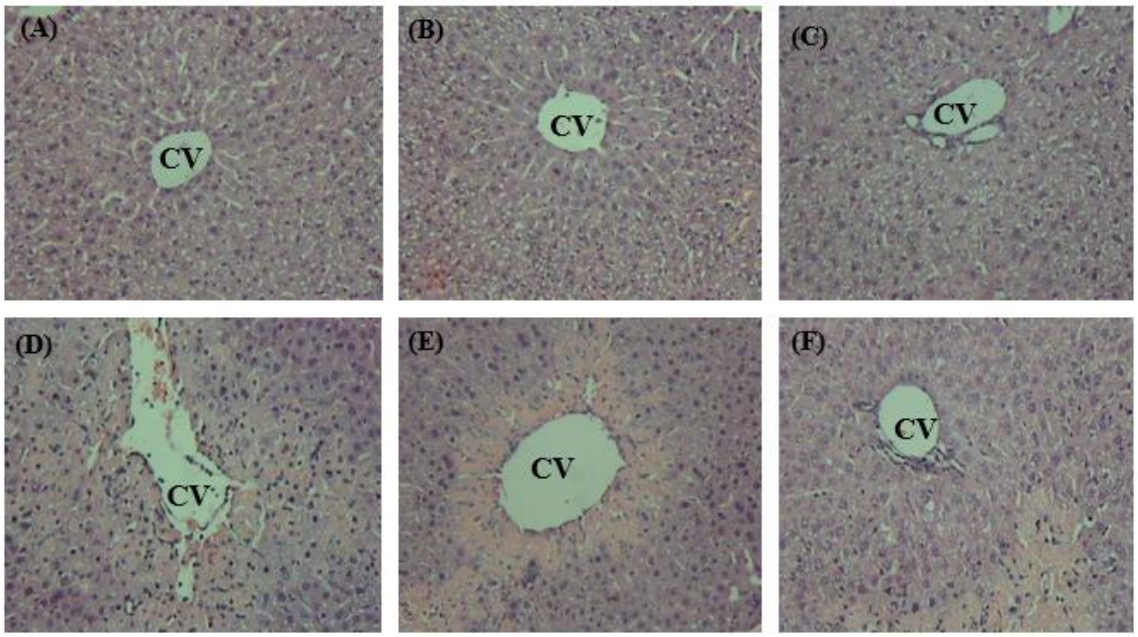

Fig. A,B,C,D,E,F

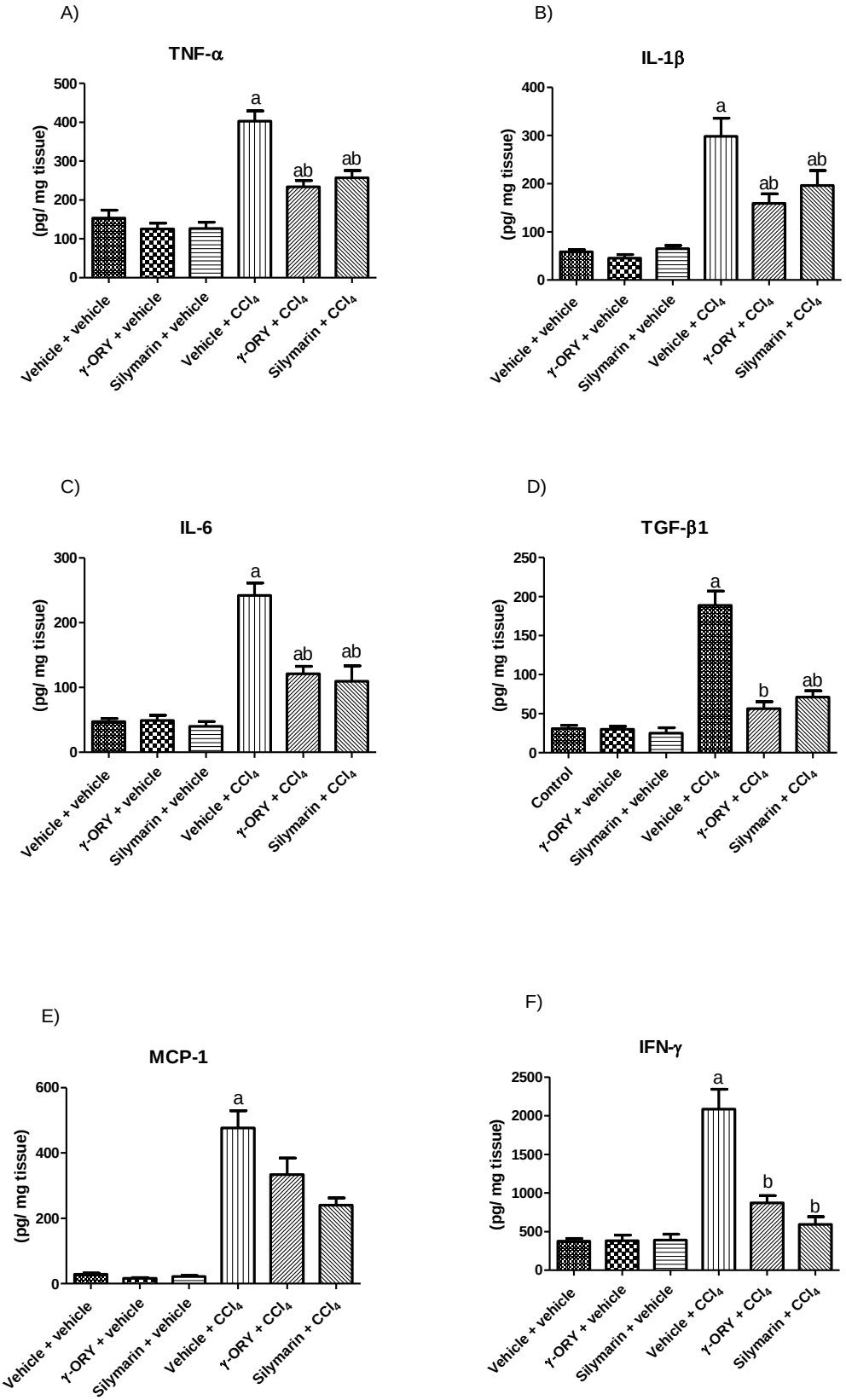
A)
TNF-α
(pg/ mg tissue)
500
400
300
200
100
0
a
ab
ab
Vehicle + vehicle
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4
B)
IL-1β
(pg/ mg tissue)
400
300
200
100
0
a
ab
ab
Vehicle + vehicle
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4
C)
IL-6
(pg/ mg tissue)
300
200
100
0
a
ab
ab
Vehicle + vehicle
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4
D)
TGF-β1
(pg/ mg tissue)
250
200
150
100
50
0
a
b
ab
Control
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4
E)
MCP-1
(pg/ mg tissue)
600
400
200
0
a
Vehicle + vehicle
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4
F)
IFN-γ
(pg/ mg tissue)
2500
2000
1500
1000
500
0
a
b
b
Vehicle + vehicle
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4

Fig 1. A,B,C,D,E,F

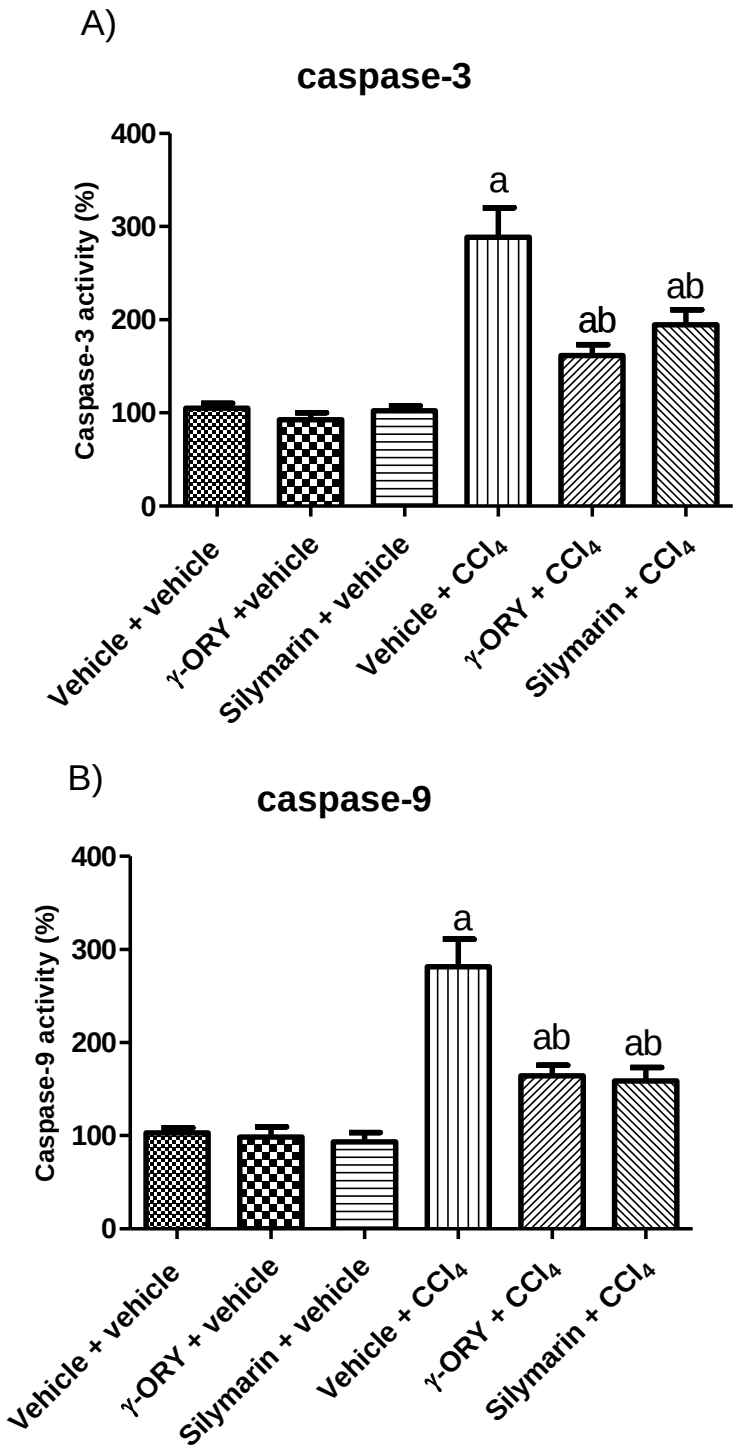
A)
caspase-3
Caspase-3 activity (%)
400
300
200
100
0
a
ab
ab
Vehicle + vehicle
γ-ORY +vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4
B)
caspase-9
Caspase-9 activity (%)
400
300
200
100
0
a
ab
ab
Vehicle + vehicle
γ-ORY + vehicle
Silymarin + vehicle
Vehicle + CCl4
γ-ORY + CCl4
Silymarin + CCl4

Fig. 2 A,B

Table 1. Effect of γ-ORY administration on enzyme MPO activity and NO levels in liver of mice exposed to CCl_4

Groups	**MPO** (U/mg liver)	**NO** (nmol NOx/g tissue)
Vehicle + vehicle	0.59 ± 0.05	19.60 ± 2.89
γ-ORY + vehicle	0.50 ± 0.06	15.20 ± 2.03
Silymarin + vehicle	0.44 ± 0.05	16.00 ± 1.41
Vehicle + CCl_4	2.93 ± 0.32[a]	37.00 ± 4.13[a]
γ-ORY + CCl_4	1.34 ± 0.22[ab]	22.00 ± 2.47
Silymarin + CCl_4	1.22 ± 0.25[ab]	23.20 ± 2.51

Table 2. Effect of γ-ORY administration on AST, ALT, ALP, GGT and LDH activities in blood of mice exposed to CCl_4

Groups	**AST** (U/L)	**ALT** (U/L)	**ALP** (U/L)	**GGT**(U/L)	**LDH** (U/L)
Vehicle + vehicle	54.00 ± 6.67	47.80 ± 6.85	210.2 ± 17.78	20.80 ± 2.97	331.2 ± 23.92
γ-ORY + vehicle	52.00 ± 8.97	54.60 ± 8.55	165.6 ± 12.50	26.20 ± 4.85	354.2 ± 61.41
Silymarin + vehicle	33.60 ± 3.82	54.20 ± 7.72	175.8 ± 20.36	27.20 ± 4.65	344.4 ± 33.63
Vehicle + CCl_4	397.0 ± 47.14^{a}	399.6 ± 51.35^{a}	1223 ± 146.8^{a}	127.0 ± 16.57^{a}	2350 ± 400.4^{a}
γ-ORY + CCl_4	131.6 ± 24.27^{b}	172.0 ± 21.39^{ab}	440.0 ± 52.64^{b}	51.20 ± 8.04^{b}	775.0 ± 78.00^{b}
Silymarin + CCl_4	188.0 ± 13.81^{ab}	199.0 ± 12.41^{ab}	406.0 ± 62.90^{b}	59.00 ± 6.00^{ab}	927.0 ± 81.36^{b}

Table 3. Effect of γ-ORY administration on TBARS, NPSH, AA in blood and Bilirubin levels in liver of mice exposed to CCl_4

Groups	TBARS (nmol MDA equivalents/g tissue)	NPSH (µmol/g tissue)	Ascorbic Acid (µg AA/g tissue)	Bilirubin (mg/dL)
Vehicle + vehicle	14.70 ± 1.04	39.80 ± 2.50	291.2 ± 10.38	58.80 ± 4.74
γ-ORY + vehicle	11.90 ± 0.86	47.76 ± 2.94	300.4 ± 16.82	45.60 ± 7.44
Silymarin + vehicle	10.92 ± 0.93	53.24 ± 3.71	277.6 ± 18.97	65.20 ± 6.53
Vehicle + CCl_4	110.6 ± 8.72[a]	16.82 ± 1.99[a]	154.0 ± 16.99[a]	298.6 ± 37.72[a]
γ-ORY + CCl_4	38.88 ± 6.93[ab]	31.90 ± 3.59[b]	209.2 ± 14.70[b]	159.4 ± 19.84[ab]
Silymarin + CCl_4	37.10 ± 6.39[ab]	34.42 ± 3.57[b]	199.6 ± 16.09[b]	196.6 ± 30.99[ab]

PARTE IV

Possible protective effect of gamma oryzanol on oxidative stress and apoptotic markers in liver injury induced by paracetamol in mice

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Possible protective effect of gamma oryzanol on oxidative stress and apoptotic markers in liver injury induced by paracetamol in mice

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Abstract

The liver is an important organ of vertebrates. Liver diseases affect thousands of people annually. Paracetamol (PCM) is an analgesic drug which is used as an overdose of liver damage model which mimics in rodents. The γ-oryzanol (γ-ORY) is one of the constituents of rice bran oil, being a bioactive compound with antioxidant activity. The aim of this study is to verify the possible hepatoprotective and anti apoptotic effect of γ-ORY in two different doses in hepatic damage caused by PCM in mice model. The animals were divided into six groups of five animals per group. The animals received γ-ORY for two days in two doses (10mg and 50mg /kg p.o.), on the third day PCM (2 g/ kg, p.o.). After 24 hours the animals were euthanized. Also the following biochemical tests: Lipid peroxidation (TBARS), Determination of non-protein thiols (NPSH), Ascorbic Acid (AA), 4-Hydroxynonenal (4-HNE). Markers of hepatic damage: Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH). Oxidative stress markers: Catalase (CAT), Glutathione S-transferase (GST), Superoxide dismutase (SOD), δ-Aminolevulic acid dehydratase (δ-ALA-D), Glutathione peroxidase (GPx), Glutathione Reductase (GR). Apoptotic markers: Caspase 3 and 9. As the histological analysis the γ-ORY was able to attenuate liver injury caused by the model. The γ-ORY was able to decrease lipid peroxidation, which was increased due to induce oxidative stress model. Also mediated the activity of marker enzymes of liver damage, proving to be hepatoprotective. With respect to oxidative stress sensitive enzymes, γ-ORY was capable of mediating the activity thereof. The activity of apoptotic markers, was able to decrease the activity thereof, both doses of γ-ORY proved effective.

Keywords: Antioxidant, Hepatoprotective, Apoptotic mediator

1. Introduction

The liver is vital for vertebrate organ as it is involved in the synthesis of proteins, carbohydrates and lipids, as well as being responsible for the metabolism of several compounds**.** Liver diseases such as viral hepatitis, alcoholic liver injury, drug-induced liver injury, liver fibrosis and autoimmune hepatitis represent major threats to human health worldwide (Herkel et al., 2005). About 25,000 deaths found every year due to liver disorders (Sharma and Sharma, 2010). Paracetamol (PCM) is a drug used as an analgesic and antipyretic. The main problem with this medication is misuse through intentional or unintentional ingestion of supratherapeutic dosages, which usually lead to hepatic necrosis (Bond et al., 2003). Most of the PCM is rapidly metabolized in the liver by conjugation with glucuronic acid (40–67%) and sulfates (20–46%) (Hung and Nelson, 2004). A smaller part is metabolized via the cytochrome P450 producing a metabolite is more reactive than N-acetyl-p-benzoquinonimine (NAPQI). However, when an overdose of PCM primary conjugation pathways become saturated and sulfation occurs, and the pathway used NAPQI more, making the detoxification of this metabolite inefficient. When high levels of NAPQI has the same covalently linking the membrane proteins of hepatocytes.

This situation leads to the formation of reactive oxygen and nitrogen species, and initiates lipid peroxidation that eventually results in destruction, necrosis or apoptosis of the liver cells (Hinson et al., 2004; James et al., 2003; Nelson, 1990). Several complex mechanisms were found to be involved in acetaminophen toxicity; oxidative stress (Ahmed and Khater, 2001; Srinivasan et al., 2001), inflammatory mediators, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) (Gardner and Laskin, 2007; Laskin and Gardner, 2007; Laskin, 2009). It is believed that the ROS may injure cell

membranes through lipid peroxidation and modify or damage biomolecules, i.e., proteins, lipids, carbohydrates and DNA *in* vitro and *in* vivo (Halliwell, 1996).

Compounds having antioxidant properties have been tested to prevent the toxicity of PCM. Thus, gamma oryzanol (γ-ORY) is a major component found in rice bran oil. γ-ORY is a mixture of esters of trans-ferulic acid. γ-ORY is one of major bioactive components in rice bran, and has been suggested to possess effects of lowering serum cholesterol levels (Rong et al., 1997; Wilson et al., 2007), anti-inflammatory effects (Akihisa et al. 2000), an anti-cancer effect (Yasukawa et al. 1998) and to function as an antioxidant (Isram et al. 2009; Xu et al. 2001). The antioxidant potency of γ-ORY makes it a good candidate for pharmaceutical drugs, cosmetic formulations, and health food (Juliano et al., 2005). The aim of this study is to verify the possible hepatoprotective by analysis of aminotransferases, enzymatic markers of oxidative stress, oxidative stress markers non-enzymaticand and apoptosis by measuring the activity of caspases of γ-ORY in two different doses in hepatic damage caused by PCM in mice model.

2. Materials and methods

2.1. Experimental animals

Male adult Swiss mice (25–35 g) from our own breeding colony were used. Animals were kept in separate animal rooms, on a 12 h light/dark cycle, at a temperature of 22±2 ◦C, food and water ad libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil. This study was approved with number of protocol 021/2014.

2.2 Reagents

γ-ORY was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PCM, glutathione reductase from baker's yeast, b-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and oxidised glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co. (USA).

2.3 Exposure

To investigate the possible protective effect of pretreatment with γ-ORY against hepatic oxidative damage induced by PCM exposure to mice, the animals were randomly divided into six groups consisting of five animals each:

Group 1: Canola oil (2.5 ml/kg, per oral p.o.) + saline (5 ml/kg, p.o.)

Group 2: γ-ORY (10 mg/kg of body weight, p.o.) + saline (5 ml/kg, p.o.)

Group 3: γ-ORY (50 mg/kg of body weight, p.o.) + saline (5 ml/kg, p.o.)

Group 4: Canola oil (2.5 ml/kg, per oral p.o.) + PCM (2 g/kg, p.o.)

Group 5: γ-ORY (10 mg/kg of body weight, p.o.) + PCM (2 g/kg, p.o.)

Group 6: γ-ORY (50 mg/kg of body weight, p.o.) + PCM (2 g/kg, p.o.)

In group I, mices received two doses of canola oil, and on the third day mices were given saline. Animals from group II were treated with two doses of γ-ORY 10 mg and, on the third day mices were given saline. In group III, the animals were treated with two doses of γ-ORY 50 mg and, on the third day mices were given saline. In group IV, mices received two doses of canola oil and 24 h after were given PCM according to pilot experiments. In group V, mices received two doses of γ-ORY 10 mg, and on the third day rats were given PCM. Group VI, the animals received two doses of γ-ORY 50

mg, and on the third day rats were given PCM. Was expected 24 hours the animals were anesthetized and blood collected by cardiac puncture.

After this procedure, mice were their euthanized using sodium pentobarbital (100 mg/kg, i.p.) and livers were removed, dissected and kept on ice until the assay of team. The liver samples were homogenized in 50 mM Tris-HCl, pH 7.4 (1/10 w /v), and centrifuged at 2400g for 15 min. The low-speed supernatants were used for biochemical analyzes. For histological a small sample was collected and kept in liver formaldehyde to retain its structure.

2.4 Histopathological analysis

Small pieces of liver tissues from individual mice were fixed in 10% formalin. For optical microscopy examination, tissues were embedded in paraffin, sectioned at 4µm, and stained with haematoxylin and eosin. Liver sections were numerically graded to assess the histological features for degree of acute hepatic injury. Centrilobular necrosis, or zonal necrosis (damage of several liver cells around the central vein), vascular congestion, megalocytosis, cell infiltration, eosinophilic cells and tumefaction of hepatocytes of liver cells were characterized (Wills and Asha, 2006).

2.5 Markers of oxidative stress

2.5.1 Thiobarbituric acid reactive species (TBARS)

The testes were rapidly homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400 x g for 15 min. An aliquot (100 µL) of homogenized w as incubated at 95 °C for 2 h. Thiobarbituric acid reactive species (TBARS) were determined as described by (Ohkawa et al., 1979). TBARS levels are expressed as nmol of MDA/mg protein.

2.5.2 Non-protein thiols (NPSH)

NPSH levels were determined by the method of (Ellman, 1959). To determine NPSH, a sample aliquot was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free–SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The colour reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue.

2.5.3 Ascorbic acid determination (AA)

AA levels are non-enzymatic antioxidant defenses that are involved in protecting against the injurious effects of RS. AA levels were determined as described by (Jacques-Silva et al., 2001) with some modifications. Briefly, S1 was precipitated in 10 % trichloroacetic acid solution. An aliquot of S1 (300 ml) at a final volume of 575 ml of solution was incubated for 3 h at 38°C, then 500 ml H2SO4 65% (v/v) was added to the medium. The reaction product was determined using a colour reagent containing 4.5 mg/ml dinitrophenyl hydrazine and CuSO4 (0.075 mg/ml) at 520 nm. The AA contente is related to the amount of tissue (mmol AA/g tissue).

2.5.4 4-Hydroxynonenal (4-HNE)

4-HNE levels in the liver were determined using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

2.6 Markers of hepatic damage

2.6.1 Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities

The enzymes activities were measured using commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil). The enzymatic activities were expressed as U/L.

2.7 Marker enzymes of oxidative stress

2.7.1 Catalase (CAT) activity

CAT is an enzymatic antioxidant defense that is involved in protecting against the injurious effects of RS. Catalase activity was assayed spectrophotometrically, as described by (Aebi, 1984), which involves monitoring the disappearance of H_2O_2 in the presence of S_1 at 240 nm. Enzymatic reaction was initiated by adding an aliquot of S_1 20 µl, and the substrate (H_2O_2) at a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units of (1 U decomposes 1 µmol of H_2O_2 per minute at pH 7 at 25°C).

2.7.2 Superoxide dismutase (SOD) activity

SOD is an enzymatic antioxidant defense that protects against active free radicals by scavenging excess superoxide. SOD activity in liver homogenate was assayed spectrophotometrically as described by (Misra and Fridovich, 1972). The method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 37 ◦C.

2.7.3 δ-Aminolevulic acid dehydratase (δ-ALA-D) activity

δ-ALA-D activity was assessed by measuring the formation of porphobilinogen (PBG), according to (Sassa, 1982) method, except that 45mM sodium phosphate buffer and 2.2mM ALA were used. Samples were homogenized in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2400 × g for 15 min. An aliquot of 50 µL of

homogenized tissue was incubated for 2 h at 37 ◦C. PBG formation was detected with the addition of modified Erlich's reagent at 555 nm.

2.7.4 Glutathione peroxidase (GPx) activity

GPx activity is measured in a system containing reduced glutathione (GSH) by (Wendel, 1981), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR). GPx acts oxidizing GSH into glutathione disulphide (GSSG). To complete the cycle, GR reduces GSSG back to GSH at the expenses of NADPH. The decline in the concentration of NADPH can be monitored at 340 nm. The activity of GPx is given by the consumption of NADPH in nmol/min/mg of protein.

2.7.5 Glutathione Reductase (GR) Activity

GR activity was determined spectrophotometrically as described by (Calberg and Mannervik, 1985). In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which is followed at 340 nm. GR activity is proportional to NADPH decay. An aliquot of 50llofS1was added in the system containing 0.15 M potassium phosphate buffer, pH7.0, 1.5 mM EDTA, 0.15 mM NADPH. After the basal reading, 50ll of 20 mM GSSG (substrate) was added. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.7.6 Glutathione S-transferase (GST) activity

GST is an enzymatic antioxidant defense that protects against RS-induced injury. GST activity was assayed through the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by (Habig et al., 1974). An aliquot, (100 µl of 0.1 M potassium phosphate buffer (pH 7.4), with CDNB as the substrate) was added to 50 mM GSH. The enzymatic activity was expressed in µmol/min mg protein.

2.8. Apoptotic markers

2.8.1 Caspase 3 and 9 activities assay

Caspase 3 and 9 activities in liver were measured using a Caspase-Glo assay kit (Promega, Madison, USA) according to the manufacturer's instructions.The activities was determined in porcentage.

2.9. Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a Two-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. The level of significance was set at $p<0.05$. The statistical analysis was performed using the software GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

3.Results

3.1 Histopathological analysis

The liver tissue from mice exposed to PCM when compared with the control group (Figure A) revealed extensive injuries, characterized by vascular congestion, megalocytosis, cell infiltration, eosinophilic cells and tumefaction (Figure D). γ-ORY in doses of 10 mg/kg and 50 mg/kg significantly reduced the liver damage and markedly ameliorated the degree of liver damage (Figure E and F). It was observed that γ-ORY administration at the dose of 10 mg/kg p.o. did not cause changes to the liver histopathological analysis in this study (Figure B). It was observed that γ-ORY administration at the dose of 50 mg/kg p.o. did not cause changes to the liver histopathological analysis in this study (Figure C).

3.2 TBARS levels

Two-way ANOVA of TBARS levels revealed a significant γ-ORY x PCM interaction ($F(2,24) = 3.72$; $p<0.03$). Post hoc comparisons showed that PCM increased TBARS levels when compared to control group ($F(1,24) = 55.16$; $p<0.001$) and treatment with γ-ORY in doses of 10 and 50 mg/kg significantly decreased lipid peroxidation levels ($F(2,24) = 3.20$; $p<0.05$) in liver of mice (Table 1).

3.3 NPSH levels

Two-way ANOVA of NPSH levels revealed no significant γ-ORY x PCM interaction ($F(2,24) = 0.21$; $p<0.81$). Post hoc comparisons showed that PCM increased TBARS levels compared to control group ($F(1,24) = 28.63$; $p<0.001$) and treatment with γ-ORY in doses of 10 and 50 mg/kg significantly decreased NPSH levels ($F(2,24) = 8.76$; $p<0.001$) in liver of mice (Table 1).

3.4 AA levels

Two-way ANOVA of AA levels in liver yielded a significant γ-ORY x PCM interaction ($F(2,24) = 4.20$; $p<0.02$). Post hoc comparisons showed that PCM decreased Ascorbic acid levels compared to control group ($F(1,24) = 12.36$; $p<0.001$) and treatment with γ-ORY at 10 and 50 mg/kg significantly increased AA levels ($F(2,24) = 7.79$; $p<0.05$) in liver of mice (Table 1).

3.5 4-HNE levels

Two-way ANOVA of 4-HNE levels revealed a significant γ-ORY x PCM interaction ($F(2,24) = 26.42$; $p<0.001$). Post hoc comparisons showed that PCM increased 4-HNE levels when compared to control group ($F(1,24) = 192.16$; $p<0.001$) and treatment with γ-ORY at 10 and 50 mg/kg significantly decreased 4-HNE levels ($F(2,24) = 39.79$; $p<0.001$) in liver of mice (Table 1).

3.6 Markers of hepatic damage: AST, ALT, ALP, GGT and LDH activities

Two-way ANOVA of plasma AST activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 16.44$; $p<0.001$). Post hoc comparisons revealed that PCM ($F(1,24) = 98.04$; $p<0.001$) increased AST activity compared to the control group. γ-ORY at 10 and 50 mg/kg ($F(2,24) = 17.03$; $p<0.001$) prevented significantly AST activity increased by PCM (Table 2).

Two-way ANOVA of plasma ALT activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 22.23$; $p<0.001$). Post hoc comparisons showed that PCM increased AST activity ($F(1,24) = 157.9$; $p<0.001$) and treatment with γ-ORY at 10 and 50 mg/kg significantly decreased ALT activity ($F(2,24) = 16.95$; $p<0.001$) in liver of mice (Table 2).

Two-way ANOVA of plasma ALP activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 16.91$; $p<0.001$). Post hoc comparisons revealed that PCM ($F(1,24) = 116.6$; $p<0.001$) increased ALP activity compared to the control group. γ-ORY at 10 and 50 mg/kg ($F(2,24) = 18.32$; $p<0.001$) prevented significantly ALP activity increased by PCM (Table 2).

Two-way ANOVA of plasma GGT activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 20.71$; $p<0.001$). Post hoc comparisons showed that PCM increased GGT activity ($F(1,24) = 152.8$; $p<0.001$) and treatment with γ-ORY at 10 and 50 mg/kg significantly decreased GGT activity ($F(2,24) = 14.56$; $p<0.001$) in liver of mice (Table 2).

Two-way ANOVA of plasma LDH activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 17.46$; $p<0.001$). Post hoc comparisons revealed that PCM ($F(1,24) = 118.6$; $p<0.001$) increased LDH activity compared to the control group. γ-

ORY at 10 and 50 mg/kg ($F(2,24) = 20.63$; $p<0.001$) prevented significantly LDH activity increased by PCM (Table 2)

3.7 Enzymes of oxidative stress: CAT, SOD, δ-ALA-D, GPx, GR and GST activities

Two-way ANOVA of CAT activity showed no significant γ-ORY x PCM interaction ($F(2,24) = 1.09$; $p<0.35$). Post hoc comparisons showed that PCM decreased significantly CAT activity ($F(1,24) = 19.65$; $p<0.001$) but treatment with γ-ORY did not modify the inhibition of enzyme activity induced by PCM (Table 3).

Two-way ANOVA of SOD activity showed no significant γ-ORY x PCM interaction ($F(2,24) = 0.16$; $p<0.84$). Post hoc comparisons showed that PCM decreased significantly SOD activity ($F(1,24) = 70.78$; $p<0.001$) but treatment with γ-ORY did not modify the inhibition of enzyme activity induced by PCM (Table 3). γ-ORY treatment was not effective in protecting against inhibition of SOD activity induced by PCM treatment (Table 3). γ-ORY, independent of the dose tested, did not alter SOD activity when compared to the control group (Table 3).

Two-way ANOVA of δ-ALA-D activity showed no significant γ-ORY x PCM interaction ($F(2,24) = 5.23$; $p<0.70$). Post hoc comparisons showed that PCM decreased significantly δ-ALA-D activity ($F(1,24) = 40.66$; $p<0.02$) but treatment with γ-ORY at 10 and 50 ($F(2,24) = 57.12$; $p<0.03$) prevented significantly δ-ALA-D activity increased by PCM (Table 3). γ-ORY, independent of the dose tested, did not alter δ-ALA-D activity when compared to the control group (Table 3).

Two-way ANOVA of plasma GST activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 20.45$; $p<0.001$). Post hoc comparisons revealed that PCM ($F(1,24) = 22.27$; $p<0.001$) increased GST activity compared to the control group. γ-ORY at 10 and 50 mg/kg ($F(2,24) = 8.04$; $p<0.002$) prevented significantly GST

activity increased by PCM (Figure 3-A). γ-ORY, independent of the dose tested, did not alter SOD activity when compared to the control group (Figure 3-A)

Two-way ANOVA of GPx activity showed no significant γ-ORY x PCM interaction ($F(2,24) = 0.48$; $p<0.62$). Post hoc comparisons showed that PCM decreased significantly GPx activity ($F(1,24) = 31.85$; $p<0.001$) but treatment with γ-ORY at 10 and 50 ($F(2,24) = 9.26$; $p<0.001$) prevented significantly GPx activity increased by PCM (Figure 3-B). γ-ORY, independent of the dose tested, did not alter GPx activity when compared to the control group (Figure 3-B).

A two-way ANOVA of plasma GR activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 6.93$; $p<0.004$). Post hoc comparisons showed that PCM increased Gr activity ($F(1,24) = 7.46$; $p<0.01$) and treatment with γ-ORY at 10 and 50 mg/kg significantly decreased GR activity ($F(2,24) = 5.51$; $p<0.01$) in liver of mice (Figure 3-C).

3.8 Apoptotic markers: Caspase 3 and 9 activities

Two-way ANOVA in liver Caspase 3 activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 18.25$; $p<0.001$). Post hoc comparisons revealed that PCM ($F(1,24) = 101.0$; $p<0.001$) increased Caspase 3 activity compared to the control group. γ-ORY at 10 and 50 mg/kg ($F(2,24) = 21.25$; $p<0.001$) prevented significantly Caspase 3 activity increased by PCM (Figure 2-A). γ-ORY, independent of the dose tested, did not alter GPx activity when compared to the control group (Figure 2-A)

Two-way ANOVA in liver Caspase 9 activity revealed a significant γ-ORY x PCM interaction ($F(2,24) = 13.10$; $p<0.001$). Post hoc comparisons revealed that PCM ($F(1,24) = 100.5$; $p<0.001$) increased Caspase 9 activity compared to the control group.

γ-ORY at 10 and 50 mg/kg ($F(2,24) = 17.16$; $p<0.001$) prevented significantly Caspase 9 activity increased by PCM (Figure 2-B).

4. Discussion

The main objective of this study was to evaluate the protective effects and anti apoptotic of γ-ORY in two different doses against PCM hepatotoxicity in mice. We demonstrated that the PCM-induced increases in serum ALT and ALT levels and hepatic histological changes were drastically attenuated by γ-ORY administered two days before the PCM injection in mice. In addition, histopathological changes, such as extensive centrilobular necrosis, hepatocyte degeneration observed in PCM hepatotoxicity, were prevented by γ-ORY at a dose of 10 mg/kg and 50 mg/kg administered two days before the PCM injection in mice. Moreover, pretreatment with γ-ORY at a dose of 10 mg/kg and 50 mg/kg administered two days before the PCM injection significantly decreased the levels of TBARS and 4-HNE, lipid peroxidation markers in PCM hepatotoxicity in mice. Therefore, it was shown that the PCM decreases the antioxidant status, and increase the activity of caspase activators of apoptosis, and γ-ORY pretreatment was able to prevent this.

Oxidative stress caused by PCM results in the release of LDH, a marker of cell damage, and the release of several soluble products, including ALT, and AST (Shireen et al., 2008). The estimation of enzymes in the serum and histological analysisis a useful quantitative marker of the extent and type of hepatocellular damage. The mice treated with an overdose of PCM developed significant hepatic damage, which was observed by a substantial increase in the concentration of serum enzymes (AST, ALT, ALP, GGT and LDH). Administration of γ-ORY at two different doses, before PCM treatment resulted in a significant reduction of PCM-induced elevation of AST, ALT, ALP, GGT and LDH and appears to be protective in reducing the injurious effect of PCM. The

MDA is a good indicator of the degree of lipid peroxidation (Gamal et al., 2003), which is closely related to PCM-induced tissue damage. In the present study, we also observed significant increase in the levels of MDA in liver, which was decreased by the pretreatment of γ-ORY at a dose of 10 mg/kg and 50 mg/kg. This might due to hydroxyl radicals scavenging activities of γ-ORY. 4-HNE is the major aldehyde formed as a consequence of the interaction of reactive oxygen species with membrane lipids and lipoproteins (Völkel et al., 2005). It is formed after oxidation of ω-6 unsaturated fatty acids and is considered a second toxic messenger of oxygen free radicals (Esterbauer et al., 1991; Eckl et al., 1993). The elevated levels of 4-HNE in liver tissue homogenate of mice treated with PCM reflected lipid peroxidation and damage to plasma membrane as a consequence of oxidative stress. γ-ORY showed the highest efficacy in restoring the elevated 4-HNE levels in PCM.

The decrease in AA content in liver certainly contributes to the toxicity of PCM (Mitra et al., 1991). The results showed that our PCM lowers the AA levels, and the pretreatment with γ-ORY in two doses was able to increase these levels. This result seems to be related to its antioxidant activity. GSH is the major nonenzymatic antioxidant and regulator of intracellular redox homeostasis, ubiquitously present in all cell types (Meister and Anderson, 1983). The depletion of cellular GSH in the liver cells is known to play an important role in PCM toxicity (Mitchell et al., 1973). PCM administration leads to a significant decrease in the glutathione level because the metabolite NAPQI is conjugated with glutathione, which can be an important factor in the PCM toxicity. The mechanism of hepatoprotection by γ-ORY against PCM toxicity might be due to restoration of the GSH level.

The toxic NAPQI metabolite of PCM is a major trigger of ROS causing oxidative stress. PCM depleted GSH and reduced the activities of SOD, CAT, $^{\delta}$-AlA-D, GST, GPx, and

GR, reflecting perturbations in normal oxidative mechanisms. Alternatively, the reactive metabolite NAPQI can also react directly with essential–SH groups of δ-ALA-D, inactivating the enzyme and causing oxidative stress as demonstrated by (Rocha et al., 2005). In δ-ALA-D enzyme activity was diminished by its being an enzyme with thiol groups, sensitive to oxidative stress, γ-ORY for both doses was able to increase the activity of enzymes δ-ALA-D but was not significant compared to the control. SOD catalyses the dismutation of superoxide anion to H_2O_2 and O_2. Because H_2O_2 is still harmful to cells, CAT and GPx further catalyse the decomposition of H_2O_2 to water (Hayes et al., 2005). Thus, the coordinate actions of various cellular antioxidants in mammalian cells are critical for effectively detoxifying free radicals (Lee et al., 2012). PCM administration to mice declined antioxidant capacity of the mice liver as evinced in decreased activity of the antioxidant enzymes (Lee et al., 2012). γ-ORY pretreatment at a dose of 10 mg/kg and 50 mg kg, was able to enhance the activities of these enzymes, but the control was not significant. Thus, prevented the reduction in the antioxidant enzyme activities and consequent oxidative damage to the liver. The activities of Gr and GST were increased in the group which was administered PCM. The γ-ORY was able to significantly decrease the activity of both enzymes in two doses, prevented liver damage.

Caspases and apoptosis play an important causal role in the mechanism of PCM-induced hepatic injury by initiating the sequence of events that ultimately lead to liver necrosis (El-Hassan et al., 2003). PCM induced the release of cytochrome c from mitochondria to the cytosol (Ferret et al., 2001). Usually the apoptotic process is activated by caspases. The main intrinsic pathway is characterized by mitochondrial dysfunction, with the release of cytochrome c, activation caspase 9, and subsequently of caspase 3 (Porter and Jänicke, 1999). PCM administration to mice caused a high activity

of caspase 3 and 9 signaling the apoptotic process. γ-ORY pretreatment for two days at a dose of 10 mg/kg and 50 mg kg, was able to significantly decrease activity, can be compared to the control. Thus, γ-ORY in two doses in two doses was able to restore enzyme activity of caspases, thereby decreasing the apoptotic process which leads to necrosis of hepatocytes result also confirmed by histology.

In conclusion, the results of our present study found that γ-ORY significantly attenuated PCM–induced liver injury in mice largely due to its antioxidant effect by with its ability to modulate the activity of caspases 3 to 9. These results indicate that the beneficial effects of treatment with the γ-ORY at a dose of 10 mg/kg and 50 mg kg, reestablishment of liver function are mediated by its antioxidant action and ability to modulate the activity of caspases. Also restored the enzymatic activity was inhibited enzyme markers of liver injury (AST, ALT, ALP, GGT and LDH), a result also demonstrated by histology. It is therefore suggested that the γ-ORY may represent a new type of protective agent against hepatic damage caused by PCM and it may provide a potent hepatoprotective effect for clinical use.

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Legends

Fig 1- Photomicrography of segment of the hepatic lobe (A) of an animal control. (B) of an animal treated with γ-Oryzanol 10 mg/kg. (C) of an animal treated with γ-Oryzanol 50 mg/kg. (D) of an animal treated with PCM showed necrosis. (E) of an animal treated with γ-Oryzanol 10 mg/kg + PCM. (F) of an animal treated with γ-Oryzanol 50 mg/kg + PCM. Note the hepatocyte strings (arrow), the centrilobular vein (VC) and sinusoid capillaries (*) with normal aspect in figures 1A, 1B and 1C. Observe around the centrilobular vein (VC) some hepatocytes with vacuolation and acidophilic cytoplasm (arrows). H.E. 100X.

Fig 2-A. Effect of administration of γ-ORY in activity (%) of caspases 3 in the liver of mice exposed to PCM. Values are mean±S.E.M. (n=5 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the PCM.

Fig 2-B. Effect of administration of γ-ORY in activity (%) of caspases 9 in the liver of mice exposed to PCM. Values are mean±S.E.M. (n=5 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the PCM.

Fig 3-A. Effect of administration of γ-ORY in GST activity in the liver of mice exposed to PCM. Values are mean±S.E.M. (n=5 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the PCM.

Fig 3-B. Effect of administration of γ-ORY in GPx activity in the liver of mice exposed to PCM. Values are mean±S.E.M. (n=5 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the PCM.

Fig 3-C. Effect of administration of γ-ORY in GR activity in the liver of mice exposed to PCM. Values are mean±S.E.M. (n=5 per group). [a] Denoted $p<0.001$ as compared to the control group. [b] Denoted $p<0.001$ as compared to the PCM.

Table 1. Data are expressed as means ± S.E.M of 5 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the PCM.

Table 2. Data are expressed as means ± S.E.M of 5 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the PCM.

Table 3. Data are expressed as means ± S.E.M of 5 animals per group (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). a Denoted $p<0.001$ as compared to the control group. b Denoted $p<0.001$ as compared to the PCM.

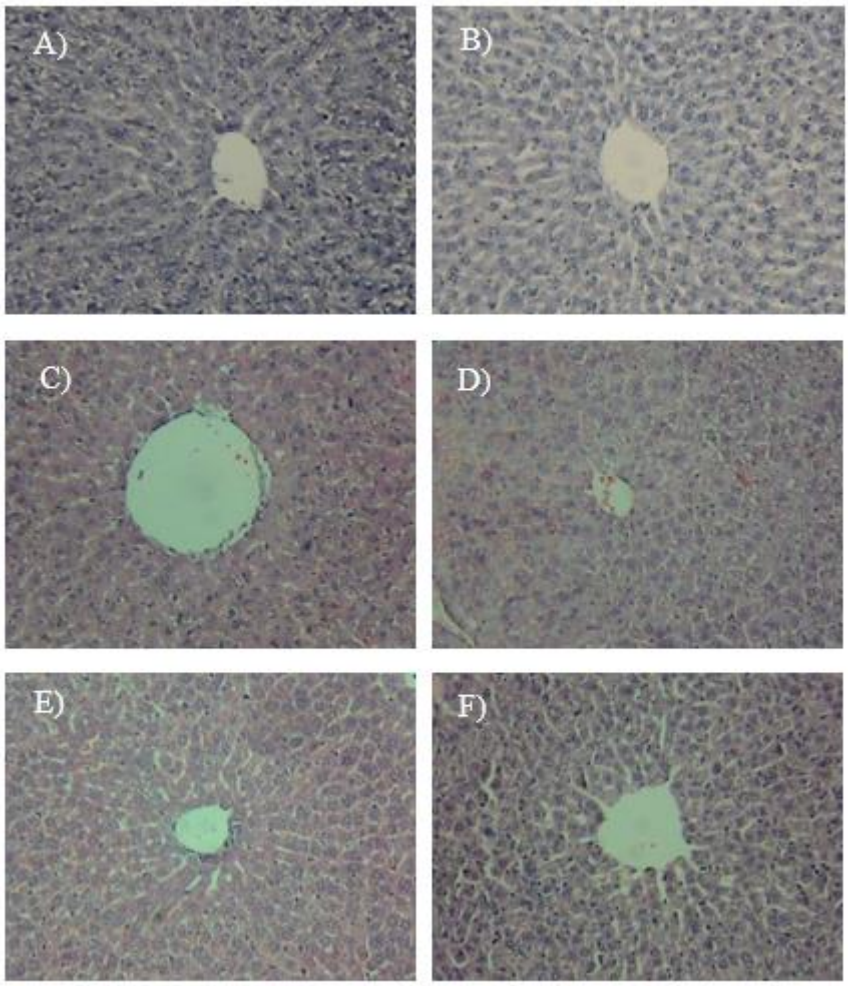
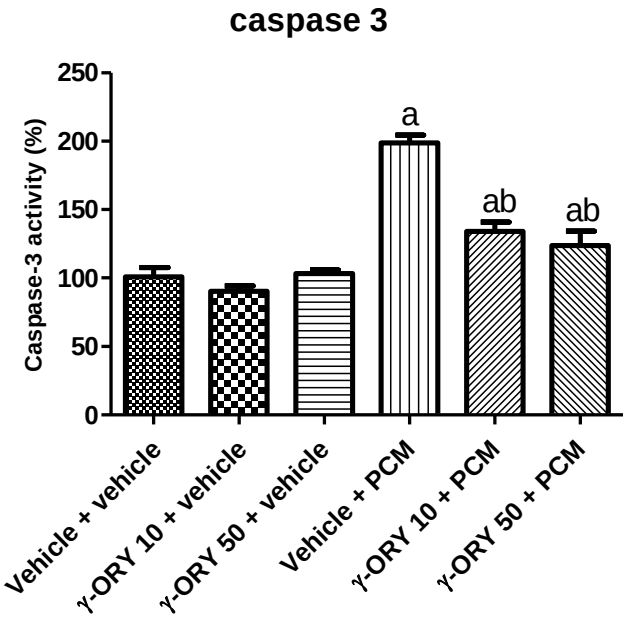


Fig. 1

A)


caspase 3
Caspase-3 activity (%)
250
200
150
100
50
0
a
ab
ab
Vehicle + vehicle
γ-ORY 10 + vehicle
γ-ORY 50 + vehicle
Vehicle + PCM
γ-ORY 10 + PCM
γ-ORY 50 + PCM

B)

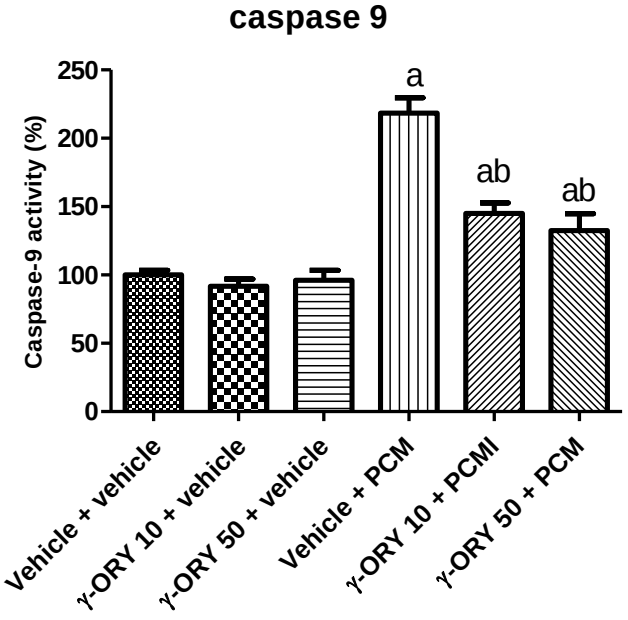

caspase 9
Caspase-9 activity (%)
250
200
150
100
50
0
a
ab
ab
Vehicle + vehicle
γ-ORY 10 + vehicle
γ-ORY 50 + vehicle
Vehicle + PCM
γ-ORY 10 + PCMI
γ-ORY 50 + PCM

Fig. 2-A,B

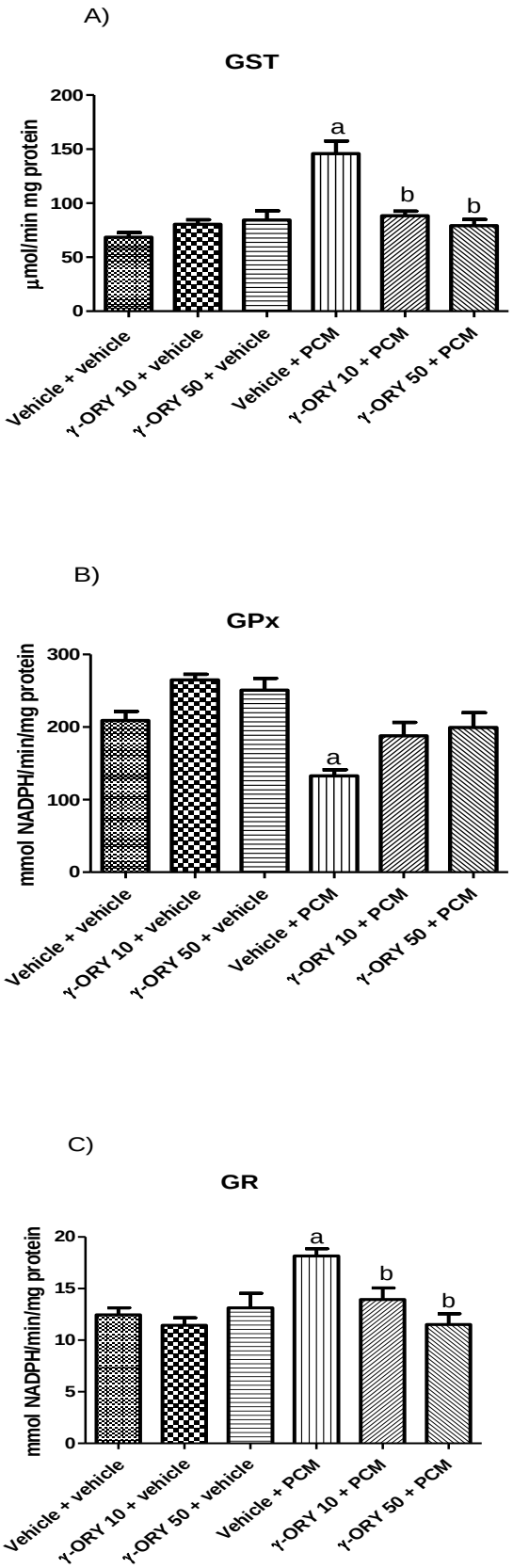

A)
GST
µmol/min mg protein
200
150
100
50
0
a
b
b
Vehicle + vehicle
ɣ-ORY 10 + vehicle
ɣ-ORY 50 + vehicle
Vehicle + PCM
ɣ-ORY 10 + PCM
ɣ-ORY 50 + PCM
B)
GPx
mmol NADPH/min/mg protein
300
200
100
0
a
Vehicle + vehicle
ɣ-ORY 10 + vehicle
ɣ-ORY 50 + vehicle
Vehicle + PCM
ɣ-ORY 10 + PCM
ɣ-ORY 50 + PCM
C)
GR
mmol NADPH/min/mg protein
20
15
10
5
0
a
b
b
Vehicle + vehicle
ɣ-ORY 10 + vehicle
ɣ-ORY 50 + vehicle
Vehicle + PCM
ɣ-ORY 10 + PCM
ɣ-ORY 50 + PCM

Fig. 3-A,B,C

Table 1. Effect of γ-ORY administration on TBARS, NPSH, AA and 4-HNE levels in liver of mice exposed to PCM

Groups	TBARS (nmol MDA equivalents/g tissue)	NPSH (µmol/g tissue)	Ascorbic Acid (µg AA/g tissue)	4-HNE (pg/ mg tissue)
Vehicle + vehicle	14.62 ± 1.22	33.80 ± 2.13	298.0 ± 13.82	1.26 ± 0.11
γ-ORY 10 + vehicle	15.48 ± 1.42	47.20 ± 4.21	295.2 ± 22.65	1.04 ± 0.06
γ-ORY 50 + vehicle	14.92 ± 1.67	48.60 ± 7.22	321.8 ± 18.79	1.10 ± 0.07
Vehicle + PCM	41.02 ± 3.37[a]	15.80± 2.47[a]	199.2 ± 12.74[a]	3.56 ± 0.21[a]
γ-ORY 10 + PCM	29.38 ± 3.99[ab]	27.00 ± 2.66[b]	279.0 ± 10.27[b]	2.16 ± 0.08[ab]
γ-ORY 50 + PCM	26.88 ± 3.98[ab]	33.60 ± 3.32[b]	299.8 ± 13.90[b]	1.72 ± 0.09[ab]

Table 2. Effect of γ-ORY administration on AST, ALT, ALP, GGT and LDH activities in liver of mice exposed to PCM

Groups	**AST** (U/L)	**ALT** (U/L)	**ALP** (U/L)	**GGT**(U/L)	**LDH** (U/L)
Vehicle + vehicle	44.40 ± 5.14	30.80 ± 4.11	237.4 ± 18.21	25.20 ± 2.88	272.4 ± 25.51
γ-ORY 10 + vehicle	35.80 ± 5.64	31.00 ± 5.16	228.0 ± 24.44	27.60 ± 3.62	277.0 ± 44.92
γ-ORY 50 + vehicle	47.60 ± 4.53	38.40 ± 3.64	229.2 ± 21.22	32.00 ± 6.04	249.2 ± 49.10
Vehicle + PCM	166.6 ± 16.52[a]	164.4 ± 14.56[a]	873.6± 45.72[a]	129.4 ± 8.98[a]	879.6 ± 51.97[a]
γ-ORY 10 + PCM	92.60 ± 6.45[ab]	99.40 ± 8.26[ab]	498.6 ± 42.28[ab]	79.40 ± 5.80[ab]	579.2 ± 25.88[ab]
γ-ORY 50 + PCM	74.60 ± 6.26[ab]	71.60 ± 3.61[ab]	413.6 ± 70.38[ab]	59.80 ± 6.98[ab]	396.4 ± 31.01[b]

Table 3. Effect of γ-ORY administration on CAT, SOD and $^{\delta}$-AlA-D activities in liver of mice exposed to PCM

Groups	**CAT** (U/mg protein)	**SOD** (U/mg protein)	**δ-AlA-D** (nmol PBG/mg protein/h)
Control	33.70 ± 2.73	11.06 ± 0.56	10.52 ± 0.97
γ-ORY 10 + vehicle	34.18 ± 1.72	11.14 ± 0.60	12.62 ± 0.92
γ-ORY 50 + vehicle	36.15 ± 3.20	12.58 ± 1.08	13.29 ± 1.83
Vehicle + PCM	21.75 ± 1.12 [a]	5.70 ± 0.67[a]	8.05 ± 0.57[a]
γ-ORY 10 + PCM	26.31 ± 2.03	6.20 ± 0.79[a]	9.34 ± 1.28
γ-ORY 50 + PCM	30.96 ± 2.39	6.74 ± 0.85[a]	12.05 ± 1.32

PARTE V

3. DISCUSSÃO

O fígado é um órgão de importância vital para os seres vertebrados, pois é responsável pela síntese e metabolismo macromoléculas essenciais para a manutenção da vida e também atua detoxificando compostos xenobióticos. As doenças hepáticas com danos aos hepatócitos são causadas geralmente por uma infecção viral, esteatose não alcoólica ou outro agente hepatotóxico e estão altamente associadas com inflamação aguda ou crônica (Tacke et al., 2009; Tseng et al., 2014). Assim, a hepatite fulminante aguda é uma patologia caracterizada por encefalopatia hepática, coagulopatia grave, icterícia e hidroperitônio. Uma alta taxa de mortalidade elevada (80-90%) ocorre em pacientes com hepatite fulminante aguda que não recebem o transplante de fígado (Lee, 1994). O estresse oxidativo contribui para a patogênese da hepatite fulminante aguda induzida pelo álcool, infecção por vírus, hemocromatose, isquemia/reperfusão, exposições tóxicas, e abuso de drogas como o PCM em overdose (Mayer et al., 1993). Assim, nos três modelos diferentes de hepatite fulminante aguda, o γ-ORY foi capaz de proteger contra o aumento na atividade enzimática causado nos marcadores clínicos. O γ-ORY, também foi capaz de proteger as enzimas e marcadores não enzimáticos sensíveis ao estresse oxidativo, os quais estavam alterados devido ao mecanismo de ação dos agentes hepatotóxicos. Os níveis das citocinas pró-inflamatórias encontravam-se alterados devido ao mecanismo inflamatório dos agentes causadores de dano hepático e o γ-ORY foi capaz de proteger contra o aumento dos níveis da citocinas pró-inflamatórias. A atividade das caspases ativadoras das vias apoptóticas se encontrava alterada devido aos mecanismos de morte celular e o γ-ORY foi capaz de modular a atividade das caspases ativadoras das vias apoptóticas. Desta forma, este trabalho demonstrou que a administração do γ-ORY induziu a um efeito hepatoprotetor em três modelos de hepatite fulminante aguda. Assim, esta atividade deve-se a modulação do status oxidante, inflamatório e apoptótico. Estes resultados, quando comparados ao controle positivo silimarina, encorajam-no ao prosseguimento

dos estudos com o γ-ORY para o desenvolvimento de formulações farmacêuticas para patologias hepáticas.

A peroxidação lipídica, mediada pelo mecanismo de EROS, está implicada na patogênese de diversas lesões hepáticas e subsequente fibrogênese hepática tanto em animais como em seres humanos (Nimela et al., 1994; Liu et al., 2006). A produção excessiva de EROS danifica a membrana biológica, o que pode levar à oxidação dos ácidos graxos poli-insaturados da membrana através da peroxidação lipídica (Mayer et al., 1993). Os níveis de TBARS nos modelos de indução de hepatite fulminante aguda estavam aumentados e o γ-ORY foi capaz de proteger contra o aumento deste marcador. Confirmando assim, que o γ-ORY protegeu contra a peroxidação lipídica causada pelos agentes hepatotóxicos, comparando-se a silimarina (um fármaco hepatoprotetor). O NPSH é constituído principalmente de GSH e se trata da primeira linha de defesa contra os radicais livres. A depleção de NPSH está diretamente associada com a elevação da peroxidação lipídica que envolve a sua proteção contra as espécies reativas (Nogueira et al., 2009). Em ambos modelos de hepatite fulminante aguda, houve uma depleção de NPSH, devido aos modelos causarem estresse oxidativo. O tratamento com γ-ORY, foi efetivo em evitar a depleção do conteúdo de NPSH hepático, nos modelos com CCl_4 e LPS/D-GalN e o γ-ORY apresentou um efeito hapatoprotetor semelhante a silimarina. Já no modelo com PCM, ambas as doses de γ-ORY foram capazes de evitar a depleção do conteúdo de NPSH. O efeito hepatoprotetor do γ-ORY é relacionado a sua atividade antioxidante demonstrada pela diminuição dos níveis de TBARS e aumento do NPSH de maneira semelhante à silimarina. Desta forma, o mecanismo de diminuição da peroxidação lipídica através da manutenção dos níveis de NPSH está envolvido na hepatoproteção exercida pelo γ-ORY.

O 4-HNE é o principal aldeído formado como consequência da interação de EROS com os lípidos da membrana e lipoproteínas (Völkel et al., 2005). É formado após a oxidação de ácidos graxos insaturados ω-6 e é considerado um segundo mensageiro tóxicos de EROS (Eckl et al., 1993). Os níveis elevados de 4-HNE no homogenato do tecido hepático dos camundongos os

quais foram administrados com PCM, demonstrando a peroxidação lipídica e os danos à membrana plasmática. Estes dados corroboram com o aumento do TBARS e indicam que nos modelos de hepatite fulminante ocorre um dano à membrana lipídica com consequente diminuição das funções fisiológicas do órgão. O tratamento com γ-ORY apresentou eficácia em restaurar os níveis elevados de 4-HNE nos animais administrados com PCM. Assim, o γ-ORY apresentou efeito hepatoprotetor em marcadores de peroxidação lipídica que são justificados através do efeito antioxidante *per se* ou do aumento das defesas antioxidantes. O AA é a maior defesa antioxidante não enzimática tendo uma ação sinérgica na eliminação de EROS (Jacques-Silva et al., 2001). O AA é considerado um marcador de estresse oxidativo e a redução indica um aumento do estresse oxidativo (Jacques-Silva et al., 2001). No modelo no qual foi utilizado o CCl_4, houve a diminuição dos níveis de AA e o γ-ORY não foi capaz de proteger contra essa diminuição totalmente, mas teve um efeito principal. Este resultado reforça o envolvimento das defesas antioxidantes no efeito hepatoprotetor do γ-ORY com igualdade comparada ao controle positivo silimarina.

O organismo dos seres vertebrados possui mecanismos de defesa para proteger contra os danos causados pelo estresse oxidativo, possuindo enzimas com essa função. A CAT, é uma enzima antioxidante, é uma das mais importantes enzimas para detoxificar os efeitos do metabolismo do oxigénio (Linares et al, 2006;. Pande et al., 2002). A CAT catalisa a detoxificação de peróxido de hidrogênio produzido na célula em O_2 e H_2O (Linares et al., 2006). A GPx catalisa também a decomposição do peróxido de hidrogênio para H_2O (Hayes et al., 2005). Porém necessita da atividade da enzima GR, a qual é é responsável pela manutenção de GSH reduzido que é substrato para GPx e GST. A GST é uma enzima citosólica envolvida na detoxificação de uma gama de compostos xenobióticos por conjugação com GSH que é essencial para a manutenção dos processos fisiológicos normais (Dagget et al., 1998). A SOD é a enzima que mantém a concentração de radicais superóxido em níveis baixos e, por conseguinte, desempenha um papel importante na defesa contra o estresse oxidativo (Fridovich, 1997). A enzima δ-ALA-D sintetiza o grupo heme presente nas hemácias e sensível ao estresse oxidativo, pois ocorre oxidação

dos grupamentos tiólicos. (Spiazzi et al., 2013). Nos modelos os quais foram utilizados PCM e LPS/D-GalN, ouve um aumento nas atividades da CAT, GPx, e δ-ALA-D. O tratamento com γ-ORY foi capaz de proteger contra ao aumento na atividade de CAT e GPx, causado por PCM e LPS/D-GalN. Em relação à enzima SOD, o tratamento com γ-ORY teve um efeito principal aumentando a atividade enzimática diminuida pelo LPS/D-GalN. Já no modelo com PCM, o γ-ORY foi capaz de proteger contra a diminuição na atividade causada pelo PCM. Foi comprovado o efeito do tratamento com γ-ORY na atividade da δ-ALA-D, pois foi capaz de proteger contra ao aumento na atividade causado por PCM e LPS/D-GalN. Os modelos com PCM e LPS/D-GalN tiveram a atividade de GST aumentada. O tratamento com γ-ORY foi capaz de proteger contra o aumento na atividade de GST. Em conclusão, o γ-ORY exerce o efeito hepatoprotetor através da modulação da atividade das enzimas antioxidante e sensíveis ao estresse oxidativo. Assim, podemos indicar que o γ-ORY possui efeito benéfico nos modelos de hepatite fulminante aguda por possuir atividade antioxidante intrínseca e por reestabelecer o status oxidativo pela modulação de parâmetros enzimáticos e não-enzimáticos.

A atividade das aminotransferases é considerada como indicadores altamente sensíveis à lesão hepática (Ghanshyam et al., 2008). A lesão causada nos hepatócitos altera a sua função de transporte e a permeabilidade da membrana, levando ao risco de extravasamento celular das enzimas (Zimmerman e Seeff, 2002). Em todos os modelos de hepatite fulminante aguda, ocorreu um aumento nas atividades enzimáticas (AST, ALT, ALT, GGT e LDH), indicando assim a hepatite e ainda, comprovada pela histologia. O γ-ORY foi capaz de proteger, reduzindo a atividade enzimática das aminotranferases nos modelos, os quais foram induzidos pela administração de CCl_4 e LPS/D-GalN, se equivalendo a silimarina. Já no modelo no qual foi utilizado o PCM, o γ-ORY reduziu a atividade das aminotranferases, evitando a lesão aguda aos hepatócitos. Além destes marcadores de função hepática, a bilirrubina também foi utilizada. Acumulam-se evidências mostrando que a bilirrubina é um eficiente eliminador de EROS e de óxido nítrico (Mancuso et al., 2006; Vitek e Ostrow, 2009), tem capacidade de diminuição dos níveis de citocinas pró-inflamatórias e inibidora a migração de células imunes (Keshavan

et al., 2005; Tang et al., 2007). Nos animais no qual o modelo foi induzido pelo CCl_4, apresentou níveis elevados de bilirrubina e o tratamento com γ-ORY foi capaz de proteger contra o aumento nos níveis de bilirrubina. Portanto, o γ-ORY demonstrou atividade hepatoprotetora pela proteção em marcadores clássicos de patologias hepáticas que são reconhecidos por serem fidedignos em relação a funcionalidade do órgão. Assim, os resultados obtidos neste estudo asseguram a eficiência do γ-ORY em modelos de hepatite fulminante aguda.

A MPO é uma enzima abundantemente presente em neutrófilos, envolvidos na catálise de H_2O_2 em ácido hipocloroso (HOCl) (Klebanoff, 2005). O HOCl contribui para implementar os danos no tecido em locais de inflamação através da reação com uma ampla gama de substratos biológicos (Davies et al., 2008). No modelo com CCl_4 houve um aumento na atividade da enzima MPO, indicando uma infiltração de neutrófilos no tecido hepático. Isto deve-se ao processo inflamatório aumentado no fígado no modelo de hepatite fulminante aguda que auxilia no aumento das EROS e do processo apoptótico. A diminuição da atividade da MPO pela administração do γ-ORY indica que há uma menor infiltração de células inflamatórias, colaborando com os achados histológicos. Além disso, o tratamento com γ-ORY diminuiu o conteúdo de citocinas pró-inflamatórias com consequente redução do estresse oxidativo e apoptose.

Em lesões hepáticas agudas, as células lesionadas liberam uma série de citocinas e estimulam as células de Kupffer para liberar mais mediadores inflamatórios e diversos EROS (Yamada et al., 1997). Alguns estudos têm demonstrado que o NO regula a expressão de citocinas pró-inflamatórias durante processos inflamatórios (Kobayashi, 2010; Sigala et al., 2012). No modelo induzido pelo CCl_4, houve um aumento nos níveis de NO, tanto o γ-ORY quanto a silimarina foram capazes de restaurar o aumento de NO. As citocinas podem ser classificadas como pró-inflamatórias, que induzem efeitos citotóxicos, tais como a ativação da síntese e secreção de proteínas de fase aguda (Gadient e Otten, 1997; Tringali et al., 2000) e dano oxidativo (Andersen et al., 1996). Nos modelos induzidos pelo CCl_4 e LPS/D-GalN, demonstrou-se

um aumento das citocinas TNF-α e IL-1β e no tecido hepático, corrobando com o aumento da atividade da MPO, dos níveis de NO e infiltração neutrófila nas lâminas histológicas. O tratamento com γ-ORY restaurou o aumento das citocinas TNF-α e IL-1β no tecido hepático. Com isto, podemos indicar uma atenuação da inflamação nos modelos de hepatite fulminante aguda induzida pelo CCl_4 e LPS/D-GalN. Assim, um dos mecanismos envolvidos do efeito hepatoprotetor do γ-ORY é o anti-inflamatório. Isto também é evidenciado em inúmeros estudos realizados em modelos de hepatite em camundongos (Xiaomin et al., 2014).

Algumas moléculas pró-inflamatórias são responsáveis por induzirem efeitos citotóxicos, tais como a ativação da síntese e secreção de proteínas de fase aguda (Gadient e Otten, 1997; Tringali et al., 2000), e causam oxidativo celular (Andersen et al., 1996). No modelo o qual o agente indutor de dano hepático foi o CCl_4, houve um aumento nos níveis das seguintes moléculas pró- inflamatórias (TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 e IFN-γ). Desta maneira, o modelo de hepatite fulminante aguda induzida pelo CCl_4 apresentou uma intensa resposta inflamatória pelo aumento de citocinas pró-inflamatórias, além dos níveis de NO, TBARS e da enzima MPO. Assim, os resultados corroboram com estudos que indicam que a hepatite fulminante é um processo composto de respostas inflamatórias, oxidativas e apoptóticas, além da alteração da estrutura tecidual (Ethel et al., 2009) O efeito hepatoprotetor do γ-ORY ocorre através da modulação da resposta inflamatória, principalmente da diminuição das citocinas pró- inflamatórias (TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 e IFN-γ).

Algumas citocinas pró-inflamatórias, como o TNF-α são mediadores apoptóticos, os quais agravam a lesão hepática, pois induzem à síntese de tecido fibroso e à perda da função celular. A apoptose ou morte celular programada é um processo importante envolvido no desenvolvimento normal e a manutenção das células. O processo apoptótico pode ser ativado por caspases. A principal via intrínseca é caracterizada por disfunção mitocondrial, com a liberação de citocromo c, ativação da caspase 9 e posteriormente, da caspase 3 (Porter e Jänicke, 1999). O aumento da atividade das caspases 3 e 9 ocorreu nos modelos induzidos por CCl_4 e PCM. Assim, o processo oxidativo

e inflamatório aumentado induzem à apoptose pelas caspases. A apoptose aumentada leva aos processos de fibrose e intenso dano tecidual que foram revelados nas lâminas histológicas. O tratamento com γ-ORY atenou o aumento das caspases 3 e 9, evidenciando um novo mecanismo envolvido no efeito hepatoprotetor. Este resultado junta-se aos efeitos antioxidante e anti-inflamatório na proteção exercida pelo γ-ORY nos diferentes modelos de hepatite fulminante aguda demonstrados neste estudo.

4. CONCLUSÃO

O presente estudo demonstrou pioneiramente o efeito hepatoprotetor do γ-ORY, sendo efetivo nos seguintes danos causados em diferentes modelos de hepatite fulminante aguda:

- Aumento nos níveis de TBARS, 4-HNE e depleção de NPSH e AA.
- Aumento na atividade enzimática de CAT, GPx, GST e δ-ALA-D e diminuição na atividade da SOD.
- Aumento na atividade de AST, ALT, ALT, GGT e LDH.
- Aumento nos níveis de bilirrubina, NO e na atividade da MPO.
- Aumento da atividade das Caspases 3 e 9.
- Aumento nos níveis de TNF-α, IL-1β, IL-6, TGF-β1, MCP-1 e IFN-γ.
- Lesões evidenciadas na análise histológica.

Em conclusão, sugerimos que o γ-ORY pode ser uma alternativa para o tratamento da hepatite fulminante aguda, pois ficou comprovado seu efeito hepatoprotetor está associado com sua capacidade antioxidante, anti-inflamatória e anti-apoptótica.

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