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EFEITOS DO ÁCIDO GÁLICO EM PARÂMETROS BIOQUÍMICOS E DE ESTRESSE OXIDATIVO EM RATOS SUBMETIDOS A UM MODELO EXPERIMENTAL DE DIABETES

DISSERTAÇÃO DE MESTRADO

Lizielle Souza de Oliveira

Santa Maria, RS, Brasil

2015

EFEITOS DO ÁCIDO GÁLICO EM PARÂMETROS BIOQUÍMICOS E DE ESTRESSE OXIDATIVO EM RATOS SUBMETIDOS A UM MODELO EXPERIMENTAL DE DIABETES

Lizielle Souza de Oliveira

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Mestre em Bioquímica Toxicológica

Orientadora: Prof.ª Dr.ª Roselia Maria Spanevello

Co-orientadora: Prof.ª Dr.ª Vera Maria Melchiors Morsch

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elaborada por
Lizielle Souza de Oliveira

como requisito parcial para a obtenção do grau de
Mestre em Bioquímica Toxicológica

COMISSÃO EXAMINADORA

Roselia Maria Spanevello

Prof[a]. Dra. Roselia Maria Spanevello
(Presidente/Orientador)

Roberta Schmatz

Prof[a]. Dra. Roberta Schmatz (IFRS)

Vania Lucia Loro

Prof[a]. Dra. Vânia Lucia Loro (UFSM)

Santa Maria, 11 de setembro de 2015.

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RESUMO

Dissertação de mestrado
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EFEITOS DO ÁCIDO GÁLICO EM PARÂMETROS BIOQUÍMICOS E DE ESTRESSE OXIDATIVO EM RATOS SUBMETIDOS A UM MODELO EXPERIMENTAL DE DIABETES

AUTORA: LIZIELLE SOUZA DE OLIVEIRA
ORIENTADORA: ROSELIA MARIA SPANEVELLO
CO-ORIENTADOR: VERA MARIA MELCHIOR MORSCH
Data e Local da Defesa: Santa Maria, 11 de setembro de 2015.

O diabetes mellitus (DM) é uma doença metabólica caracterizada por hiperglicemia associada com o aumento de radicais livres e diminuição da resposta antioxidante. O ácido gálico tem inúmeras propriedades biológicas, dentre elas pode-se destacar sua função antioxidante que pode fornecer uma série de benefícios para os pacientes com DM. Neste contexto, o objetivo deste estudo foi avaliar o efeito do tratamento com ácido gálico em parâmetros bioquímicos, biomarcadores de estresse oxidativo e histológicos em fígado e rim de ratos diabéticos induzidos com estreptozotocina. No presente estudo, foram utilizados 40 ratos wistar machos que foram divididos em quatro grupos: I - controle, II - ácido gálico (30mg/kg), III - diabético, IV - diabético + ácido gálico (30mg/kg). O DM foi induzido nos animais através de injeção intraperitoneal de estreptozotocina (65 mg/kg). Após a confirmação da hiperglicemia, iniciou-se o tratamento com ácido gálico (30mg/kg) que foi administrado por via oral durante 21 dias. Após este período os animais foram submetidos à eutanásia e o sangue, fígado e rim foram coletados para as posteriores análises. Os resultados obtidos demonstraram que o tratamento com ácido gálico foi capaz de prevenir a formação de espécies reativas, reduzir os níveis de peroxidação lipídica e aumentar a atividade das enzimas superóxido dismutase e delta-aminolevulinato ácido desidratase no fígado e rim de ratos diabéticos ($P<0.05$) Além disso, este composto também preveniu a diminuição na atividade da superóxido dismutase, glutationa S- transferase e dos níveis de vitamina C no fígado de ratos diabéticos ($P<0.05$). O tratamento com ácido gálico preveniu o aumento da atividade da aspartato aminotransferase e no tecido hepático reduziu o número de núcleos e aumentou a área nuclear, e no tecido renal aumentou a área glomerular. Estes resultados indicam que o ácido gálico pode proteger contra danos induzidos pelo estresse oxidativo no estado diabético. Portanto, pode-se sugerir que o ácido gálico pode ser um candidato potencial na terapia complementar no diabetes, devido às suas ações antioxidantes, em combinação com fármacos hipoglicêmicos.

Palavras-chave: antioxidantes; ácido gálico; delta-aminolevulinato ácido desidratase; diabetes mellitus.

ABSTRACT

Dissertation of Master's Digree
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EFFECTS OF GALLIC ACID ON BIOCHEMICAL PARAMETERS AND OXIDATIVE STRESS IN RATS SUBMITTED TO THE EXPERIMENTAL MODEL OF DIABETES

AUTHOR: LIZIELLE SOUZA DE OLIVEIRA
ADVISER: ROSELIA MARIA SPANEVELLO
CO-ADVISER: VERA MARIA MORSCH
Date and place of the defense: Santa Maria, September, 11th, 2015.

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia associated with the increase of free radicals and decrease in the antioxidant response. Gallic acid has many biological properties, among them we can highlight its antioxidant function that can provide a number of benefits for patients with DM. In this context, the aim of this study was to evaluate the treatment effect of gallic acid in biochemical parameters of oxidative stress and histological biomarkers in liver and kidney of diabetic rats induced with streptozotocin. In the present study, we used 40 male Wistar rats were divided into four groups: I - Control II - gallic acid (30mg / kg), III - diabetic IV - Diabetic + gallic acid (30mg / kg). Diabetes mellitus was induced in the animals by intraperitoneal injection of streptozotocin (65 mg / kg). After confirmation of hyperglycemia, began treatment with gallic acid (30mg / kg) which was administered orally for 21 days. After this period the animals were euthanized and blood, liver and kidney were collected for further analysis. The results demonstrated that treatment with gallic acid was able to prevent the formation of reactive species to reduce the levels of lipid peroxidation and increase the activity of superoxide dismutase and delta-aminolevulinic acid dehydratase in the liver and kidney of diabetic rats ($P < 0.05$) Further, this compound also prevented the decrease in activity of superoxide dismutase, glutathione S-transferase and Vitamin C levels in the livers of diabetic mice ($P <0.05$). Gallic acid treatment prevented the increase of aspartate aminotransferase activity in liver tissue and reduced the number of nuclei and increased nuclear area, and increased glomerular renal tissue area. These results indicate that the gallic acid may protect against oxidative stress-induced damage in the diabetic state. Therefore, it can be suggested that the gallic acid may be a potential candidate in adjunct therapy in diabetes, due to its antioxidant action, in combination with hypoglycemic drugs.

Keywords: antioxidant; delta-aminolevulinic acid dehydratase; diabetes mellitus; gallic acid.

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SUMÁRIO

1. INTRODUÇÃO

O Diabetes mellitus (DM) é uma doença metabólica de etiologia múltipla, o qual tem sido considerado um dos principais problemas de saúde pública mundial. Segundo Danaei et al. (2011), esta doença afeta cerca de 346 milhões de pessoas no mundo. Hoje, o Brasil é o 4º país com maior número de diabéticos, ficando atrás apenas da China, Índia e Estados Unidos, respectivamente. Em 2013 havia cerca de 12 milhões pessoas (6% da população brasileira), na faixa etária de 20 a 79 anos, com diagnóstico de diabetes no Brasil. Estes elevados índices são resultantes da obesidade, sedentarismo, envelhecimento e crescimento populacional, bem como maior sobrevida dos pacientes com esta patologia (SOCIEDADE BRASILEIRA DE DIABETES, 2014).

A hiperglicemia é a principal característica do DM e está associada a distúrbios no metabolismo de carboidratos, lipídeos e proteínas. O aumento nos níveis de glicose sanguínea (hiperglicemia) ocorre quando o organismo não consegue produzir insulina ou não consegue utilizá-la de modo eficaz. A insulina é um hormônio produzido no pâncreas que permite que as células do organismo utilizem a glicose, obtida a partir de alimentos, como fonte de energia para o correto funcionamento (AMERICAN DIABETES ASSOCIATION, 2012) (Figura 1A). Assim, indivíduos com diabetes não conseguem absorver a glicose da circulação sanguínea, gerando um quadro hiperglicêmico que danifica tecidos com o passar do tempo.

O DM é classificado com base em sua etiologia, sendo que a atual classificação recomendada pela International Diabetes Federation (2013) inclui três classes clínicas: DM tipo 1 (Figura 1B), DM tipo 2 (Figura 1C) e DM gestacional (Figura 1C). O DM tipo 1 compreende entre 5 e 10% dos casos, com maior prevalência em crianças e adolescentes, e caracteriza-se pela ausência da secreção de insulina causada pela destruição das células β pancreáticas. O DM tipo 1 pode ser dividido quanto a origem etiológica em imunomediado e idiopático. O DM tipo 1 imunomediado (maioria) é causado por uma reação autoimune, onde o sistema de defesa do organismo ataca a células β pancreáticas (INTERNATIONAL DIABETES FEDERATION, 2013; AMERICAN DIABETES ASSOCIATION, 2014). Já o DM tipo 1 idiopático apresenta associação com determinados genes do sistema antígeno leucocitário humano (HLA) no cromossomo 6 e uma relação com a destruição

autoimune das células β pancreáticas (SOCIEDADE BRASILEIRA DE DIABETES, 2014). Em ambos os casos, a taxa de destruição das células beta é variável, sendo, em geral, mais rápida entre as crianças, e seus principais sintomas são: sede anormal, boca seca, micção frequente, falta de energia, cansaço extremo, fome, perda de peso, infecções recorrentes e visão turva (INTERNATIONAL DIABETES FEDERATION, 2013).

O DM tipo 2 compreende entre 90 e 95% dos casos, nesta situação o organismo é capaz de produzir insulina, no entanto, ocorre defeito na secreção ou resistência deste hormônio, o que leva ao acúmulo de glicose na corrente sanguínea (SOCIEDADE BRASILEIRA DE DIABETES, 2014). A maioria dos casos ocorre devido ao excesso de peso e sedentarismo (GIACCO; BROWNLEE, 2010). Este tipo é frequentemente diagnosticado apenas quando as complicações diabéticas já se desenvolveram (INTERNATIONAL DIABETES FEDERATION, 2013).

Assim como o DM tipo 2, o DM gestacional está associado com a resistência à insulina e/ou diminuição da função das células β pancreáticas. Este tipo caracteriza-se por alterações na glicemia durante a gravidez e normalmente, desaparece após o parto (AMERICAN DIABETES ASSOCIATION, 2014).

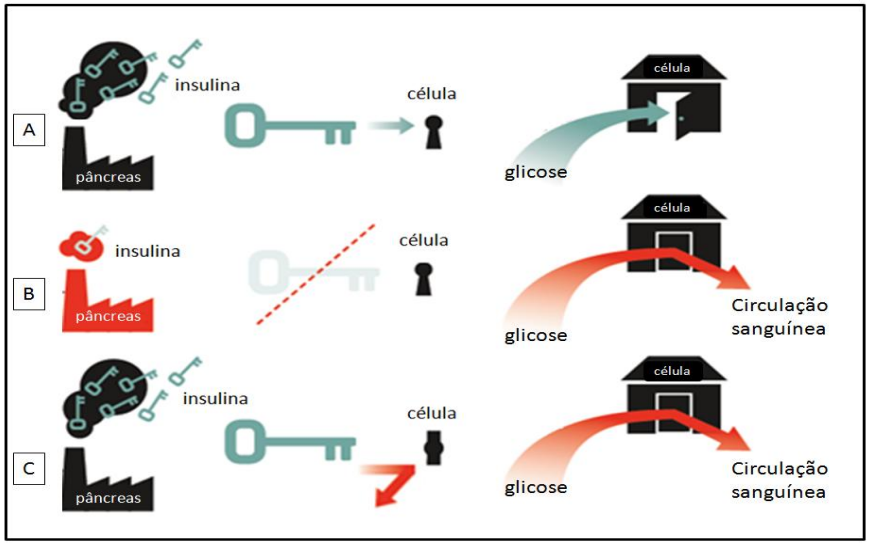

Figura 1: Esquema demonstrando o mecanismo de ação da insulina: normal (A), DM tipo 1 (B) e DM tipo 2 ou gestacional (C).
Fonte: AMERICAN DIABETES ASSOCIATION (2014), adaptado.

Além da hiperglicemia outros sintomas tais como polidpsia, polifagia, poliúria, perda de peso, câimbras, cansaço, perda de visão e aumento da susceptibilidade a determinadas infecções são característicos desta patologia (CANIVELL; GOMIS, 2014). Além disso, essa doença traz consigo uma grande sobrecarga econômica à sociedade devido à perda da força de trabalho e do elevado custo de tratamento, além do sofrimento individual associado ao curso prolongado desta doença. Os valores gastos pelos países no tratamento do diabetes variam de acordo com a incidência e o tipo de tratamento utilizado, podendo atingir até 15 % do orçamento total disponível para saúde (SOCIEDADE BRASILEIRA DE DIABETES, 2014).

Estudos em modelos animais, os quais mimetizam sintomas e alterações metabólicas características do DM têm colaborado para a compreensão dos mecanismos envolvidos na etiologia e progressão desta patologia bem como na busca de novas intervenções terapêuticas (ISLAS-ANDRADE et al., 2000; SZKUDELSKI, 2001; WU;HUAN, 2008). Agentes diabetogênicos como a estreptozotocina e aloxano, quando administrados em animais, causam alterações metabólicas e clinicas idênticas as que ocorrem no DM (SZKUDELSKI, 2001). A estreptozotocina (STZ) também denominada quimicamente de 2-deoxi-2-(3-metil-3-nitrosoureído)-D-glicopiranose é um antibiótico produzido por bactérias Streptomycetes achromogenes (XIANG et al., 2010; SZKUDELSKI, 2001; DELFINO et al., 2002). Esta substância quando administrada de forma intraperitoneal age sobre as células β das ilhotas de Langherans, o qual gera alterações nos níveis de insulina que por sua vez promove a instalação de um quadro hiperglicêmico (BEDOYA et al., 1996; MARITIM et al., 2003; WU;HUAN, 2008). A STZ quando administrada na dose entre 50 e 100 mg/kg de peso corporal, causa necrose das células β, o que promove o desenvolvimento do DM tipo 1, cerca de dois dias após a aplicação intraperitoneal da droga, constituindo-se assim em um modelo animal de DM já bem estabelecido na literatura (HOUNSOM, 1998).

A STZ exerce seus efeitos tóxicos devido à similaridade de sua estrutura com a da molécula de glicose (Figura 2). Em consequência a esta similaridade estrutural, a STZ atravessa ás células β pancreáticas por meio do transportador de glicose GLUT 2 (ISLAS-ANDRADE et al., 2000; WU;HUAN, 2008). O mecanismo citotóxico da STZ está relacionado com a alcalização do DNA celular, ativação da poli-ADP ribose sintetase, depleção de NAD nas células pancreáticas, baixos níveis de ATP e

inibição da síntese e secreção da insulina (YAMAMOTO et al., 1981, MORGAN et al., 1994). Por outro lado, sabe-se que a produção excessiva de espécies reativas de oxigênio (ERO), acarreta na privação da produção de insulina e é essencial para seu efeito diabetogênico (WU;HUAN, 2008; AGGARWAL;HARIKUMAR, 2009).

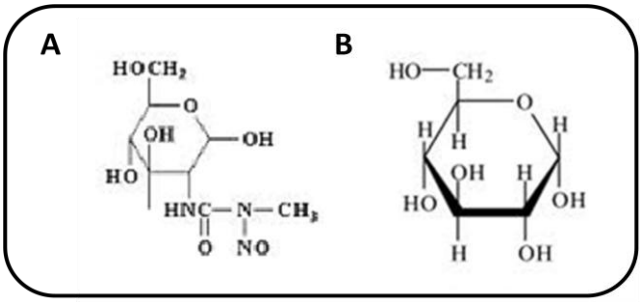

Figura 2. A estrutura química da molécula de estreptozotocina (A) e da molécula de glicose (B).

Estudos com modelos experimentais de diabetes bem como com pacientes diabéticos têm sugerido que o estresse oxidativo está envolvido em complicações associadas ao DM (MATOUGH et al., 2003; SCHMATZ et al., 2012; ESTEGHAMATI et al., 2013; STEFANELLO et al., 2015). O estresse oxidativo é caracterizado pelo desequilíbrio que ocorre entre produção de espécies reativas e os sistemas de defesa antioxidantes (VALKO et al., 2007). Quando espécies reativas são produzidas em excesso, elas exercem efeitos citotóxicos sobre fosfolipídios de membrana resultando em peroxidação lipídica, oxidação proteica, diminuição do conteúdo de ácido ascórbico e alterações na atividade de enzimas antioxidantes (GARG; BANSAL, 2000; ANANTHAN et al., 2003; SEVEN et al., 2004).

As espécies reativas são compostas por radicais livres (compostos que possuem um elétron desemparelhado em sua órbita externa) e outras substâncias que não possuem elétrons desemparelhados, mas facilmente sofrem reações químicas formando radicais livres como, por exemplo, o H_2O_2 (BARREIROS;DAVID, 2006). Entre as espécies reativas de oxigênio (ERO) destacam-se o ânion radical superóxido ($O2^{\bullet-}$), radical hidroxila (OH^-), radical peroxila (ROO•) e peróxido de hidrogênio (H_2O_2). Estas ERO podem atacar moléculas biológicas podendo acarretar danos no DNA, lipídeos e proteínas (Figura 3) (BARREIROS; DAVID, 2006; AVERY, 2011).

Algumas destas espécies reativas (ER) são formadas naturalmente nos organismos aeróbios. Durante a respiração celular cerca de 98% do oxigênio molecular é reduzido a H_2O (HALLIWELL;GUTTERIDGE, 2007). Entretanto, uma pequena fração do oxigênio molecular (2%) sofre redução incompleta, o que acarreta na formação de ER como, por exemplo, o $O2^{\bullet-}$ (BARREIROS;DAVID, 2006). Segundo Fridovic (1989), a primeira ER formada após a redução do oxigênio, por passos de um elétron, é $O2^{\bullet-}$ o qual pode agir como oxidante ou como redutor, formando outras espécies reativas.

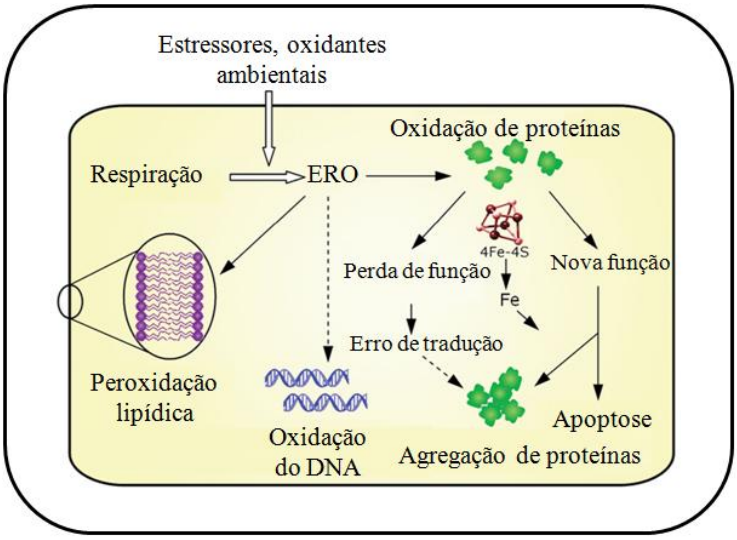

Figura 3: Dano oxidativo às macromoléculas biológicas.
Fonte: AVERY (2011), com adaptações.

Os $O2^{\bullet-}$ são eliminados por uma reação catalisada pela enzima superóxido dismutase (SOD), o qual gera H_2O_2. Desta forma, o H_2O_2 é formado a partir da redução parcial do oxigênio molecular por dois elétrons. O H_2O_2 é pouco reativo com moléculas orgânicas, contudo, ele é capaz de transpor as membranas celulares e reagir com metais de transição o que promove a geração do OH^- (BARREIROS; DAVID, 2006). O radical OH^- pode ser gerado também a partir da reação do $O_2^{\bullet-}$ com o H_2O_2. Este OH^- é altamente reativo, apesar de seu baixo tempo de vida e baixa concentração intracelular (BARREIROS; DAVID, 2006). Assim, este radical livre promove danos aos constituintes celulares, moléculas orgânicas, proteínas e DNA. Além disso, os radicais OH^- podem atacar os lipídeos de membrana,

promovendo a peroxidação lipídica (HALLIWELL; GUTERIDGE, 2000; STARK, 2005).

A peroxidação lipídica tem início pela ação do radical OH^- sobre os ácidos graxos poliinsaturados da membrana plasmática, promovendo perda de elétron. Desta forma, o ácido graxo com um elétron desemparelhado reage com o oxigênio molecular, formando radical peroxil. Este radical pode se combinar com outros radicais semelhantes, propagando os danos às estruturas biológicas (GATÉ et al., 1999). Um dos produtos da lipoperoxidação é o malondialdeído (MDA), um dialdeído altamente reativo que eventualmente reage com o amino grupo de proteínas, fosfolipídios ou ácido nucleicos, induzindo modificações estruturais das moléculas (DRÖGE, 2002; CHERUBINI et al., 2005).

Os danos oxidativos promovidos pelas espécies reativas são minimizados pela ação do sistema de defesa antioxidante. Antioxidantes são compostos que protegem os sistemas biológicos contra os efeitos deletérios dos processos ou das reações que levam à oxidação de macromoléculas ou estruturas celulares (VALKO et al., 2007). Este sistema de defesa antioxidante é dividido em enzimático e não-enzimático. Entre os principais antioxidantes não enzimáticos destacam-se os tióis protéicos e não protéicos (NPSH), vitamina C (VIT C) e vitamina E (VIT E) (HALLIWELL;GUTTERIDGE, 2000; ALMEIDA, 2008). Já as enzimas superóxido dismutase (SOD), catalase (CAT) e a glutationa peroxidase (GPx), são as principais representantes do sistema de defesa antioxidante enzimático (BARREIROS;DAVID, 2006).

A enzima SOD (EC 1.15.1.1), uma metaloenzima, foi a primeira enzima antioxidante descoberta e , constitui a primeira linha de defesa contra o excesso de oxidantes (GATÉ et al., 1999; DRÖGE, 2002). Existem 3 classes de SOD: Fe-SOD, CuZn-SOD (citosólica) e Mn-SOD (mitocondrial). A CuZn-SOD e a Mn-SOD encontram-se em eucariotos e a Fe-SOD apenas em procariotos. Sua função nas células eucarióticas é catalisar a dismutação do ánion superóxido ($O2^{\bullet-}$), produzindo H_2O_2 (Figura 4). Assim, a ação da enzima SOD impede o acúmulo do $O2^{\bullet-}$, o que evita sua reação com o óxido nítrico (NO•), inibindo a formação do peroxinitrito ($ONOO^-$) (GATÉ et al., 1999).

A CAT (EC 1.11.1.6) é uma hemoproteína com estrutura tetramérica, ou seja, é composta por quatro subunidades idênticas (monômeros) com um grupo ferro

protoporfirina, e massa molecular de 240 KDa. A CAT é encontrada em vários tecidos animais, principalmente no sangue, medula óssea, mucosas, rim e fígado. No fígado, esta enzima está presente nos peroxissomos e mitocôndrias e sua atividade depende de NADPH (FERREIRA; MATSUBARA, 1997; VALKO et al., 2006). Sua ação dá continuidade ao processo de eliminação de ERO decompondo tanto o H_2O_2 gerado pela redução do O_2, quanto o da dismutação do $O2^{\bullet-}$ em H_2O e O_2. (HALLIWELL;GUTTERIDGE, 2000) (Figura 4). Devido ao seu elevado K_m, são necessárias duas moléculas de H_2O_2 no sitio ativo para que sua atividade seja completa. O H_2O_2 tem vida longa, é capaz de atravessar camadas lipídicas, podendo reagir com a membrana eritrocitária e com proteínas ligadas ao Fe^{2+}. Apesar de não ser um radical livre, pela ausência de elétrons desemparelhados na última camada, o H_2O_2 é um metabólito do oxigênio extremamente deletério, porque participa da reação que produz o radical $OH^{\bullet}$ (HALLIWELL;GUTTERIDGE, 2000, WIERNSPERGER, 2003)

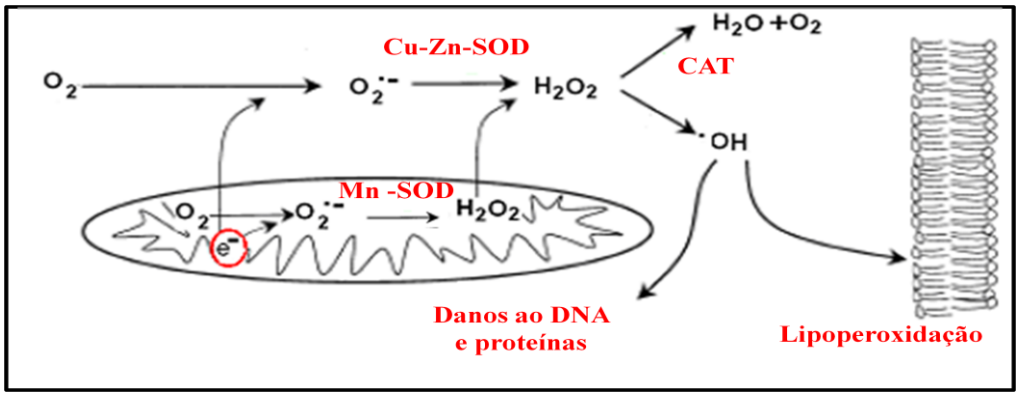

Figura 4: Mecanismo antioxidante das enzimas superóxido dismutase e catalase. Fonte: NORDBERG;ARNER (2001), com adaptações.

Assim o equilíbrio entre a atividade das enzimas SOD e da CAT é de fundamental importância na determinação do equilíbrio dos níveis de radical $O_2^{\bullet-}$ e do H_2O_2 nas células a fim de evitar a formação do radical $OH^{\bullet}$ altamente reativo (SANTINI et al., 1997; BRIONES;TOUYZ, 2010). Outra importante enzima que atua na eliminação do H_2O_2 é a glutationa peroxidase (GPx) (Figura 5). Nesta eliminação a glutationa opera em ciclos entre sua forma oxidada e reduzida. Na presença da enzima GPx, a glutationa reduzida (GSH) reduz o H_2O_2 a H_2O, formando glutationa

oxidada (GSSH). A GSSH é reciclada, por uma reação catalisada pela glutationa redutase (GR), reação esta dependente de NADPH (BARREIROS;DAVID, 2006). Além disso, a enzima glutationa S- transferase catalisa a conjugação da glutationa com diversos compostos produzidos na presença do estresse oxidativo (FRANKLIN,2010).

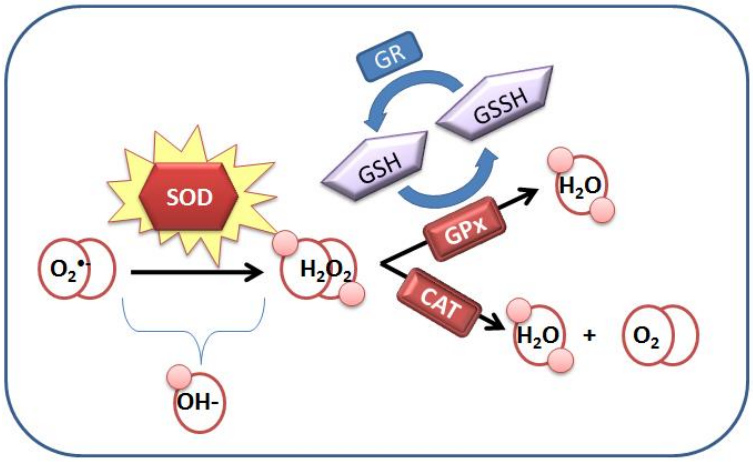

Figura 5: Interação do sistema de defesa enzimático.

Outra importante enzima que tem sido relacionada com o estresse oxidativo é a delta-aminolevulinato ácido desidratase (δ-ALA-D) (EC 4.2.1.24). Esta enzima sulfidrílica participa da biossíntese de moléculas tetrapirrólicas de importantes proteínas fisiológicas como a hemoglobina e os citocromos. Assim, a inibição da δ-ALA-D pode resultar no acúmulo do seu substrato δ-ALA, o qual está relacionado com a superprodução de ERO (PEREIRA et al., 1992; SASSA et al., 1998). Assim, a δ-ALA-D em conjunto com outros biomarcadores de estresse oxidativo, pode atuar como um marcador de dano oxidativo (VALENTINI et al., 2008).

Além destas enzimas antioxidantes existem sistemas de defesa não enzimáticos que desempenham um importante papel no organismo celular. Segundo Halliwell e Gutteridge (2000), o organismo pode produzir compostos com capacidade de defesa antioxidante, os quais ajudam a manter a homeostase celular. A glutationa reduzida (GSH) é um dos mais importantes tióis intracelulares, sendo o tiol não-protéico mais abundante nas células animais (LI et al., 2001). A GSH é formada por cisteína, glicina e resíduos de ácido glutâmico e cuja capacidade redutora é determinada pelo grupamento - SH presente na cisteína. A vitamina C é outro

importante antioxidante. Este composto hidrossolúvel tem a capacidade de neutralizar ER ao participar de reações de oxi-redução como transportador de hidrogênio ou como aceptor de moléculas isoladas de oxigênio (ALMEIDA, 2008).

Alteração em vários parâmetros de estresse oxidativo já tem sido descritos tanto em humanos quanto em modelos experimentais de DM. A hiperglicemia parece ser um dos fatores mais importantes relacionados ao aumento do estresse oxidativo nesta patologia. O aumento na produção de ERO pode ser associado a auto-oxidação da glicose, formação de produtos finais de glicação avançada e problemas nos sistemas de defesa antioxidante do organismo (RAINS;JAIN, 2011). Além disso, a oxidação de lipídeos e proteínas contribui para o desenvolvimento e progressão das complicações diabéticas (REIS et al., 2008).

Dados da literatura já têm demonstrado um aumento significativo nos níveis de peroxidação lipídica em vários tecidos como rim, pâncreas, soro e eritrócitos de animais submetidos a modelos experimentais de diabetes (YILMAZ et al., 2004; LIKIDLILID et al., 2010; PUNITHAVATHI et al., 2011; STEFANELLO et al., 2015). Além disso, também já foram descritas alterações nas defesas antioxidantes como inibição das enzimas CAT, SOD no fígado, rim e pâncreas, juntamente com a diminuição dos níveis de tióis não protéicos e vitamina C no fígado e rim (ARULSELVAN;SUBRAMANIAN, 2007; KADE et al., 2009; PUNITHAVATHI et al., 2011; SCHMATZ, et al., 2012; STEFANELLO et al., 2015). É importante destacar que estudos já demonstraram também uma inibição da enzima δ-ALA-D em fígado e rim de animais diabéticos (SCHMATZ et al. 2012; STEFANELLO et al., 2015). Esses achados sustentam fortemente a hipótese de que o estresse oxidativo está envolvido no desenvolvimento e progressão de várias alterações neuroquímicas, hepáticas, renais e pancreáticas que fazem parte da etiopatogenia desta doença.

Considerando o papel do estresse oxidativo no desenvolvimento das complicações diabéticas, o uso de substâncias com atividades antioxidantes têm sido uma estratégia promissora associada ao tratamento convencional da doença (ROCHA et al., 2006). Antioxidantes naturais identificados em plantas têm mostrado efeitos benéficos no combate do diabetes e parecem representar uma oportunidade empolgante para o desenvolvimento de novas classes terapêuticas (ANDRADE – CETTO; WIEDENFILED, 2001; NEGRI, 2005; ROCHA et al., 2006).

Dados da literatura têm demonstrado que compostos fenólicos presentes na dieta possuem muitas atividades biológicas importantes tais como ação antioxidante, antiinflamatória, antimicrobiana, anticarcinogênica, ansiolítica e hipoglicêmica (KONO et al., 1998; SANTOS et al., 2006; BASSOLI et al., 2008; FARAH;DONANGELO, 2006).

Os compostos fenólicos são derivados do metabolismo secundário de plantas, sendo importante para suas funções de crescimento e reprodução. Estes compostos também são formados frente a situações agressoras como infecções, ferimentos, radiações UV, entre outras (NACZK;SHAHIDI, 2004). As principais fontes destes compostos são frutas (limão, laranja, cereja, uva, ameixa, pêra, maçã), vegetais, legumes e cereais (PIMENTEL et al., 2005; FARAH;DONANGELO, 2006). Além disso, compostos fenólicos são componentes importantes nos vinhos, contribuindo para as características sensoriais como cor, sabor, adstringência e dureza (ABE et al., 2007; GIEHL et al., 2007).

Quimicamente, os compostos fenólicos são definidos como substâncias que possuem pelo menos um anel aromático com um ou mais grupamentos hidroxílicos. Além disso, sua síntese ocorre principalmente pela via do ácido chiquímico (SANTOS, 2007). Apresentam estrutura variável, desde moléculas simples até moléculas com alto grau de polimerização, o que os conferem uma variedade de características multifuncionais. Dentre os fenólicos, destacam-se os flavonóides, os ácidos fenólicos, os tocoferóis e os taninos.

Os flavonóides são compostos de baixo peso molecular caracterizados por um esqueleto base contendo 15 átomos de carbono (C6-C3-C6). A sua estrutura química consiste em dois anéis aromáticos unidos por 3 carbonos que formam um anel heterocíclico (Figura 6A). Fazem parte deste grupo os flavanóis (catequina, epicatequina e epigalocatequina), flavonóis (caempferol, quercetina, rutina e miricetina) e as antocianinas (malvidina e cianidina) (MAMEDE; PASTORE, 2004). Também fazem parte do grupo dos flavonóides, a classe dos dihidroflavonóis e as flavonas das folhas da parreira (GONZALEZ-PARAMAS et al., 2004).

Os taninos possuem o peso molecular relativamente alto, constituem uma classe de polifenóis e, segundo a estrutura química, são classificados em taninos hidrolisáveis e taninos condensáveis (OSZMIANSKI et al., 2007).

Os tocoferóis são compostos monofenólicos. Eles estão divididos em dois grupos com estruturas semelhantes os quais são denominados de tocoferóis (Figura 6 D) e tocotrienos (Figura 6 E) (ANGELO; JORGE, 2007).

Os ácidos fenólicos podem ser divididos em dois grupos. O primeiro é composto pelos derivados do ácido hidroxibenzóico (Figura 6B) e são os ácidos fenólicos mais simples encontrados na natureza. Este grupo é representado pelos ácidos gálico, p-hidroxibenzóico, protocatecuico, vanílico e siríngico. O segundo é formado pelos derivados do ácido hidroxicinâmico (Figura 6C), sendo o ácido caféico, ferúlico, p-cumárico e sináptico os seus principais representantes (ANGELO; JORGE, 2007).

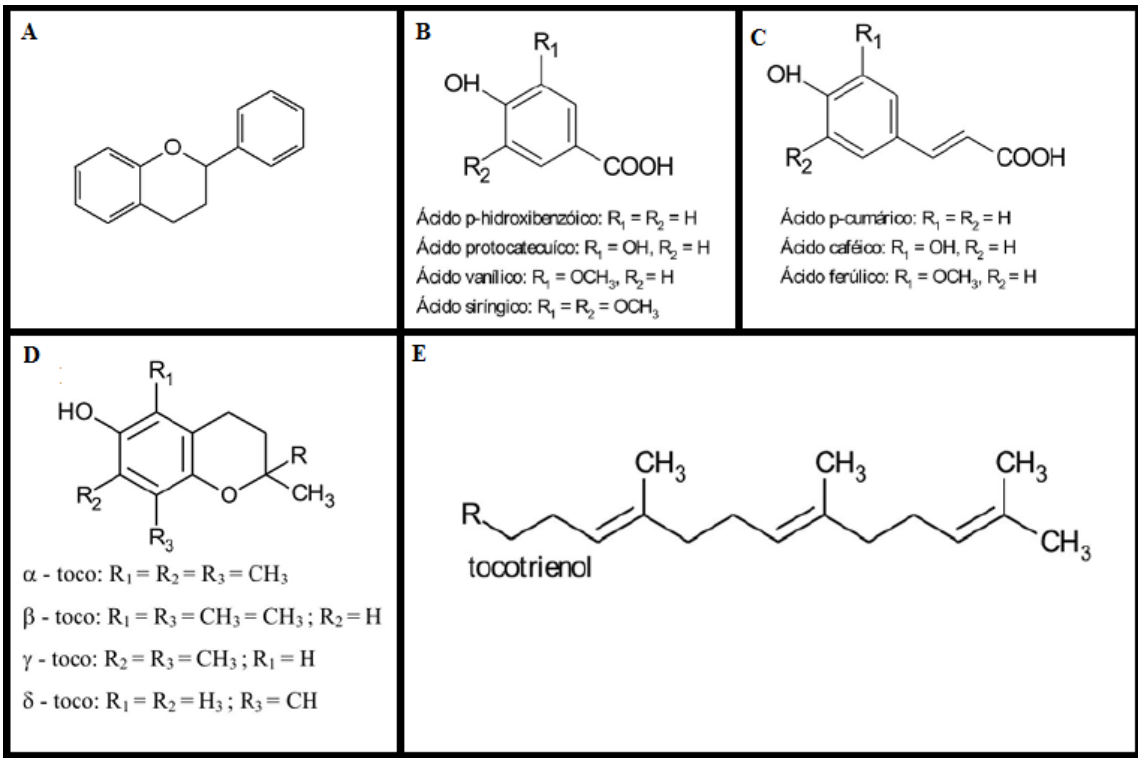

Figura 6: Estrutura química de compostos fenólicos: A – flavonóides; B- ácido hidrobenzóico; C- ácido hidrocinâmico; D- tocoferol; E- tocotrienos.
Fonte: Angelo;Jorge (2007), com adaptações.

Um importante composto fenólico que tem despertado interesse de pesquisadores é o ácido gálico. O ácido gálico é um ácido fenólico derivado do ácido hidroxibenzóico, o qual é produzido através da via do ácido chiquímico, sendo um intermediário do metabolismo secundário (GRUNDHOFER et al., 2001). O ácido

gálico é uma molécula plana, quimicamente denominado de ácido 3,4,5-triidroxibenzóico (Figura 7). Sua estrutura consiste de um anel aromático, três grupos hidroxila e um grupo ácido carboxílico (PUNITHAVATHI et al., 2011). Os três grupos hidroxila estão ligados ao aromático anel em posição orto em relação um ao outro, o que é imprescindível para a forte capacidade antioxidante de compostos fenólicos (SROKA; CISOWSKi, 2003).

Seus ésteres n-alquílicos, também conhecidos como galatos, em especial o galato de propila, octila e dodecila, são utilizados como aditivos antioxidantes em alimentos, para prevenir mudanças no sabor e no valor nutritivo devido à oxidação de gorduras insaturadas (VAN DER HEIJDEN; JANSSEN; STRIK, 1986; KUBO et al., 2002; MUNOZ et al., 2002; OW; STUPANS, 2003).

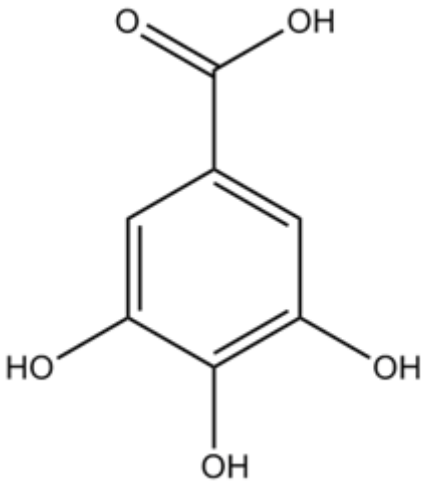

Figura 7: Estrutura química do ácido gálico.
Fonte: Punithavathi et al, 2011.

O ácido gálico é muito bem absorvido em humanos sendo que concentrações micromolares de formas livres e glicuronados têm sido observadas em plasma de humanos após ingestão de alimentos ricos em ácido gálico (MANACH et al., 2004; SHAHRZAD et al, 2001). No reino vegetal, o ácido gálico é encontrado principalmente em sua forma livre ou ligada a açúcares. As principais fontes de ácido gálico são nozes, chá, uvas, casca de carvalho, mel, romã, manga entre outras frutas, legumes e bebidas (SHAHRZAD et al., 2001; SAMANIDOU et al., 2012)

Várias atividades biológicas do ácido gálico têm sido relatadas na literatura. A sua capacidade anticancerígena, é devido à ação pró-oxidante de seus compostos galatos que induzem apoptose de células tumorais, deixando intactas as células saudáveis (INOUE et al., 1995; CHIA et al., 2010; INOUE et al., 2000).

A atividade antiinflamatória é desencadeada por dois mecanismos: o primeiro está relacionado com a inibição da enzima mieloperoxidase, devido ao bloqueio da produção de ácido hipocloroso; já o segundo está relacionado com a eliminação de ERO (KIM et al., 2006; ROSSO et al., 2006). Outra ação desenvolvida pelo ácido gálico é a inibição da tirosinase, que evita a hiperpigmentação da pele, agindo como protetor (KIM et al., 2007).

O AG também desempenha capacidade antibacteriana (KANG et al., 2008), antiviral (KRATZ et al., 2008), antialérgica (ABE et al., 2000), neuroprotetora (QI et al., 2005), nefro e hepatoproterora (NABAVI et al., 2013), e antioxidante (KIM et al., 2006).

Existem várias possibilidades para o AG atuar como antioxidante. Uma delas é a partir da restauração dos níveis de antioxidantes, como SOD, CAT, GST. Há relatos que o tratamento com AG é capaz de restabelecer a atividade da enzima CAT e diminuir a peroxidação lipídica no fígado e rim, bem como aumentar os níveis de GSH, vitamina C (LI et al., 2005). Ele também, devido principalmente as propriedades redox, pode agir como agente redutor, doador de hidrogênio e supressor de oxigênio singleto (KÄHKÖNEN et al., 1999).

Alguns estudos já demonstram que compostos fenólicos como ácido clorogênico e resveratrol foram capazes de reverter alterações em parâmetros de estresse oxidativo em um modelo experimental de diabetes (SCHMATZ et al., 2012; STEFANELLO et al., 2015). Desta forma, considerando que o ácido gálico também possui muitas atividades biológicas já descritas, como por exemplo, ação antioxidante, torna-se importante avaliar os efeitos deste composto sobre parâmetros bioquímicos e de estresse oxidativo em um modelo experimental de diabetes tipo 1, a fim de contribuir para a busca de novas terapias a base de compostos naturais que possam beneficiar pacientes com esta endocrinopatia.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar os efeitos do ácido gálico em parâmetros bioquímicos e de estresse oxidativo em fígado e rim de animais diabéticos induzidos por estreptozotocina.

2.2. Objetivos específicos

Em ratos diabéticos induzidos por estreptozotocina submetidos ao tratamento com ácido gálico:

- Avaliar parâmetros bioquímicos como glicose, colesterol total, lipoproteína de alta densidade, lipoproteína de baixa densidade, triglicerídeos, alanina aminotransferase, aspartato amino transferase e uréia no soro.

- Determinar o nível de espécies reativas em sobrenadante de fígado e rim

- Determinar os níveis de peroxidação lipídica em sobrenadante fígado e rim.

- Avaliar a atividade das enzimas SOD, CAT e GST em sobrenadante fígado e rim.

- Determinar a atividade da enzima δ-ALA-D em sobrenadante fígado e rim.

- Determinar os níveis de vitamina C em sobrenadante fígado e rim.

- Analisar parâmetros histológicos no fígado e rim.

3. METODOLOGIA E RESULTADOS

Os resultados desta dissertação estão apresentados na forma de um manuscrito. Os itens materiais e métodos, resultados, discussão e referências bibliográficas encontram-se no próprio manuscrito e representam a íntegra deste estudo. O manuscrito está formatado de acordo com as normas para publicação da revista *Food and Chemical Toxicology.*

Manuscrito: Effects of gallic acid on biochemical, histological and oxidative stress parameters in liver and kidney from streptozotocin-induced diabetic rats.

3.1. Manuscrito

Effects of gallic acid on biochemical, histological and oxidative stress parameters in the liver and kidney of streptozotocin-induced diabetic rats

Lizielle Souza de Oliveira[a], Gustavo Roberto Thomé[a], Thauan Faccin Lopes[a], Karine Paula Reichert[a], Juliana Sorraila de Oliveira[a], Aline da Silva Pereira[a], Cristina da Costa Krewer[b], Vera Maria Morsch[a], Maria Rosa Chitolina Schetinger[a], Roselia Maria Spanevello[c]*

[a] Programa de Pós Graduação em Bioquímica Toxicológica, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

[b] Departamento de Morfologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

[c] Programa de Pós Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário, Capão do Leão, 96010-900 Pelotas, RS, Brazil

***** Corresponding author. Phone: +55 53 39217233

E-mail: rspanevello@gmail.com

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; δ-ALA, δ-aminolevulinic acid; δ-ALA-D, delta-aminolevulinic acid dehydratase; DCF, dichlorofluorescein; DCFH-DA, dichloro-dihydro-fluorescein diacetate; DM, diabetes melitus; DNPH, dinitrophenyl hydrazine; GA, gallic acid; GST, glutathione S-transferase; HDL, high density lipoprotein; LDL, low density lipoprotein; PBG, porphobilinogen; ROS, reactive oxygen species; RS, reactive species; SD, standard deviation; SDS, sodium dodecylsulfate; SOD, superoxide dismutase; STZ, streptozotocin; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TC, total cholesterol; TCA, trichloroacetic acid; TG, triglycerides.

Abstract

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycaemia associated with the increase of free radicals and decrease of the antioxidant response. It is well established that gallic acid has potent antioxidant properties. The aim of the present study was to evaluate the effect of gallic acid on the biochemical and histological parameters and oxidative stress biomarkers in the liver and kidney of streptozotocin-induced diabetic rats. Forty male Wistar rats were divided in four groups: control/saline, control/gallic acid, diabetic/saline and diabetic/gallic acid. DM was induced in the animals by intraperitoneal injection of streptozotocin (65 mg/kg). Gallic acid (30 mg/kg) was administered orally for 21 days. Our results showed an increase in reactive species levels and lipid peroxidation, and a decrease in activity of the enzymes superoxide dismutase and delta-aminolevulinic acid dehydratase in the liver and kidney of the diabetic animals ($P<0.05$). Gallic acid treatment showed protective effects in these parameters evaluated, and also prevented a decrease in the activity of catalase and glutathione S-transferase, and vitamin C levels in the liver of diabetic rats. In addition, treatment with gallic acid prevented an increase of aspartate aminotransferase activity, reduced the number of nuclei and increased the area of the core in hepatic tissue, and increased the glomerular area in renal tissue. These results indicate that gallic acid can protect against oxidative stress-induced damage in the diabetic state. Therefore, we suggest that gallic acid could be a potential candidate for complementary therapy in diabetes due to its antioxidant action, in combination with hypoglycaemic drugs.

Keywords: Antioxidant, Delta-aminolevulinic acid dehydratase, Diabetes mellitus, Gallic acid, Liver, Kidney

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that affects about 346 million people worldwide and it is considered one of the major public health problems (Danaei et al., 2011). It is characterised by hyperglycaemia, resulting from defects in the action and/or secretion of insulin. The main DM symptoms are polydipsia, polyphagia, polyuria, weight loss, fatigue and loss of vision. In most cases, symptoms are mild or can even be absent and, consequently, hyperglycaemia is sufficient to cause pathological and functional changes in the short and long term (American Diabetes Association, 2014).

The etiology of DM is multiple; however, studies have related that oxidative stress plays a central role in the pathogenesis and complications of the disease (Yang et al., 2011). This hypothesis is supported by evidence that persistent hyperglycaemia increases the production of reactive oxygen species (ROS), through mechanisms of glucose auto-oxidation and protein glycosylation (Rains and Jain, 2011). In addition, alterations in oxidative stress parameters such as increased free radical production and diminished antioxidant status have been observed in human and animal diabetics (Turk et al., 2002; Memisogullari et al., 2003; Schmatz, et al., 2012; Palma et al., 2014; Stefanello et al., 2015).

Oxidative stress is defined as an imbalance between the production of free radicals and the antioxidant defence status (Valko et al., 2007). This imbalance can cause oxidation and damage of macromolecules such as DNA, proteins and lipids, resulting in cell dysfunction and tissue injury. Defence against free radical damage includes non-enzymatic systems such as vitamins C and E, carotenoids, flavonoids and glutathione, and enzymatic antioxidant systems that are represented by the enzymes superoxide dismutase, catalase and glutathione peroxidase (Halliwell and

Gutteridge, 2010). These antioxidant defences are extremely important since they represent the direct removal of free radicals and have a key role in protecting cells against oxidative stress (Valko et al., 2007).

Another important enzyme which has been related to oxidative stress is delta-aminolevulinic acid dehydratase (δ-ALA-D), which catalyses the synthesis of porphobilinogen (PBG) from two molecules of δ-aminolevulinic acid (δ-ALA) (Fujihara et al., 2009). Its inhibition may result in the accumulation of its substrate δ-ALA, which is associated with the overproduction of ROS (Pereira et al., 1992; Sassa, 1998). Of particular importance, a decrease in the activity δ-ALA-D has been observed in the liver and kidney of diabetic rats (Schmatz et al., 2012; Stefanello et al., 2015).

Many studies have demonstrated that the use of compounds with antioxidant properties has beneficial effects in the treatment of complications associated with the diabetic state (Kim et al., 2006; Bhooshan et al., 2009; Schmatz et al., 2012; Palma et al., 2014). Gallic acid (GA) is a phenolic acid, chemically known as 3,4,5-trihydroxybenzoic acid (Figure 1). The chemical structure of GA consists of one aromatic ring with three hydroxyl groups and one carboxylic acid group. The hydroxyl groups are bonded to the aromatic ring in the ortho position which is essential for the strong antioxidant capacity of phenolic compounds (Sroka and Cisowski, 2003). In this respect, the antioxidant propriety of GA is well established in the literature; however, evidence has demonstrated that this compound also possesses anti-inflammatory (Pandurangam et al., 2015), antibacterial (Kang et al., 2008), antiviral (Kratz et al., 2008) and anticancer (Chia et al., 2010) properties. Based on these findings, GA has become attractive as a therapeutic agent in the treatment of many pathological conditions.

Therefore, considering that GA is a natural compound present in the diet, and it possesses an antioxidant action which might contribute to prevention of the progression of diabetic complications, the aim of this study was to evaluate the effect of this compound on biochemical and histological parameters and biomarkers of oxidative stress in the liver and kidney of streptozotocin (STZ)-induced diabetic rats.

2. Materials and Methods

2.1. Chemicals

DNPH (dinitrophenyl hydrazine), TCA (trichloroacetic acid), δ–ALA, hydrogen peroxide, epinephrine, STZ, GA, thiobarbituric acid (TBA), tris (hydroxymethyl)-aminomethane and Coomassie Brilliant Blue G were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and high purity.

2.2. Animals

Forty adult male Wistar rats were used in this experiment. They were kept on a 12 h light/12 h dark cycle at a temperature of 22 ± 2 °C, with free access to food and water, according to the guidelines of the Council for Animal Experiments Control (CONCEA), which is in accordance with international guidelines. All animal procedures were previously approved by the Animal Ethics Committee of the Federal University of Santa Maria (protocol number: 007/2015).

2.3. Experimental induction of diabetes

A type 1 diabetes model was induced by a single intraperitoneal injection of STZ at a concentration of 65 mg/kg, diluted in 0.1 M sodium citrate buffer (pH 4.5).

The control group received an equivalent dose of sodium citrate buffer. Animals submitted to STZ injection received 5% glucose solution instead of water for 24 h after diabetes induction, in order to reduce mortality due to hypoglycaemic shock. Blood samples were drawn five days after STZ/vehicle injection through the tail vein in order to assess blood glucose. Glucose levels were determined with a portable glucometer (ADVANTAGE, Boehringer Mannheim, MO, USA). Only animals with a high fasting glucose (250 mg/dL) were considered as diabetic and used for the present study. The weight of the animals and glucose levels were determined on the first and last (21st) days of treatment.

2.4. Treatment with gallic acid

The animals were randomly divided into four groups (n=10): control/saline, control/gallic acid, diabetic/saline and diabetic/gallic acid. GA treatment started one week after the induction of diabetes. Control/saline and diabetic/saline animals received saline solution for oral via. The animals of the control/gallic acid and diabetic/gallic acid groups received GA by oral administration at a concentration of 30 mg/kg/day, once a day at 11:00 am for 21 days. The time and dose were chosen based on previous studies in the literature (Punithavathi et al., 2011).

2.5. Tissue preparation for biochemical tests

Twenty-four hours after the last day of treatment, the animals were anaesthetised with halothane then submitted to euthanasia. Blood was collected by cardiac puncture, and the liver and kidneys were removed for biochemical determination. Samples of liver and kidney were placed on ice and homogenised in 50 mM Tris-HCl pH 7.4 (1/10, w/v). The homogenates were centrifuged at

2000 rpm for 10 min, and the supernatant (S1) was used for determination of vitamin C, ROS, thiobarbituric acid reactive substances (TBARS) and glutathione S-transferase (GST) activity.

The samples of liver and kidney for superoxide dismutase (SOD) and catalase (CAT) activity were diluted in 50 mM Tris-HCl pH 7.4 (1/50, w/v), homogenised and centrifuged at 2000 rpm for 10 min. The samples of liver and kidney for evaluating activity of δ-ALA-D were diluted in 0.9% sodium chloride solution (1/10 w/v).

2.6. Biochemical parameters

Blood was collected in tubes without an anticoagulant system and centrifuged at 1800 rpm for 10 min at room temperature. The clot was discarded and the serum was used for the determination of triglycerides (TG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and urea using commercial kits (Labtest® Diagnóstica S.A. MG, Brazil).

2.7. Reactive species quantification

The formation of reactive species (RS) was determined according to Ali et al. (1992), with some modifications. In this assay, the oxidation of dichloro-dihydro-fluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) was measured for the detection of intracellular RS. DCF fluorescence intensity emission was recorded at 525 and 488 nm excitation 60 min after the addition of DCFH-DA to the medium. RS levels were expressed as µmol/g of tissue.

2.8 Lipid peroxidation

Lipid peroxidation in the liver and kidney were estimated colourimetrically by measuring TBARS according to the method of Ohkawa et al. (1979). The reaction mixture contained 200 µL of samples of liver or kidney or standard (MDA – malondialdehyde, 0.03 mM), 100 µL water, 200 µL of 8.1% sodium dodecylsulfate (SDS), 500 µL of acetic acid solution (1.5 M, pH 3.4) and 500 µL of 0.8% TBA. This mixture was incubated at 95 °C for 2 h forming a purple coloured product which was measured at 532 nm, and the absorbance was compared to that of a standard curve obtained using MDA. TBARS tissue levels were expressed as nmol MDA/mg of protein.

2.9. Catalase and superoxide dismutase activity

CAT activity was determined by the method of Nelson and Kiesow (1972). In this assay the reaction mixture contained 1910 µL of 50 mM potassium phosphate buffer (pH 7.5), 20 µL of sample (S1) and 70 µL of 10 mM hydrogen peroxide (H_2O_2). The reaction was monitored for 2 min at 240 nm and involved monitoring the disappearance of H_2O_2 in the presence of the homogenate. CAT activity was expressed as units of CAT/mg of protein.

The measurement of SOD activity was based on the inhibition of the radical superoxide ($O_2^{\bullet-}$) reaction with epinephrine, as described by Misra and Fridovich (1972). The epinephrine undergoes auto-oxidation at pH 10.5 to produce adrenochrome, a coloured product detected at 480 nm. The addition of samples (20, 40, 60 µL) containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored for 2 min. The SOD activity was expressed in units of SOD/mg of protein.

2.10. Glutathione S-transferase activity

GST activity in the liver and kidney was determined as previously described by Habig et al. (1974), with minor modifications. Enzyme activity was calculated using the molar extinction coefficient of 9.6/mM/cm. GST activity was expressed as mmol/min/mg of protein.

2.11. δ-aminolevulinic dehydratase activity

δ-ALA-D activity was determined in the liver and kidney according to the method of Sassa (1981) by measuring the rate of PBG formation. The reaction was initiated by addition of δ-ALA to a final concentration of 2.2 mM in a phosphate buffered solution. An aliquot of 200 µL of sample (S1) was incubated for 30 min (liver) and 1 h (kidney) at 37 °C. The reaction was stopped by adding 250 µL TCA. The reaction product was determined at 555 nm using Ehrlich's reagent. δ-ALA-D activity was expressed as nmol PBG/mg protein/h.

2.12. Vitamin C levels

Vitamin C (ascorbic acid) levels in the liver and kidney were estimated as described by Jacques-Silva et al. (2001). The samples were deproteinised with TCA to 10% using 0.5 mL sample (S1) and 0.5 mL TCA and subjected to centrifugation; 300 µL of supernatant (S2) was removed and incubated at 37 °C in a medium containing 4.5 mg/mL DNPH, 0.6 mg/mL thiourea, 0.075 mg/mL $CuSO_4$ and 0.675 mol/L H_2SO_4. After 3 h of incubation, 1 mL of 65% H_2SO_4 was added. The test was read spectrophotometrically at 520 nm. Ascorbic acid content is represented in µg ascorbic acid/g tissue.

2.13. Protein determination

The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

2.14. Histology analysis

Renal and hepatic samples were collected, fixed in buffered formalin (10%) and preserved in alcohol (70%) for histological processing. Tissues were dehydrated, cleared and embedded in paraffin. Histological sections were made with intervals of 6 µm (three blades per animal) and stained using the hematoxylin-eosin method. One section was selected for analysis, where 12 renal corpuscles in six random fields were photographed. Areas of the glomerulus and corpuscle were analysed by Image-Pro Plus software (version 4.1 for Windows). Through the subtraction of these areas, the subcapsular space (μm^2) area was determined. In hepatic samples, nuclei were counted in a random space of 205 µm of their measurement area.

2.14. Statistical analysis

All procedures were performed in duplicate or triplicate and the measures analysed statistically through one-way analysis of variance followed by Tukey's post-test. $P<0.05$ was considered to represent a significant difference in the analysis used. All data are expressed as mean ± standard deviation (SD).

3. Results

3.1. Blood glucose and body weight

The results for body weight and blood glucose levels are shown in Table 1. These parameters were determined at the onset and the end of the GA treatment.

Our results showed that, at the onset and the end of the experiment, diabetic rats had a significant increase in glucose levels and decrease in body weight when compared with the control groups (Table 1, $P<0.05$). The treatment of diabetic rats with GA was not capable of decreasing hyperglycaemia (Table 1). No differences in glucose levels and body weight were observed in animals treated only with GA (Table 1).

3.2. Biochemical parameters

Table 2 shows the biochemical parameters. In TG, TC and LDL there was a significant decrease in the control/gallic acid group, when compared to control/saline ($P<0.05$). In the urea levels, as can be observed, the diabetic/saline and diabetic/gallic acid groups had a significant increase when compared with to the control/saline group ($P<0.05$). The activities of serum enzymes AST and ALT were significantly higher in the diabetic/saline group when compared to the control/saline group ($P<0.05$). However, treatment with GA was able to decrease only the activity of AST in the serum of rats ($P<0.05$).

3.3. Reactive species levels and lipid peroxidation

The RS levels in liver (A) and kidney (B) are shown in Figure 2. The RS levels were significantly increased in the liver and kidney of the diabetic/saline group when compared to the control group (Figures 2A and B, $P=0.001$). However, when diabetic rats were treated with GA (30 mg/kg) a decrease in RS levels in both liver and kidney was observed when compared with the diabetic/saline group (Figures 2A and B).

In relation to lipid peroxidation, our results also showed an increase in TBARS levels in the liver and kidney of diabetic/saline rats (Figures 3A and B, P=0.006 and P=0.001, respectively). However, treatment with GA was capable of preventing the increase in lipid peroxidation in both tissues in the diabetic/gallic acid group when compared to the diabetic/saline group (Figures 3A and B).

3.4. Superoxide dismutase, catalase, glutathione S-transferase and δ-aminolevulinic dehydratase activity

Figures 4 and 5 show the effect of treatment with GA on the activity of antioxidant enzymes SOD and CAT in hepatic and renal tissue. The diabetic/saline group presented a significant decrease in SOD activity in liver (P=0.001) and kidney (P=0.005), and GA treatment (30 mg/kg) was able to prevent this alteration (Figures 4A and B). Similar results were obtained in relation to CAT activity. In the diabetic/saline group, a decrease in the activity of this enzyme in both hepatic and renal tissue (Figures 5A and B, P<0.001) was also observed. Treatment with GA was capable of preventing the alterations in CAT activity in the diabetic/gallic acid group only in the liver (Figures 5A and B).

In the liver, GST activity was decreased in the diabetic/saline group (Figure 6A, P=0.003) and administration of GA for 21 days was capable of preventing this alteration. In the kidney, no statistical difference was observed between the diabetic/saline and control/saline groups. However, an increase in GST activity was observed in the animals treated with GA (Figure 6B, P<0.05).

Our findings also showed alterations in δ-ALA-D in diabetic/saline rats. In the liver (Figure 7A, P=0.0001) and kidney (Figure 7B, P=0.0019) of the diabetic/saline group, a significant decrease in δ-ALA-D activity was observed. However, treatment

with GA was able to prevent the decrease in δ-ALA-D activity in the liver and kidney caused by diabetes (Figures 7A and B, respectively).

3.5. Vitamin C levels

Figure 8 shows the vitamin C levels in the liver (A) and kidney (B). In liver and kidney tissue, vitamin C levels were significantly reduced in rats of the diabetic/saline group when compared to the control/saline group (Figures 8A and B, $P<0.05$). GA (30 mg/kg) was capable of preventing the reduction of vitamin C levels in the diabetic/gallic acid group only in hepatic tissue (Figures 8A and B).

3.6. Histology

Table 3 shows the morphometric analysis of hepatocytes in the diabetic rats and/or those treated with GA. In the diabetic/saline group, no significant difference was observed in the area of the core when compared to the control/saline group ($P<0.05$). However, treatment of the control and diabetic groups with GA presented a significant increase in the area of the core when compared to the control/saline and diabetic/saline groups (Figures 9C and D). In relation to the number of nuclei in hepatocytes, our results showed a significant increase in the diabetic/saline group ($P<0.05$). Furthermore, treatment with GA was able to reduce this effect caused by diabetes.

Morphometric analysis of the kidney revealed no significant difference between the groups in the glomerular and renal corpuscle areas (Table 4). However, the subcapsular area in the control/gallic acid group was significantly increased when

compared with the other groups (Figures 9A and B). It is worth noting that the subcapsular area of the control/saline, diabetic/saline and diabetic/gallic acid groups did not differ statistically.

4. Discussion

STZ-induced diabetes is a well-characterised model of type 1 diabetes. As expected, our results showed that diabetic animals displayed hyperglycaemia and weight loss (Table 1). STZ causes degeneration in Langerhans islet β cells, reducing the synthesis and release of insulin as a consequence of glucose level increase in the animals (Maritim et al., 2003; Wu and Huan, 2008). The loss of body weight in this diabetes model can be associated with dehydration, catabolism of fats, increased muscle wasting and loss of tissue proteins (Rajagopal and Sasikala, 2008).

In the present study the antihyperglycaemic effect of GA (30 mg/kg) was not observed. Previous studies have related that oral administration of GA in doses of 10, 20 and 25 mg/kg decreased the levels of blood glucose and increased the levels of insulin in diabetic rats (Punithavathi et al., 2011; Kade and Rocha, 2013). This discrepancy in the relation of these findings can be explained by the doses of STZ administered. Besides this, in other studies GA treatment started three days after STZ injection and in the present study we induced a type 1 diabetes model and the animals were treated after seven days of STZ administration. In this context, we can suggest that this compound is not capable of improving glucose levels when administered in more advanced stages of this disease.

Studies in experimental models of diabetes and in diabetic patients have suggested that oxidative stress is involved in complications associated with the disease (Schmatz et al., 2012; Esteghamati et al., 2013; Stefanello et al., 2015). In

our study we demonstrated an increase in RS levels in the liver and kidney of diabetic animals. This increase in RS production can be associated with auto-oxidation of glucose, formation of advanced glycation end products and problems in antioxidant defence systems (Rains and Jain, 2011). As a consequence, the overproduction of RS can result in the initiation and propagation of lipid peroxidation, causing damage in hepatic and renal tissue.

Corroborating this hypothesis, our results showed an increase in TBARS levels in the liver and kidney of diabetic rats. Our findings are consistent with other studies where an increase in lipid peroxidation in many tissues such as liver, kidney, pancreas and brain of rats submitted to an experimental model of diabetes was described (Maritim et al., 2003; Punithavathi et al., 2011; Schmatz et al., 2012; Palma et al., 2014; Stefanello et al., 2014). Lipid peroxidation promotes serious changes to the cell membrane, causing loss of fluidity, a decrease in membrane potential, increased permeability and eventual cell rupture (Gutteridge, 1995). In this respect, oxidation of lipids or lipid peroxidation has crucial importance in the pathogenesis and complications of diabetes.

GA treatment (30 mg/kg) prevented the increase in RS and TBARS levels in the liver and kidney of STZ-induced diabetic rats. Punithavathi et al. (2011) also showed a decrease in lipid peroxidation in the pancreas of diabetic rats after 21 days of GA treatment (10 and 20 mg/kg). Similar results were described in the brain of diabetic animals after administration of GA (25 mg/kg) for 42 days (Kade and Rocha, 2013). This antioxidant effect of GA is due to its ability to scavenge free radicals via various electron transfers and chelate metals such as iron (Kähkönen et al., 1999; Rao et al., 2010; Marino et al., 2014). Thus, when acting as an iron chelator, GA inhibits the Fenton reaction, decreasing the formation of free radical

species and also reducing the amount of iron available to form complexes with oxygen, which is responsible for initiating lipid peroxidation.

Many reports have demonstrated that antioxidant enzyme activity is depleted in diabetes (Punithavathi et al., 2011; Schmatz et al., 2012; Palma et al., 2014; Stefanello et al., 2015). Consistent with the results of previous studies, we also observed a decrease in SOD and CAT activity in the liver and kidney of diabetic rats. The balance between the activity of SOD and CAT is of fundamental importance in determining the balance of $O2^{\bullet-}$ and H_2O_2 levels (Santini et al., 1997; Briones and Touyz, 2010). Based on this, our results suggest that the decrease in SOD and CAT activity contributes to the increase in RS levels and lipid peroxidation in the liver and kidney of STZ-induced diabetic rats.

In addition, it is important to note that the activity of the enzyme GST was also decreased in the liver of diabetic rats. GST is an enzyme of crucial importance, mainly in the liver, which catalyses the conjugation of reduced glutathione with toxic substances, especially those with an oxidant effect (Franklin, 2010). Inhibition of GST activity in diabetic animals can cause alterations in the major cellular defence strategies against xenobiotics. This alteration in GST activity may contribute to the liver becoming more susceptible to oxidative damage.

On the other hand, our results demonstrated that when diabetic rats were treated with GA this compound prevented the reduction in SOD, CAT and GST activity. Rassol et al. (2010) also showed that GA treatment (100 mg/kg) reverted changes in antioxidant enzymes in paracetamol-induced liver damage in rats. Of particular importance, when diabetic rats were treated with GA (10 and 20 mg/kg), an increase in SOD, CAT and glutathione peroxidase activity was observed in pancreatic tissue (Punithavathi et al., 2011). It can be suggested that, in addition to

preventing a decrease in the activity of these enzymes, GA also increases SOD and CAT expression. Studies have shown that the use of GA increases SOD and CAT mRNA expression in cardiac and hepatic tissue (Yeh and Yen, 2006; Yeh et al., 2009). Taken together, these findings suggest that GA can strengthen the antioxidant enzymatic defence system, reduce free radicals and alleviate liver and kidney damage caused by oxidative stress in diabetic rats.

δ-ALA-D, or PBG synthase, is a sulfhydryl-containing enzyme that catalyses the condensation of two molecules of aminolevulinate to produce PBG, which is the precursor of heme (Gibson et al., 1955). Our findings showed inhibition of the activity of this enzyme in hepatic and renal tissue of diabetic animals. Inhibition of δ-ALA-D activity has also been described in previous studies in the liver, kidney, brain and pancreas of rats submitted to experimental diabetes (Kade et al., 2009; Schmatz et al., 2012; Palma et al., 2014; Stefanello et al., 2015). The inhibition of δ-ALA-D may affect the heme biosynthesis pathway. Furthermore, the accumulation of its substrate δ-ALA is associated with the overproduction of ROS (Pereira et al., 1992; Sassa et al., 1998). Treatment with 30 mg/kg GA was able to prevent the decrease of δ-ALA-D activity in the liver and kidney of diabetic animals. Considering that GA decreases oxidative stress, the effect of this compound on δ-ALA-D activity can be associated with the prevention of oxidation of the essential sulfhydryl groups present in the active site of this enzyme.

Another important aspect to be discussed is that vitamin C levels were also decreased in the liver and kidney of diabetic rats. Vitamin C has the capacity to capture and neutralise ROS-eliminating RS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. GA was able to prevent a decrease in

vitamin C levels demonstrating that attenuation of oxidative stress by this compound occurred by enzymatic and non-enzymatic antioxidant mechanisms.

In the present study we also evaluated biochemical parameters such as TG, TC, LDL, HDL, AST, ALT and urea. GA promotes an improvement in the lipid profile of the control animals, because it reduces levels of TG, TC and LDL. Our results showed an increase in ALT and AST in the serum of diabetic rats. Abnormal elevation of aminotransferases is consistent with injury and inflammation of the liver (El-Demerdash et al., 2005). In addition to hepatic damage, diabetic rats also showed renal dysfunction evidenced by an increase in serum urea levels. GA treatment (30 mg/kg) was not capable of preventing alterations in ALT and urea levels. More studies are necessary to understand this response of GA in these biochemical parameters.

Previous studies have evaluated histological changes in the liver two, four and six weeks after STZ-induction of diabetes in rats. Two weeks after STZ injection, the liver showed several alterations including a mild degree of fatty changes, mild infiltration of lymphocytes and haemorrhage. However, after four and six weeks more progressive changes such as congestion, necrosis, severe hydropic degeneration and Kupffer cell hyperplasia have been observed (Salih et al., 2009). In our study, morphometric analysis of hepatocytes showed an increase in the number of nuclei in the diabetic/saline group (Table 3). This could be associated with an increase in the activity of antioxidant enzymes in the liver. The increase in the number of hepatocyte nuclei associated with the same cell area could predict that there is a decrease in cytoplasmic area, the area responsible for synthesis, metabolism and cellular detoxification. Another important aspect to note is that treatment with GA increases the cytoplasmic area which allows us to suggest that this compound, in addition to

preventing a decrease in the activity of antioxidant enzymes, also acts by stimulating enzymatic synthesis in the liver.

In the kidney, the increase in subcapsular space observed in the control/gallic acid group is associated with the high filtration activity of these animals. Studies have demonstrated that capsular space morphometry is an important evidence of filtration activity (Yaliali et al., 2015).

In conclusion, the results of this study showed that GA may provide effective protection against oxidative stress damage in the liver and kidney in rats with diabetes induced by STZ. GA may reduce lipid peroxidation and free radical levels and increase enzymatic and non-enzymatic antioxidant defence in these tissues. These findings provide evidence that GA may be useful for the treatment of hepatic and renal complications associated with DM and raise the possibility of a new application as a complementary therapy associated with hypoglycaemic drugs.

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Table 1 - Blood glucose levels and body weight of the diabetic and control rats at the onset and the end of the gallic acid treatment (30 mg/kg) for 21days.

Groups	Glucose (mg/dL)		Body weight (g)	
	Onset	End	Onset	End
Control saline	86.6 ± 6.3	155.6 ± 21.3	377.1 ± 53.1	426.5 ± 34.5
Control gallic acid	88.40 ± 6.8	125.0 ± 40.1	373.7 ± 30.8	405.9 ± 26.8
Diabetic saline	379.1 ± 27.0*	402.3 ± 63.8*	307.1 ± 31.8*	291.2 ± 26.50*
Diabetic gallic acid	355.0 ± 30.8*	442.0 ± 65.3*	323.1 ± 31.1*	299.0 ± 28.4*

Values are expressed as mean ± SD. * indicates a significant difference in relation to the control saline group ($P<0.05$, n=10).

Table 2 – Biochemical parameters in serum of STZ - induced diabetic rats and treated with gallic acid (30 mg/kg).

Groups	Control saline	Control gallic acid	Diabetic saline	Diabetic gallic acid
Tryglycerides (mg/dL)	216.0 ± 15.1	162.1 ± 28.6*	234.4 ± 46.7	170.2 ± 34.66
Total Cholesterol (mg/dL)	123.4 ± 18.5	84.8 ± 19.1*	87.2 ± 14.8*	112.9 ± 27.8
HDL (mg/dL)	57.9 ± 5.8	51.7 ± 9.2	55.8 ± 1.35	49.9 ± 16.6
LDL (mg/dL)	34.8 ± 1.5	8.9 ± 5.3*	22.4 ± 18.44	31.6 ± 14.59
AST (U/l)	6.4 ± 0.9	9.6 ± 1.3	12.9 ± 1.6*	9.7 ± 1.5
ALT (U/l)	5.9 ± 1.8	9.0 ± 0.7	11.2 ± 0.7*	10.4 ± 1.7*
Ureia (mg/dL)	18.6 ± 5.9	32.2 ± 12.7	68.6 ± 7.2*	67.7 ± 4.1*

Values are expressed as mean ± SD. * indicates a significant difference in relation to the control group ($P<0.05$, n=5).

Table 3 - Morphometric analysis of hepatocytes the diabetic rats treated with gallic acid (30 mg/kg) and the controls groups.

Groups	Area of the core (µm)	Number of nuclei
Control saline	41.1 ± 13.0	17.0 ± 4.1
Control gallic acid	49.0 ± 13.0*	15.0 ± 2.6
Diabetic saline	40.0 ± 13.0	21.0 ± 3.7*
Diabetic gallic acid	49.0 ± 20.0*	17.0 ± 4.0

Values are expressed as mean ± SD. * indicates a significant difference in relation to the control saline group ($P<0.05$).

Table 4 - Mean area renal parameters of the diabetic rats treated with gallic acid (30 mg/kg) and the controls groups.

Group	Glomerulus (μm^2)	Renal Corpuscle (μm^2)	Subcapsular área (μm^2)
Control saline	7474.0 ± 2252.0	10462.0 ± 3113.0	2988.0 ± 1548.0
Control gallic acid	7849.0 ± 2560.0	11690.0 ± 3117.0	3840.0 ± 1607.0*
Diabetic saline	7961.0 ± 2551.0	11269.0 ± 3132.0	3309.0 ± 1458.0
Diabetic gallic acid	8712.0 ± 2793.0*	11654.0 ± 3421.0	2942.0 ±1466.0

Values are expressed as mean ± SD. * indicates a significant difference in relation to the control saline group ($P<0.05$)

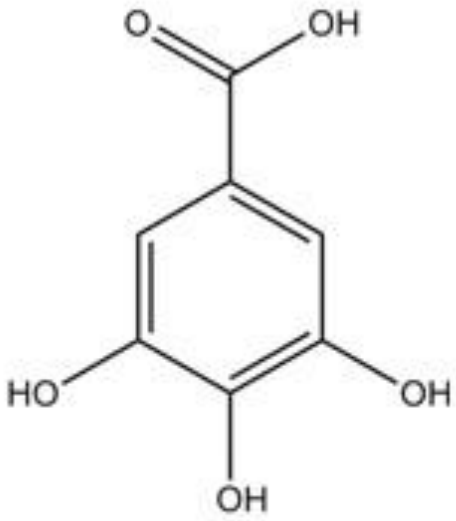
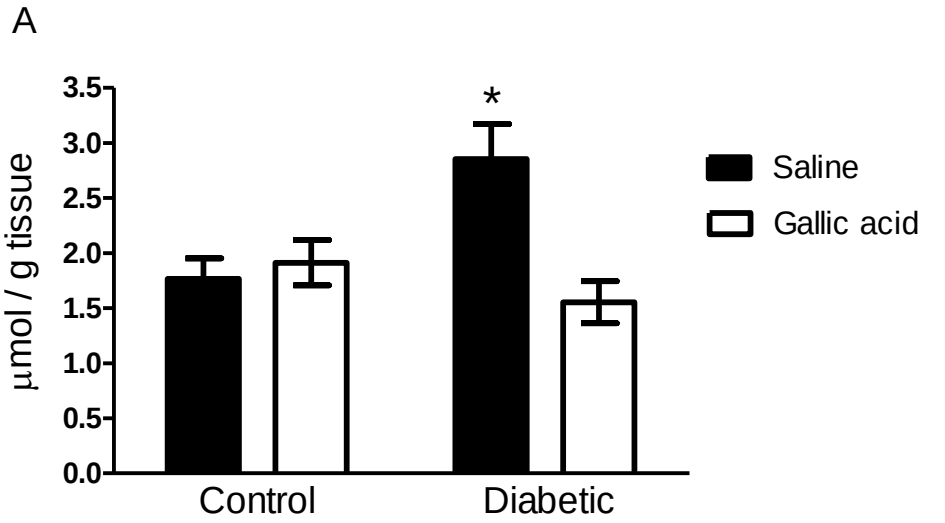


Figure 1: Chemical structure of Gallic acid (3,4,5-trihydroxybenzoic acid).



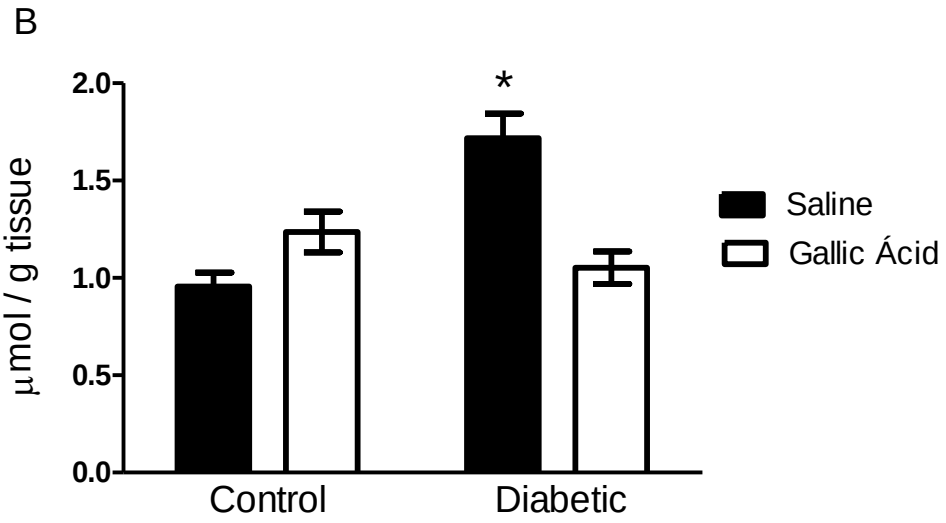
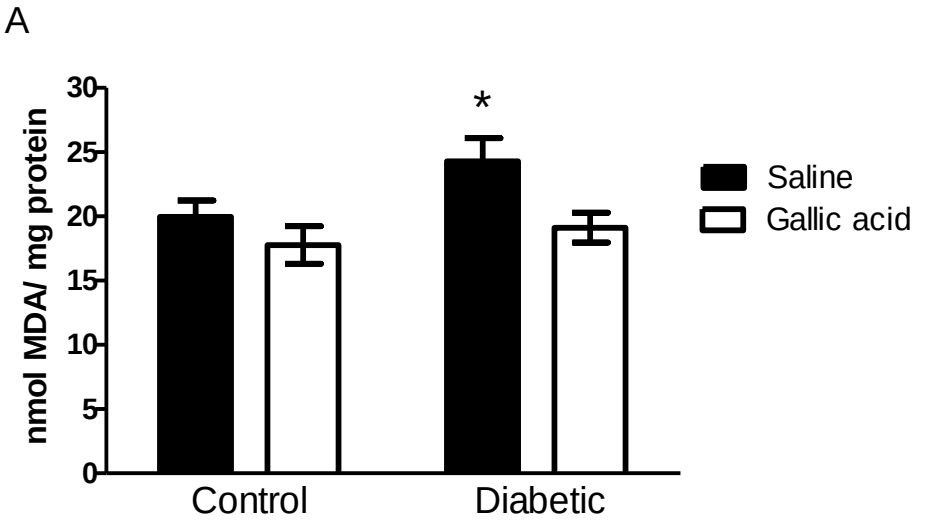


Figure 2: Reactive species levels on liver (A) and kidney (B) from STZ-induced diabetic rats and of animals treated with Gallic Acid (30 mg/kg). Bars Represent means ± S.D. * Different the others groups (P<0.05, n = 10).



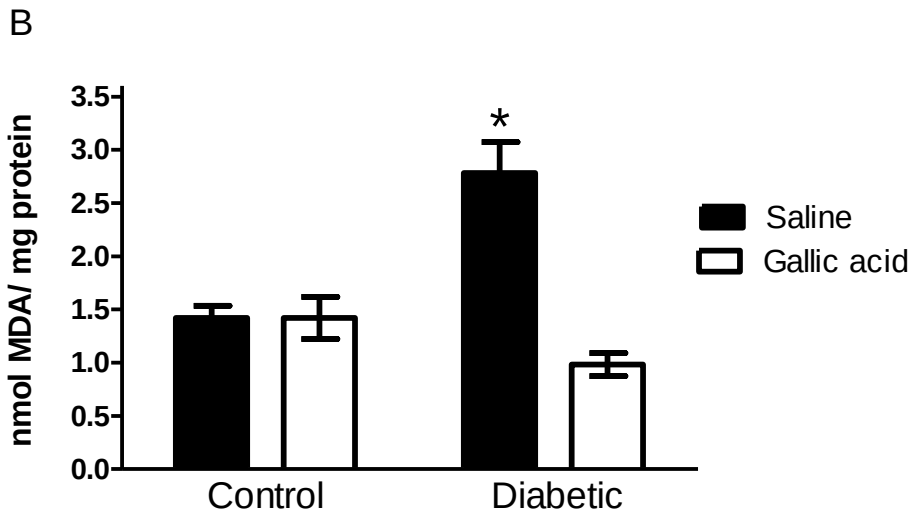


Figure 3: Levels of thiobarbituric acid reactive substances (TBARS) on liver (A) and kidney (B) from STZ- induced diabetic rats and in animals treated with Gallic Acid (30mg/kg). Bars Represent means ± S.D. * Different the others groups. ($P<0.05$, n = 10).

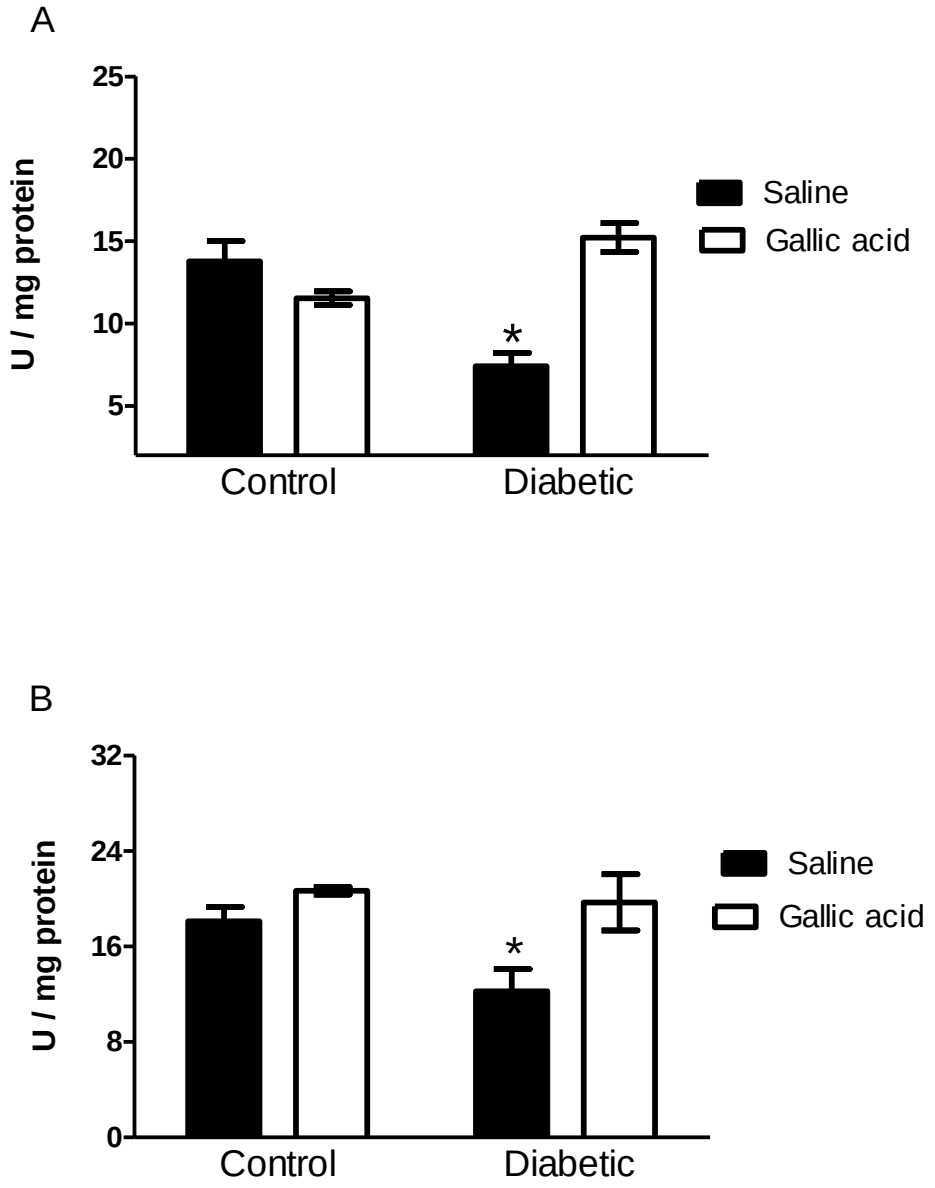


Figure 4: Superoxide dismutase activity on liver (A) and kidney (B) from STZ-induced diabetic rats and of animals treated with Gallic Acid (30mg/kg). Bars Represent means ± S.D. * Different the others groups. ($P<0.05$, n = 10).

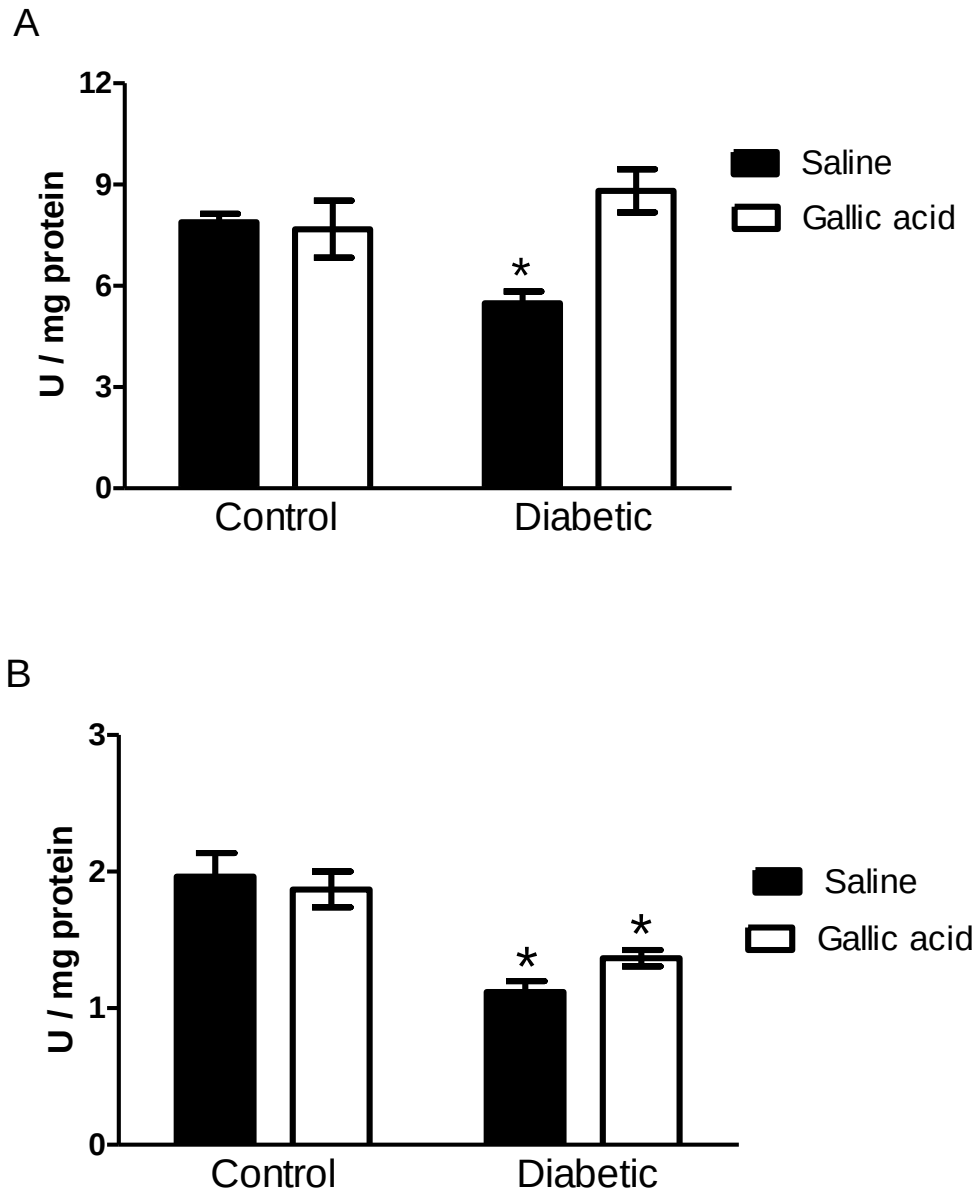


Figure 5: Catalase activity on liver (A) and kidney (B) of STZ-induced diabetic rats and of animals treated with Gallic Acid (30 mg/kg). Bars Represent means ± S.D. * Different the others groups. ($P<0.05$, n = 10).

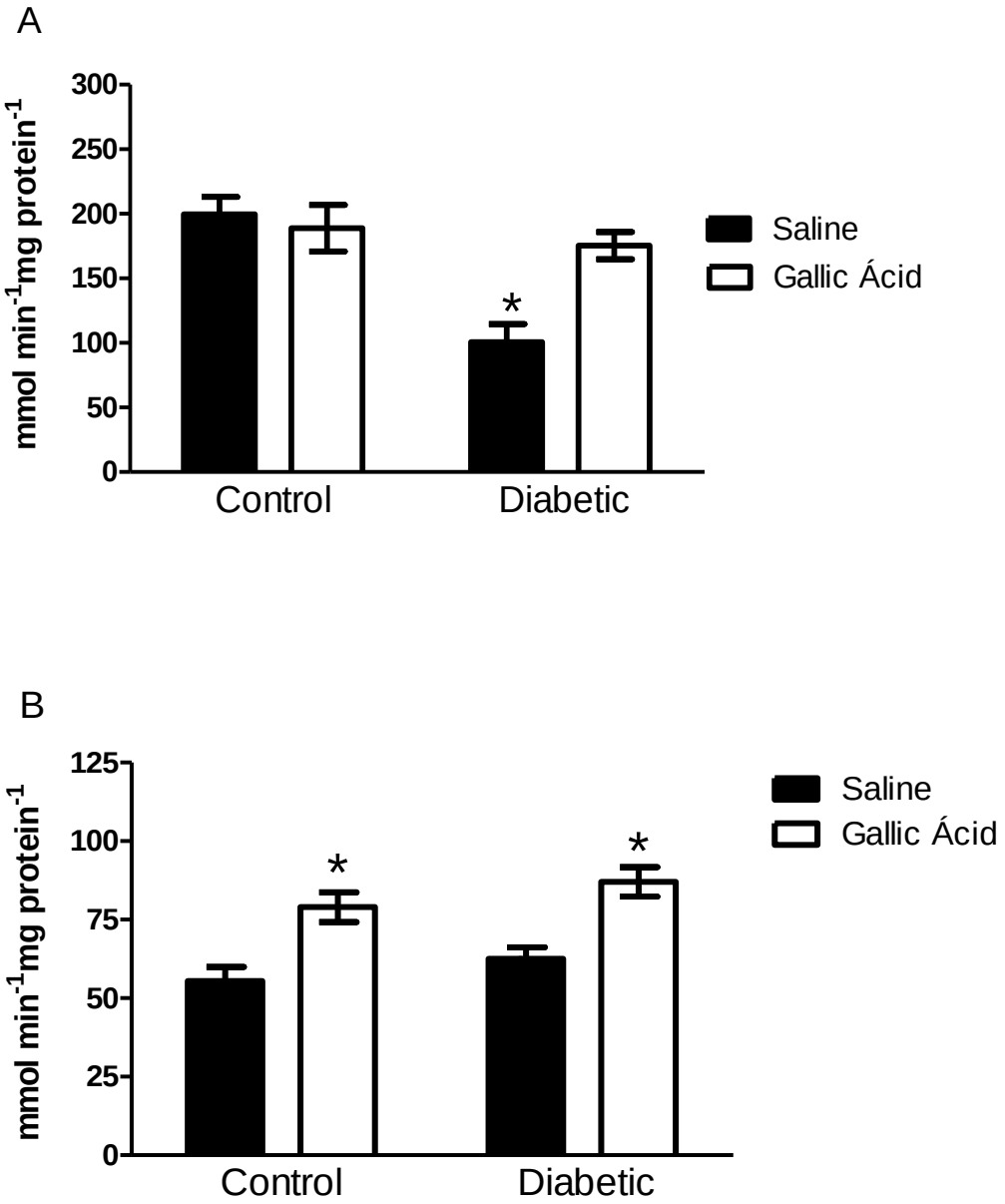


Figure 6: Glutathione S - transferase (GST) activity on liver (A) and kidney (B) from STZ-induced diabetic rats and of animals treated with Gallic Acid (30 mg/kg). Bars Represent means ± S.D. * Different the others groups (P<0.05, n = 10).

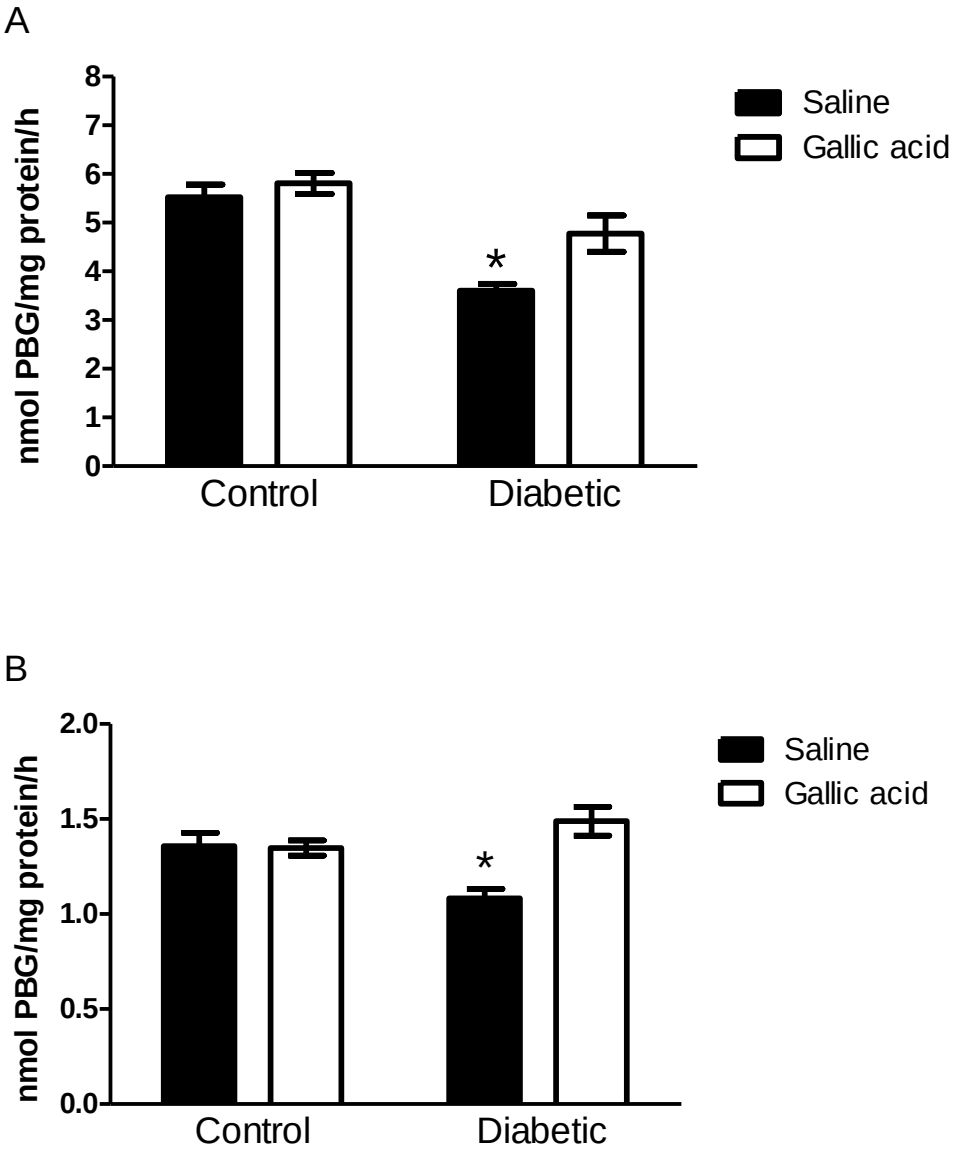


Figure 7: δ -aminolevulinic dehydratase (δ ALA-D) activity on liver (A) and kidney (B) from STZ-induced diabetic rats and of animals treated with Gallic Acid (30 mg/kg). Bars Represent means ± S.D. * Different the others groups (P<0.05, n = 10).

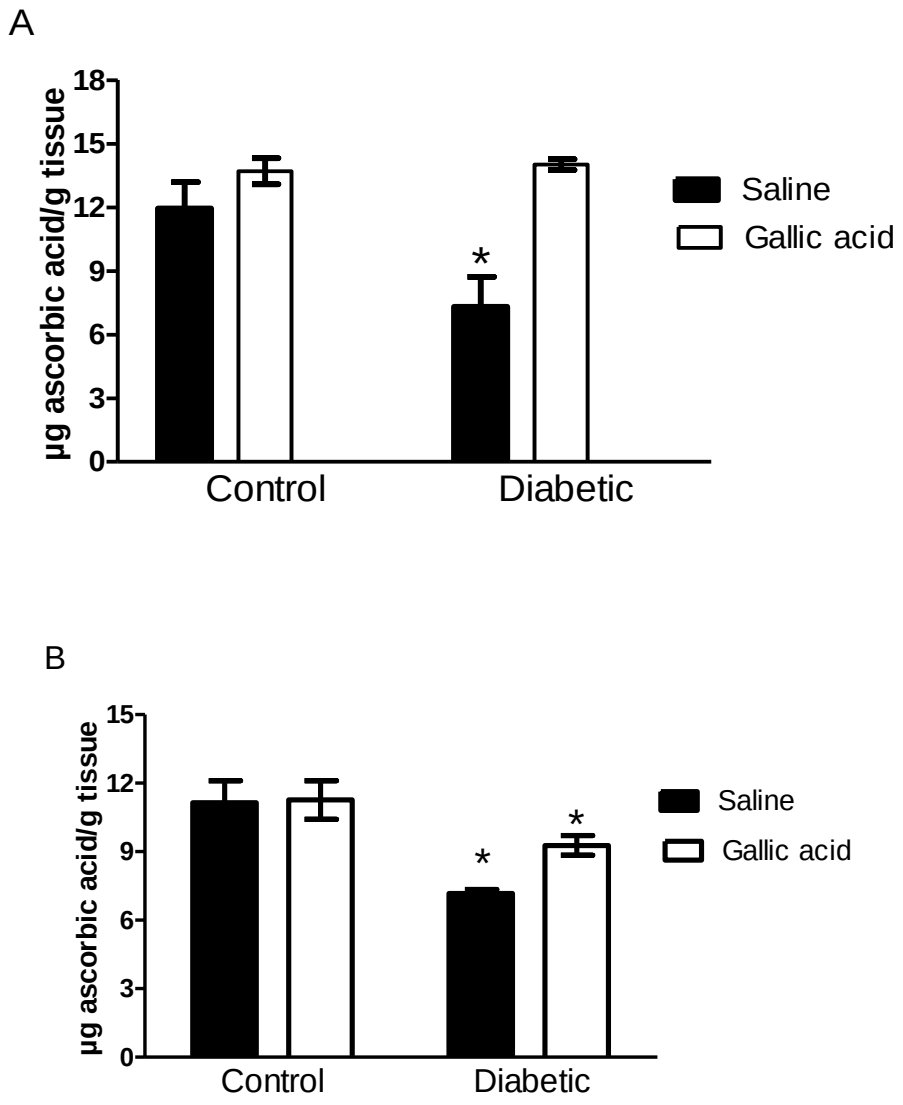


Figure 8: Vitamin C levels on liver (A) and kidney (B) from STZ-induced diabetic rats and of animals treated with Gallic Acid (30mg/kg). Bars Represent means ± S.D. * Different the others groups. ($P<0.05$, n = 10).

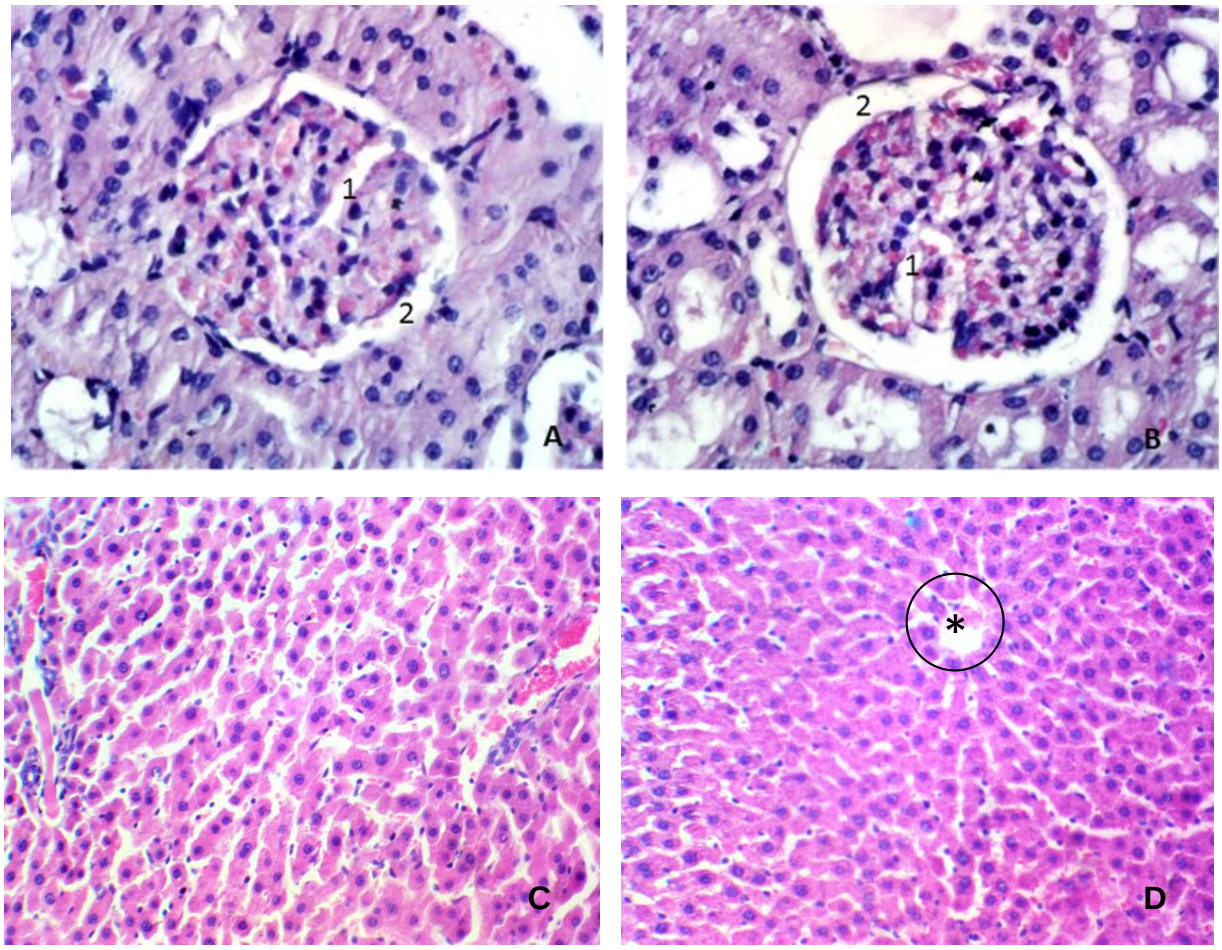


Figure 9. Renal photomicrographs (hematoxylin and eosin; original magnification 400x). A: Control saline group; B: Control gallic acid group; 1: Glomerular area; 2: Subcapsular space. Liver photomicrographs (hematoxylin and eosin; original magnification 400x). C: control saline group; D: Diabetic gallic acid group, *: nuclear area.

4. CONCLUSÃO

- A glicemia dos animais diabéticos se manteve elevada durante todo o experimento, o que demonstra a efetividade na indução do modelo experimental. Além disso, devido aos altos níveis de glicemia, os animais diabéticos tiveram menor peso corporal em relação aos controles. Este fato é devido á desidratação e aumento no catabolismo de lipídeos e proteínas. Ademais, neste estudo não foi evidenciado o efeito antihiperglicêmico do ácido gálico.

- Não ocorrem alterações no perfil lipídico de animais diabéticos. No entanto, nota-se aumento na atividade de enzimas utilizadas como marcadores de dano hepático (ALT e AST) e nos níveis de um marcador de dano renal (uréia). Contudo, o ácido gálico apenas exerceu efeitos significativos na atividade da aspartato aminotransferase (AST).

- O aumento nos níveis de espécies reativas e peroxidação lipídica, associado com o decréscimo na atividade das enzimas SOD, CAT, GST e nos níveis de vitamina C demonstram a ocorrência de um quadro de estresse oxidativo no diabetes. No entanto, o tratamento com ácido gálico foi capaz diminuir os níveis de espécies reativas, bem como seus danos lipídicos, e de restabelecer a atividade destas importantes enzimas antioxidantes e os níveis de vitamina C (importante antioxidante não-enzimático). Assim, pode-se afirmar que o ácido gálico é capaz de diminuir o estresse oxidativo provocado por esta patologia no fígado e rim.

- O decréscimo na atividade da δ-ALA-D, pode sugerir a ocorrência de um quadro de estresse oxidativo nos animais diabéticos, tendo em vista que esta enzima é altamente sensível a agentes oxidantes. Após o tratamento com ácido gálico a atividade da δ-ALA-D foi restaurada. Este dado salienta que o composto em estudo age minimizando o estresse oxidativo.

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