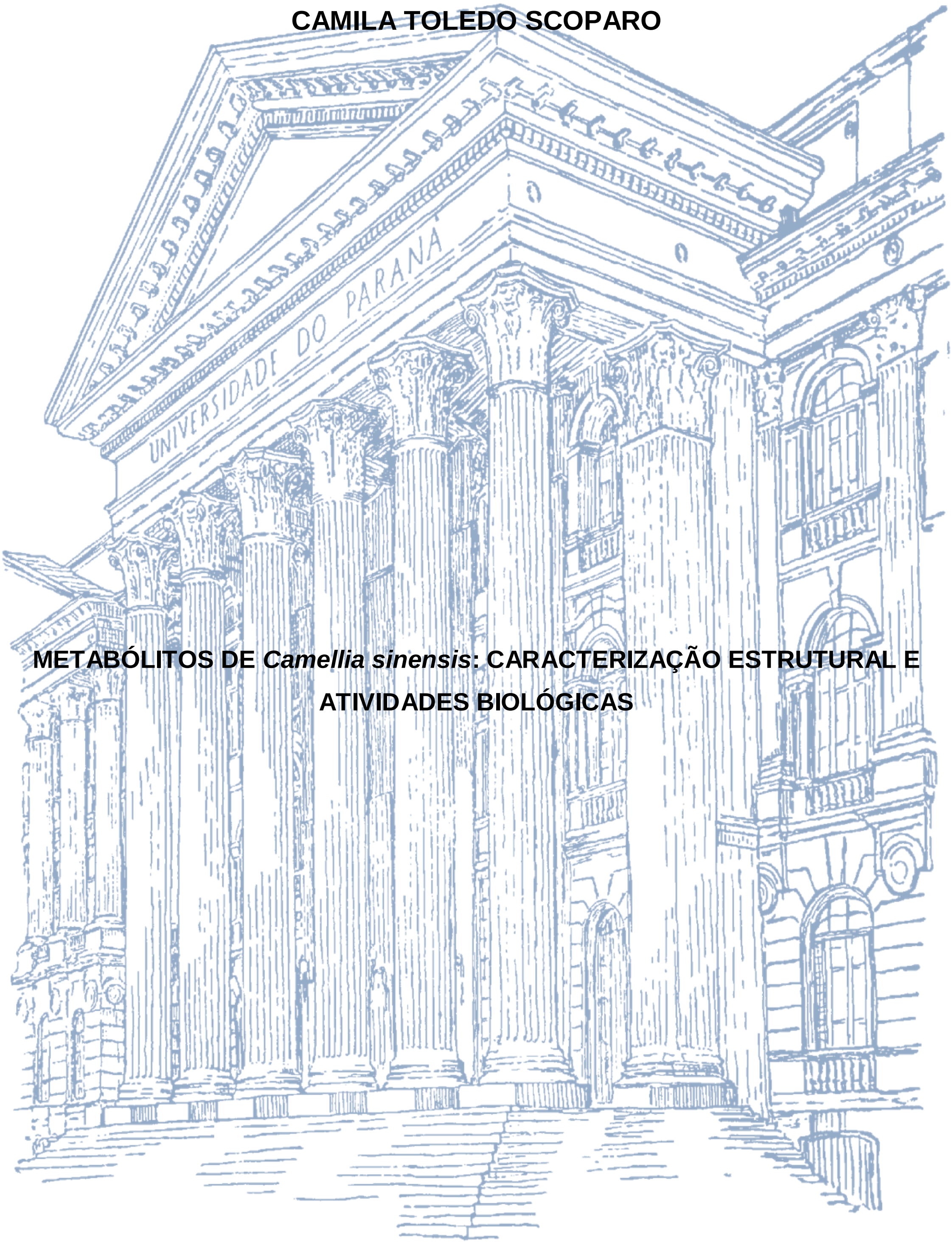
UNIVERSIDADE FEDERAL DO PARANÁ

CAMILA TOLEDO SCOPARO



METABÓLITOS DE *Camellia sinensis*: CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADES BIOLÓGICAS

CURITIBA
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METABÓLITOS DE *Camellia sinensis*: CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADES BIOLÓGICAS

Tese apresentada ao Programa de Pós-Graduação em Ciências - Bioquímica, do Departamento de Bioquímica e Biologia Molecular, do Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requisito parcial para a obtenção do título de Doutor em Ciências - Bioquímica.

Orientador: Prof. Dr. Marcello Iacomini

Co-orientador: Dr. Lauro Mera de Souza

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TERMO DE APROVAÇÃO

CAMILA TOLEDO SCOPARO

METABÓLITOS DE *C. sinensis*: CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADES BIOLÓGICAS

Tese aprovada como requisito parcial para obtenção do grau de Doutor no curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, pela seguinte banca examinadora:

Prof. Dr. Marcello Iacomini – Orientador
Departamento de Bioquímica, UFPR

Prof. Dr. Thales Ricardo Cipriani
Departamento de Bioquímica, UFPR

Prof.ª Dr.ª Yanna Dantas Rattman
Departamento de Saúde Comunitária, UFPR

Prof. Dr. Anderson Barison
Departamento de Química, UFPR

Prof.ª Dr.ª Eliana Barreto Bergter
Departamento de Microbiologia Geral, UFRJ

Curitiba, 24 de fevereiro de 2016.

Dedico esse trabalho...

Aos meus pais José Roberto Scoparo e Tânia Regina Montanha Toledo Scoparo, vocês são meu porto seguro e meus guias por toda a minha vida.

À minha irmã Carina Toledo Scoparo, por ser minha metade, minha amiga, minha companheira, minha vida.

Ao meu companheiro, amigo, amor, José Antonio Assad e Faria Jr, você tornou meus dias muito melhores.

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Amo todos vocês!

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“A mente que se abre a uma nova ideia,
jamais voltará ao seu tamanho original”
(Albert Einstein)

NOTA EXPLICATIVA

Nesta tese foram inseridos os artigos publicados no decorrer do doutoramento, utilizando-se do formato alternativo de tese de acordo com as normas estabelecidas no Programa de Pós-Graduação em Ciências - Bioquímica, da Universidade Federal do Paraná. Desta forma, este trabalho apresenta-se com a seguinte estrutura básica: introdução, revisão bibliográfica, justificativa, objetivo geral e objetivos específicos do trabalho, conclusões e os artigos científicos originados deste trabalho.

RESUMO

Existem vários tipos de chás obtidos de C*amellia sinensis*, sendo que a principal diferença entre eles está baseada no processo de auto-oxidação enzimática, sendo classificados em não oxidados (chá verde e branco), semi-oxidado (chá oolong), oxidado (chá preto) e pós-fermentado (chá pu-erh). No presente trabalho foram avaliados os polissacarídeos obtidos do extrato aquoso e alcalino dos chás verde e preto, assim como os compostos de baixa massa molecular presentes no extrato hidroalcoólico e na infusão dos mesmos. Os polissacarídeos obtidos da extração aquosa dos chás verde e preto (GSP e BSP) foram identificados como ramnogalacturonanas, com uma cadeia principal formada por unidades de α-D-Gal*p*A (1$\rightarrow$4)-ligadas, intercaladas por unidades de α-L-Rha*p*, e substituída por cadeias laterais de arabinogalactanas do tipo II. GSP e BSP foram testados quanto à sua ação farmacológica contra septicemia, reduzindo significativamente a letalidade dos camundongos e interferindo também na infiltração de neutrófilos para os pulmões e o consequente dano causado por eles. Os polissacarídeos obtidos da extração alcalina dos chás verde e preto foram identificados como arabinogalactanas proteínas tipo II (GTPS e BTPS), além de heteroxilanas altamente ramificadas (GTPI e BTPI) com uma cadeia principal de (1$\rightarrow$4)-β-Xyl*p*. A avaliação da atividade gastroprotetora de GTPS, BTPS, GTPI e BTPI demonstrou a redução das lesões gástricas induzidas por etanol, providenciada pela manutenção dos níveis de muco gástrico e glutationa reduzida (GSH). Os compostos de baixa massa molecular obtidos por meio de extração hidroalcoólica foram fracionados por particionamento líquido/líquido, sendo possível identificar uma vasta gama de metabólitos, tais como as catequinas e seus galatos (ésteres) ou derivados de oxidação, flavonoides glicosilados e outros compostos fenólicos, assim como lípidos, saponinas e alcaloides. Neste estudo foi empregada análise bio-guiada, utilizando o modelo de lesões gástricas agudas induzidas por etanol em ratos, em que os extratos hidroalcoólicos (GEt e BEt), as frações particionadas e compostos majoritários identificados (EGCG e Rutina) no chá-verde e de chá preto, protegeram a mucosa gástrica com a participação de muco gástrico e glutationa reduzida (GSH). Com relação à extração por infusão, após particionamento líquido/líquido, inúmeros compostos foram caracterizados para os extratos brutos (GTI e BTI) e as frações acetato de etila (GEAF e BEAF) de ambos os chás, sendo principalmente, catequinas e seus derivados galato e produtos de oxidação (teaflavinas e teasinensinas), além de proantocianidinas, flavonoides glicosilados, alcaloides, ácidos hidroxibenzóicos e hidroxicinâmicos, bem como saponinas. Além disso, os extratos GTI e BTI, as frações GEAF e BEAF e os compostos identificados a partir de ambos os chás (EGCG, teaflavinas e cafeína) foram analisados quanto à sua atividade na sepse murina, resultando em uma diminuição da mortalidade causada pela sepse. Este efeito de protecção pode ser promovido, em parte, pela redução da atividade da MPO, a redução dos níveis das citocinas pró-inflamatórias (IL-6 e TNF-α) e um aumento dos níveis das citocinas anti-inflamatórias (IL-10) e também uma diminuição da expressão de ambas as enzimas (iNOS e COX-2), demonstrando a possibilidade de controle da complexa cascata inflamatória e, assim, a modulação dos efeitos da sepse.

Palavras-chaves: chá verde / chá preto / polissacarídeos / metabólitos secundários/ gastroproteção / sepse / anti-inflamatória

ABSTRACT

There are several types of teas made from Camellia sinensis, and the main difference between them is based on the enzymatic auto-oxidation process, so they are classified as non-oxidized (green and white tea), semi-oxidized (oolong), oxidized (tea black) and post-fermented (pu-erh tea). In the present study we evaluated the polysaccharides obtained from aqueous and alkaline extract of green and black teas, as well as compounds of low molecular weight present in the hydroalcoholic extract and infusion thereof. The polysaccharides obtained from aqueous extraction of green and black teas (GSP and BSP) have been identified as ramnogalacturonanas with a backbone composed of units α-D-GalpA ($1\rightarrow4$) -ligadas, interspersed by units of α-L-Rhap and replaced by side chains of type II arabinogalactan. GSP and BSP were tested for their pharmacological activity against murine sepsis, significantly reducing the lethality of mice, and also interfering in the neutrophil infiltration into the lungs and the subsequent damage for them. The polysaccharides obtained from the alkaline extraction of green and black teas were identified as type II arabinogalactan proteins (GTPS and BTPS), and also highly branched heteroxilanas (GTPI and BTPI) with a backbone ($1\rightarrow4$) -β-Xylp. The evaluation of the gastroprotective activity of GTPS, BTPS, GTPI and BTPI showed a reduction in gastric lesions induced by ethanol provided the maintenance of gastric mucous levels and reduced glutathione (GSH). The compounds of low molecular weight obtained by hydroalcoholic extraction were fractionated by liquid/liquid partitioning, and we can identify a wide range of metabolites, such as catechins and their gallates (esters) or derivatives oxidation, glycosylated flavonoids and other phenolic compounds as well as lipids, saponins and alkaloids. This study employed bio-guided analysis using the model of acute gastric lesions induced by ethanol in rats, wherein the hydroalcoholic extracts (GEt and BEt), partitioned fractions and identified major compounds (EGCG and rutin) in green tea and black tea, protected the gastric mucosa with the participation of gastric mucus and reduced glutathione (GSH). With respect to the extraction by infusion, after liquid/liquid partitioning, several compounds were characterized for crude extracts (IGTI and BTI) and fractions ethyl acetate (GEAF and beaf) of both teas, being mainly catechins and their gallate derivatives and oxidation products (theaflavins and teasinensins), as well as proanthocyanidins, flavonoids, glycosides, alkaloids, hydroxybenzoic and hydroxycinnamic acids, as well as saponins. Moreover, GTI and BTI extracts, GEAF and BEAF fractions and majors compounds identified from both tea (EGCG theaflavins and caffeine) were analyzed for their activity in murine sepsis, resulting in a decrease of mortality caused by sepsis. This protective effect may be promoted in part by reduced MPO activity, reduced levels of the proinflammatory cytokines (IL-6 and TNF-α) and increased levels of anti-inflammatory cytokines (IL - 10) and also a reduction of expression of both enzymes (iNOS and COX-2), demonstrating the possibility of inflammatory cascade of complex control and thus modulating the effects of sepsis.

Keywords: green tea / black tea / polysaccharide / secondary metabolites / gastroprotection / sepsis / anti-inflammatory

LISTA DE FIGURAS

REVISÃO LITERÁRIA

LISTA DE QUADROS

LISTA DE ABREVIATURAS, SÍMBOLOS, SIGLAS E TERMOS*

Compostos químicos

AcOH	- Ácido acético
Akt	- *Serine threonine kinase protein* (proteína serina treonina quinase)
Ara*f*	- *Furanosidic arabinose* (arabinose furanosídica)
CLP	- *Cecal ligation and puncture* (ligação e perfuração do ceco)
COX-2	- *Cyclooxygenase-2* (cicloxigenase-2)

DMSO	- *Dimethyl sulfoxide* (dimetilsulfóxido)
eNOS	- *Constitutive nitric oxide synthase* (óxido nitric sintase constitutiva)
EtOH	- *Ethanol* (etanol)
Gal*p*	- *Pyranosidic galactose* (galactose piranosídica)
Gal*p*A	- *Pyranosidic galacturonic acid* (ácido galacurônico piranosidico)
GSH	- *Reduced glutathione* (glutationa reduzida)
H_2O_2	- *Hydrogen peroxide* (peróxido de hidrogênio)
IL-1β	- *Interleukin - 1β* (interleucina - 1β)
iNOS	- *Inducible nitric oxide synthase* (óxido nitrico sintase induzida)
LPS	- *Lipopolysaccharide* (lipopolissacarídeo)
LTA	- *Lipoteichoic acid* (ácido lipoteicóico)
MODS (SDMO)	- *Multiple organ dysfunction syndrome* (síndrome da disfunção de múltiplos órgãos)
MPO	- *Myeloperoxidase* (mieloperoxidade)
NF-κB	- *Nuclear factor-kappa B* (*fator nuclear capa B)*
NO	- *Nitric oxide* (óxido nítrico)
O_2^-	- *Superoxide anion* (anion superóxido)
PGA	- *Polygalacturonic acid* (acido poligalacturônico)
PGI_2	- *Prostacyclin* (prostaciclina)
PGE-2	- *Prostaglandin E2* (prostaglandina E2)
PMAA	- *Partially O-methylated alditol acetate* (alditol acetato parcialmente *O*-metilado)
RGI	- *Tipe I Rhamnogalacturonan* (ramnogalacturonana tipo I)
RGII	- *Tipe II Rhamnogalacturonan* (ramnogalacturonana tipo II)
Rha*p*	- *Pyranosidic rhamnose* (ramnose piranosídica)
ROS	- *Reactive Oxygen Species* (espécies reativas de oxigênio)

SIRS (SRIS)	- *Systemic inflammatory response syndrome* (síndrome da resposta inflamatória sistêmica)
SOD	- *Superoxide dismutase* (superóxido dismutase)
PTK	- *Protein tyrosine kinase* (proteína tirosina quinase)
TBS-T	- *Tris-buffered saline solution and 0.1% Tween 20* (solução salina tamponada com tris e 0,1% de Tween 20)
TFA	- *Trifluoroacetic acid* (ácido trifluoracético)
TMSP-d_4	- *2,2,3,3- tetradeuterium-3-trimethylilsilylpropionate* (2,2,3,3-tetradeuterio-3-trimetilsililpropionato)
TNF-α	- *Tumor necrosis factor - α* (fator de necrose tumoral – α)

Amostras, extratos e Frações

GSP	- *Green soluble polysaccharide (after freeze-thawing)* (polissacarídeo da extração aquosa do chá verde solúvel após congelamento e descongelamento)
BSP	- *Black soluble polysaccharide (after freeze-thawing)* (polissacarídeo da extração aquosa do chá preto solúvel após congelamento e descongelamento)
GEt	-*Green-tea hydro-alcoholic extract* (extrato hidroalcoólico do chá verde)
BEt	-*Black-tea hydro-alcoholic extract* (extrato hidroalcoólico do chá preto)
GCl	-*Green-tea chloroform fraction* (fração clorofórmica do extrato hidroalcoólico do chá verde)
BCl	-*Black-tea chloroform fraction* (fração clorofórmica do extrato hidroalcoólico do chá preto)
GEAc	- *Green-tea ethyl acetate fraction* (fração acetato de etila do extrato hidroalcoólico do chá verde)
BEAc	- *Black -tea ethyl acetate fraction* (fração acetato de etila do extrato hidroalcoólico do chá preto)
GBu	-*Green-tea butanolic fraction* (fração butanólica do extrato hidroalcoólico do chá verde)
BBu	-*Black-tea butanolic fraction* (fração butanólica do extrato hidroalcoólico do chá preto)
GAq	-*Green-tea aqueous fraction* (fração aquosa do extrato

	hidroalcoólico do chá verde)
BAq	-*Black-tea aqueous fraction* (fração aquosa do extrato hidroalcoólico do chá preto)
GTPS	-*Green tea polysaccharide soluble* (polyssacarídeo do chá verde obtido da extração alcalina solúvel após congelamento e degelo)
BTPS	-*Black tea polysaccharide soluble* (polyssacarídeo do chá preto obtido da extração alcalina solúvel após congelamento e degelo)
GTPI	-*Green tea* polysaccharide insoluble (polyssacarídeo do chá verde obtido da extração alcalina insolúvel após congelamento e degelo)
BTPI	-*Black tea polysaccharide insoluble* (polyssacarídeo do chá preto obtido da extração alcalina insolúvel após congelamento e degelo)
GTI	-*Green tea infusion* (extrato da infusão do chá verde)
BTI	-*Black tea infusion* (extrato da infusão do chá preto)
GEAF	-*Green tea ethyl acetate fraction* (fração acetato de etila da infusão do chá verde)
BEAF	-*Black tea ethyl acetate fraction* (fração acetato de etila da infusão do chá preto)
GBF	-*Green tea butanolic fraction* (fração butanólica da infusão do chá verde)
BBF	-*Black tea butanolic fraction* (fração butanólica da infusão do chá preto)
GAF	-*Green tea aqueous fraction* (fração aquosa da infusão do chá verde)
BAF	-*Black tea aqueous fraction* (fração aquosa da infusão do chá preto)

Técnicas e termos associados as técnicas de análise

EI	- *Electron ionization* (ionização de elétron)
ESI-MS	- *Electrospray Ionization* (ionização por spray de elétrons)
GC-MS	- *Gas chromatogaphy-mass spectrometry* (cromatografia gasosa acoplada à espectrometria de massas)

HMBC	- *Heteronuclear multiple bond correlation*
HPLC	- *High performance liquid chromatography* (cromatografia líquida de alta eficiência)
UHPLC	- *Ultra High performance liquid chromatography* (cromatografia líquida de alta eficiência)
HPSEC	- *High pressure size exclusion chromatography-* (cromatografia de exclusão estérica)
HSQC	- *Heteronuclear single quantum coherence* (coerência heteronuclear simples quântica)
m/z	- *Mass to charge ration* (relação massa/carga)
MS	- *Mass spectrometry* (espectrometria de massas)
MS^2	- Espectro de fragmentação de massa (*tandem*-MS)
NMR	- *Nuclear magnetic ressonance* (ressonância magnética nuclear)
Offline	- Análise por espectrometria de massas com infusão direta
Online	- Análise por espectrometria de massas combinada com a separação por cromatografia
PAGE	- *Polyacrylamide gel electrophoresis* (eletroforese com gel de poliacrilamida)
R_t	- *Retention time* (tempo de retenção relativo)
SEC	- *Size exclusion chromatography* (cromatografia de exclusão estérica)
TIC	- *Total ions current* (análise de íons totais)
TLC	- *Thin layer chromatography* (cromatografia em camada delgada)

Análises Estatísticas

ANOVA	- *Analysis of variance* (análise de variância)

*Para as nomenclaturas dos termos optou-se pelo uso da linguagem inglesa e portuguesa, visto que os mesmos aparecem descritos em ambas as formas no decorrer da tese.

SUMÁRIO

1. INTRODUÇÃO

Camellia sinensis é uma espécie de planta cujas folhas são utilizadas amplamente na obtenção de infusões popularmente conhecidas como “chá”, sendo o mais conhecido o chá verde e o chá preto. Existem vários tipos de chás obtidos de *C. sinensis*, sendo que a principal diferença entre eles está baseada no processo de auto-oxidação catalisado pelas enzimas polifenol-oxidase e peroxidase. Assim, os chás são classificados em não oxidados (chá verde e branco), semi-oxidado (chá oolong), oxidado (chá preto) e pós-fermentado (chá pu-erh). Estima-se que o chá é consumido há mais de cinquenta séculos e, depois da água, é a bebida não alcoólica mais consumida no mundo (CHANTRE; LAIRON, 2002; ENGELHARDT, 2010).

Diversas classes de compostos têm sido identificadas nos diferentes tipos de chás de *C. sinensis*, destacando-se os produtos do metabolismo secundário, como: terpenos, compostos fenólicos, alcaloides e vitaminas (ENGELHARDT, 2010; SCOPARO, *et al*., 2012). Também já foram identificados produtos do metabolismo primário como polissacarídeos, lipídeos e proteínas (CHEN *et al*., 2004; NIE *et al.,* 2008; YANG *et al*., 2010; NIE; XIE, 2011).

Os compostos encontrados nos chás apresentam-se com diferentes propriedades biológicas. Estas propriedades são mostradas por vários estudos *in vivo* e *in vitro*, sendo as principais: antioxidantes, anti-inflamatórias, antialérgicas, antimutagênicas, anticancerígenas, antidiabéticas, antiarteroscleróticos, antibacteriana, antiobesidade, imunoestumulante e proteção ultravioleta para a pele (COOPER *et al.*, 2005; YANG, 2007; GONZALEZ, *et al*., 2009, CAO, 2013).

Existem diversas técnicas de análise para determinação das estruturas químicas dos mais variados tipos de metabólitos de plantas, dentre as quais destacam-se a Ressonância Magnética Nuclear (NMR-*Nuclear Magnetic Ressonance*), a Espectrometria de Massas (MS-*Mass Spectrometry*) e a Cromatografia, sendo que os métodos cromatográficos são os procedimentos mais amplamente utilizados na atualidade.

Dentro deste contexto e considerando o crescente interesse científico em sua complexa e diversificada composição química e suas propriedades terapêuticas, a *Camellia sinensis* apresenta-se como um importante propósito de estudo. E, embora sejam conhecidas diversas propriedades terapêuticas atribuídas à *C. sinensis*, considera-se extremamente relevante o desenvolvimento de uma investigação científica mais aprofundada e detalhada acerca desta planta, correlacionando a identificação de seus constituintes com suas propriedades biológicas. Desta forma, este estudo teve como objetivo o desenvolvimento de novas metodologias de extração, purificação e análise fitoquímica, e a avaliação de atividades biológicas dos compostos identificados.

2. REVISÃO BIBLIOGRÁFICA

2.1 BREVE HISTÓRICO SOBRE A UTILIZAÇÃO DAS PLANTAS MEDICINAIS

O conhecimento empírico dos benefícios trazidos pelas plantas medicinais é milenar, despertando o interesse de muitos pesquisadores com o objetivo de adquirir maior e melhor ciência sobre novas moléculas que possam ser usadas na terapêutica (ALMEIDA, 1993). Em civilizações primitivas, o homem já despertava o interesse por plantas, das agradáveis ao paladar às que apresentavam algum risco à saúde, até as que demostravam algum poder curativo (GOMES, 2010).

As pesquisas relacionadas à plantas medicinais, usualmente relacionam-se à etnofarmacologia ou estudos dirigidos isto é, as informações adquiridas da população em comunidades que fazem uso da diversidade vegetal local, com estudos químicos e/ou farmacológicos. Este método é permissivo à elaboração de hipóteses relacionadas às atividades farmacológicas observadas pela população que faz uso das plantas, sendo muito utilizado para construção de um arquivo sobre práticas do conhecimento tradicional e o uso de plantas medicinais na produção de medicamentos para o tratamento de enfermidades. Por fim, as informações desse arquivo são submetidas a estudos fitoquímicos, para comprovação ou não de atividade biológica (GOMES, 2010).

Assim posto, este conhecimento popular é utilizado a fim de desenvolver novos fármacos, por meio da descoberta de moléculas direcionadas ao tratamento de patologias emergentes, para as quais ainda não existe uma terapêutica eficiente, ou ainda patologias em que os tratamentos atuais são onerosos, seja para qualidade de vida do paciente ou para os elevados custos envolvidos (FERREIRA, 1998).

O Brasil possui a maior biodiversidade do planeta, que, consoante à rica diversidade étnica, torna o país o cenário ideal para o desenvolvimento de estudos voltados à comprovação de usos populares de plantas. O desenvolvimento de novos medicamentos, baseados em plantas, é considerado uma área emergente de grande potencialidade (LAPA *et al.*, 2003; CALIXTO, 2003). A razão desta afirmação é facilmente comprovada quando se analisa o número de fármacos obtidos direta ou indiretamente a partir de produtos naturais, já que cerca de um terço dos medicamentos mais vendidos no mundo foram desenvolvidos a partir de plantas medicinais utilizadas na medicina popular para os mais diversos fins (CALIXTO, 2003).

Considerando o acima exposto, uma área que tem apresentado um intenso desenvolvimento é aquela voltada para análises que permitam a identificação dos compostos presentes nas plantas medicinais (produtos naturais). No entanto, muitos desses compostos apresentam estruturas muito complexas, dificultando eminentemente sua síntese em laboratórios (EL-SHEMY *et al.*, 2007).

Os compostos naturais produzidos pelos vegetais são agrupados em dois grandes grupos: os metabólitos primários, tais como carboidratos, proteínas e lipídeos; e os metabólitos secundários, como compostos fenólicos, terpenóides e alcaloides, que são compostos sintetizados a partir dos metabólitos primários, e os principais responsáveis pelos efeitos medicinais, ou tóxicos, das plantas, apresentando também grande importância ecológica, uma vez que podem atuar na atração de polinizadores, ou representar uma defesa química contra estresses ambientais (BALADRIN *et al*., 1985; Di STASI, 1995).

2.2 CAMELLIA SINENSIS

2.2.1 Características botânicas da planta

A planta do chá (*C. sinensis*) foi descrita taxonomicamente pela primeira vez em 1753 por Carl Linnaeus em *Species plantarum*. Ele referiu-se a planta do chá como *Thea* e posteriormente refinado a espécie em chá preto (*Thea bohea*) e chá verde (*Thea viridis*). Até o início dos anos 1900, os taxonomistas reconheceram que o chá verde e o chá preto eram ambos da mesma espécie, *Camellia sinensis* (L.) O. Kuntze, da família Theaceae (BANERJEE, 1992).

O gênero *Camellia* caracteriza-se por agrupar espécies arbustivas, de folhas longas, escuras, lustrosas, com nervuras bem marcadas nas superfícies as quais são curtamente pecioladas e serrilhadas, sendo que as folhas mais novas são cobertas de pequenos tricomas brancos. As flores são brancas ou amarelas com cálice e corola pentâmero-espiralados, surgindo sozinhas ou aos pares entre as folhas, possuindo muitos estames e um pistilo com 3 estigmas. Já os frutos são cápsulas pequenas e globosas, com 1 a 3 sementes também globosas, que possuem um óleo que pode ser utilizado para o consumo humano (BANERJEE, 1992; DUARTE *et al*., 2006) (FIGURA 1).

FIGURA 1: ILUSTRAÇÃO DE UM RAMO DE *Camellia sinensis* COM **A)** SECÇÃO TRANSVERSAL DA FLOR E **B)** SEMENTES.
Fonte: Thomas Schoepke, 2006.

O cultivo da *C. sinensis* se dá preferencialmente por meio de estacas e depende de solo fértil, ácido e bem irrigado, sob sol pleno ou até mesmo ao abrigo deste. Necessita de temperatura moderada (aproximadamente 20 °C), por isso tem sido plantada em altitudes consideráveis, junto a montanhas e planaltos com até 1600 metros de altitude (BANERJEE, 1992).

2.2.2 Origem do chá

Em relação à origem exata da planta do chá, alguns autores sugerem as cordilheiras de montanhas entre Yunnan (Sudeste da China) e Assam (Nordeste da Índia), mas estudos indicam uma área mais extensa compreendendo a China, o Japão, Burma, Índia, Sri Lanka e outras regiões da Ásia Oriental (ENGELHARDT, 2010).

Os portugueses quando contataram com esses povos do Oriente conheceram a bebida do chá, já usual nesses territórios há mais de 2 mil anos. Adotaram a designação de Cantão-tcha que deu origem à palavra chá. Outros povos europeus adotaram a designação de Tonquim-té, que, por exemplo, em espanhol deu origem à palavra “te” e em inglês “tea”. Foi então apenas no século XVI que esta bebida e esta planta chegaram ao conhecimento dos Europeus. Logo, foi gradualmente introduzida em muitos países tropicais e subtropicais. Atualmente, a espécie é cultivada comercialmente na Ásia, África e América do Sul. Os principais produtores da cultura incluem China, Índia, Quênia, Sri Lanka e Indonésia (ENGELHARDT, 2010).

2.2.2 Principais formas de consumo e utilização da planta

Historicamente, o chá tem sido usado como um remédio tradicional na China por mais de 1000 anos. Na atualidade, o chá é usado como bebida e, como um ingrediente em bebidas, tais como bebidas prontas para o consumo, sendo uma das bebidas mais populares do mundo, sendo a mais consumida depois da água. O chá também é usado em produtos cosméticos devido às suas propriedades antienvelhecimento. Tradicionalmente, em alguns países, como a China e o Japão, o chá verde é mais consumido em comparação com o chá preto. Já na Índia e no Sri Lanka, o chá preto é predominante no consumo. Com relação à Europa e Estados Unidos, o consumo sofreu alterações ao longo dos últimos 10-15 anos, sendo que o chá verde, inicialmente não muito comum, hoje em dia o consumo atingiu valores de até 20% do consumo total de chá, devido aos inúmeros benefícios à saúde comprovados em diversos estudos científicos (ENGELHARDT, 2010)

As partes mais utilizadas da *C. sinensis* na produção dos diversos tipos de chás são as folhas e os botões, pois possuem cerca de 30% de compostos polifenólicos, os quais têm sido alvos de muitos estudos por serem indicados como os principais responsáveis pelas propriedades biológicas atribuídas à planta (SIMÕES *et al.,* 2004). Isto posto, diversas são as formas de utilização da planta, que vão desde a infusão, até extratos aquosos, hidroalcoólicos, entre outros, incorporados em diferentes formas farmacêuticas e cosméticas, utilizadas tanto de forma oral, quanto via tópica na forma de cremes, loções, géis e outros (ENGELHARDT, 2010).

2.3 PROCESSAMENTO DOS CHÁS DE *CAMELLIA SINENSIS*

Existem diferentes tipos de chás, como o chá branco, verde, oolong, preto e pu-erh. Todos os tipos de chá são preparados da *C. sinensis*, sendo que as principais variedades são *C. sinensis* var. *sinensis* e var. *assamica.*

As diferenças entre os tipos de chás estão, principalmente, no processamento pós-colheita, obtido pela auto-oxidação enzimática das folhas, ocorrendo por ação das enzimas polifenol-oxidase e peroxidase presente nos vacúolos das células (TAKEO, 1992; MACKENZIE *et al*., 2007). Uma técnica de fermentação microbiana anaeróbica também tem sido empregada (OKADA *et al*., 1996).

Os dois métodos principais de processamento das folhas dos chás são o tradicional (ortodoxo), que é utilizado para fazer os chás a granel, e o método CTC ("Crush, Tear, Curl" - esmagar, rasgar e enrolar) utilizado para fazer os chás em saquinhos (HAMPTON, 1992). Estes dois métodos serão descritos em detalhes.

2.3.1 Processo ortodoxo ou tradicional

O procedimento tradicional é utilizado para fazer chá a granel, onde as folhas, dependendo do tipo de chá desejado, devem ser submetidas basicamente aos processos de: vaporização ou tratamento com calor seco, murchamento, seguido da rolagem, oxidação e secagem final (ENGELHARDT, 2010). Um esquema deste tipo de processamento está ilustrado na FIGURA 2.

2.3.1.1 Vaporização ou tratamento com calor seco

Tem como finalidade a inativação das enzimas polifenol-oxidase (PFO) e peroxidase. As folhas são colocadas em grandes panelas de metal, sobre vapor d´água (vaporização) ou uma chama quente (calor seco), onde se tornam maleáveis, essa súbita exposição ao calor destrói as enzimas que levariam à oxidação. Esta é a primeira etapa de preparação do chá verde (ENGELHARDT, 2010).

2.3.1.2 Murchamento

O objetivo do murchamento é reduzir a umidade das folhas do chá em até 70% e permitir uma leve oxidação. As folhas de chá são dispostas em bandejas no sol ou em ambientes arejados, sob fluxo de ar através das folhas, para que a umidade seja removida de uma maneira uniforme. Este processo leva entre 12 e 17 horas, até que as folhas fiquem flexíveis para o processo de rolagem (ENGELHARDT, 2010).

2.3.1.3 Rolagem

Nesta etapa, as folhas de chá são colocadas sobre uma mesa, em que uma máquina de rolamento gira horizontalmente sobre ela. Esta ação faz com que as folhas de chá sejam esmagadas. Durante o processo de produção do chá verde a rolagem é feita com aquecimento (100 ^{o}C) para redução da umidade das folhas (ENGELHARDT, 2010).

2.3.1.4 Oxidação

O processo de oxidação é o que leva à diferenciação dos tipos de chás. O chá preto é o mais oxidado, o chá oolong é parcialmente oxidado e os chás verde e branco não são oxidados. Esta etapa é fundamental para a qualidade e o sabor final do chá. Para que seja possível a ocorrência da oxidação, as folhas são colocadas sobre bandejas em um quarto frio e úmido. A oxidação ocorre quando as enzimas endógenas (PFO e peroxidade) entram em contato com o oxigênio (de 2 a 6 h). Uma vez que as folhas atingiram o nível desejado de oxidação, as folhas são secas em secadores mecânicos (ENGELHARDT, 2010).

2.3.1.5 Secagem

A secagem é realizada para que ocorra a interrupção do processo de oxidação e a redução do teor de umidade total para cerca de 3%. Neste processo, o chá é passado por secadores mecânicos com ar aquecido a, aproximadamente, 100 °C (ENGELHARDT, 2010).

2.3.2 CTC (“*Crush, Tear e Curl”* - esmagar, rasgar e enrolar)

O CTC foi inventado durante a Segunda Guerra Mundial para aumentar a quantidade de chá a ser armazenado. O processo de produção segue o modo tradicional até a fase de rolagem, a qual é substituída pela técnica CTC, em que as folhas são conduzidas por uma série de rolos cilíndricos com centenas de pequenos "dentes" que cortam, rasgam e enrolam as folhas. Isso origina fragmentos de folhas com tamanhos muito reduzidos, ideal para fabricação de chás em saquinhos. Vale ressaltar que o método CTC não altera a qualidade das folhas do chá. Após essa etapa, as folhas seguem os mesmos processos de oxidação e secagem, conforme descrito anteriormente nesta seção (HAMPTON, 1992; ENGELHARDT, 2010).

Levando-se em consideração que o processo ao qual as folhas de *C. sinensis* são submetidas após a colheita leva à diferenciação dos tipos de chás, estes serão abordados separadamente com relação ao seu processamento e suas características principais.

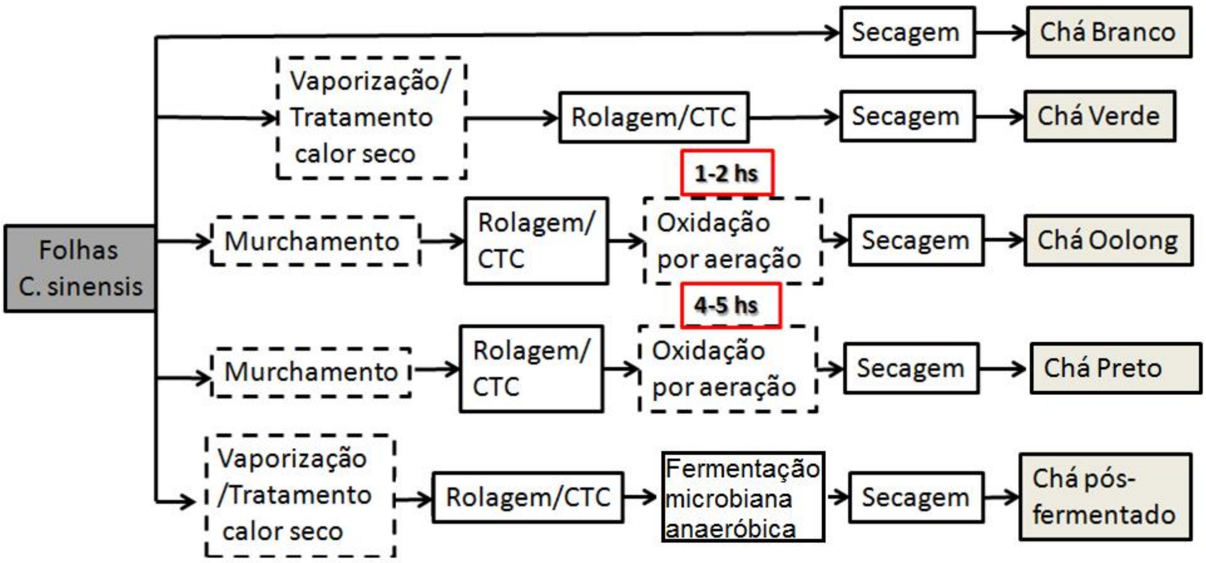

FIGURA 2: PROCESSAMENTO DOS TIPOS DE CHÁS DE *Camellia sinensis*
FONTE: Adaptado de Engelhardt (2010) e Scoparo (2012)

2.3.3 Chá branco

O chá branco é preparado a partir de folhas jovens e botões que são retirados antes que as flores desabrochem. Para tanto, os botões foliares estão protegidos da luz do sol, impedindo-se assim, a formação de clorofila. O processo básico para preparação do chá branco consiste apenas na secagem mecânica, corte e mistura das folhas, sendo que estas não sofrem nenhum tipo de oxidação (GONZALEZ *et al*., 2009).

2.3.4 Chá verde

O chá verde também não é submetido ao processo de oxidação. A fim de assegurar que as folhas recém-colhidas não oxidem, elas são expostas a vapor de água (vaporização) ou tratamento com altas temperaturas (calor seco) para inativar as enzimas PFO e peroxidase, e então são submetidas à etapa de rolagem, a qual ocorre em temperaturas elevadas, e por fim são secas mecanicamente com aquecimento (OWUOR, 2003; KARORI, *et al*., 2007; ENGELHARDT, 2010).

2.3.5 Chá oolong

O chá oolong é submetido a um processo de oxidação mediana. A primeira etapa de seu processamento é o murchamento, logo em seguida passa pelo processo de rolagem, sem aquecimento, onde ocorre a liberação das enzimas polifenol-oxidase e peroxidase e, posteriormente, ocorre a etapa de oxidação. Nesta etapa, as folhas permanecem sobre grandes bandejas em local frio e úmido, por um tempo aproximado de 1 a 2 horas, momento no qual o chá irá adquirir a sua coloração característica (escurecimento) e, finalmente, as folhas são submetidas à secagem final (OWUOR, 2003; KARORI *et al*., 2007; ENGELHARDT, 2010).

2.3.6 Chá preto

As principais etapas para a preparação do chá preto são basicamente as mesmas do chá oolong, sendo a principal diferença o aumento do tempo em que as folhas ficam submetidas ao processo de oxidação (4 a 5 h) (ENGELHARDT, 2010). Quando comparado aos demais chás provenientes da *C. sinensis*, o chá preto é o mais oxidado e apresenta um sabor mais forte e acentuado (BALENTINE *et al*., 1997).

2.3.7 Chá pu-ehr (pós-fermentado)

O chá pós-fermentado é inicialmente submetido ao mesmo processamento do chá verde, no entanto é realizada uma etapa de fermentação microbiana, onde as folhas são mantidas em contato com microrganismos como *Aspergillus fumigatus*, *Aspergillus niger* e *Penicillium* e então submetidas aos secadores mecânicos (GONG *et al*., 1996; OKADA *et al*., 1996).

2.4 COMPOSIÇÃO FITOQUÍMICA

Nas células estão presentes os compostos orgânicos e inorgânicos, que se encontram em constante fluxo nos compartimentos celulares, locais em que passam por contínuas transformações químicas. Este conjunto de reações químicas que continuamente ocorrem em cada célula vegetal é chamado de metabolismo. A presença de enzimas específicas garante certa direção a essas reações, denominadas rotas metabólicas. As reações enzimáticas podem ser do tipo anabólica, catabólica e biotransformação. As rotas metabólicas visam primariamente a obtenção de nutrientes para a necessidade da célula, como energia (ATP), poder redutor (NADPH) e biossíntese de compostos essenciais à sua sobrevivência (macromoléculas, como carboidratos, lipídios e proteínas) (SIMÕES *et al.*, 2004).

Os processos essenciais à vida e comuns nos vegetais são denominados de metabolismo primário, que se caracteriza por grande produção, distribuição universal e com funções essenciais. O metabolismo secundário caracteriza-se pela biossíntese de micromoléculas com diversidade e complexidade estrutural, produção em pequena escala, distribuição restrita e especificidade, tendo papel adaptativo ao meio, defesa contra herbívoros e microrganismos, proteção contra raios UV, atração de polinizadores e atração de animais dispersores de sementes (SIMÕES *et al.*, 2004).

A literatura remete à descrição do processo oxidativo a que as folhas de *C. sinensis* são submetidas, relatando inúmeras alterações na composição química dos seus metabólitos, tanto primários quanto secundários que podem ocorrer durante o processamento. Isto posto, os principais compostos relatados na literatura para o chá verde e o chá preto serão melhor discutidos a seguir.

2.4.1 Metabólitos primários

Conforme descrição anterior, fazem parte dos compostos do metabolismo primário, os lipídeos, as proteínas e os carboidratos.

2.4.1.1 Carboidratos

Nas plantas, os carboidratos existem como monossacarídeos, oligossacarídeos, polissacarídeos e seus derivados, tais como os glicosídeos cianogênicos e fenólicos e os flavonoides glicosilados. Os carboidratos são classificados em dois grandes grupos, que são os de reserva e os estruturais (AVIGAD; DEY, 1997).

Com relação aos carboidratos de reserva, o amido e as frutanas são os carboidratos mais abundantes e sintetizados em plantas. Contudo, as plantas produzem outros polissacarídeos que também podem ser considerados de reserva, como as galactomananas, constituintes de sementes de muitos vegetais (AVIGAD; DEY, 1997).

Os carboidratos chamados de estruturais, são os compostos naturais mais abundantes na Terra, sendo a mais importante fonte de reciclagem de carbono. Estes carboidratos encontram-se principalmente nas paredes celulares, que envolvem as células vegetais (REID, 1997) (FIGURA 3). A parede celular das plantas é altamente organizada, apresentando muitos polissacarídeos diferentes, proteínas e substâncias aromáticas. Destes, os polissacarídeos são os principais componentes da parede celular e de suas armações estruturais, sendo divididos em pectinas, hemiceluloses e celulose (CARPITA; MCCANN, 2000). A composição química da parede celular primária de dicotiledôneas é de aproximadamente 25-40% de celulose, 15-25% de hemicelulose, 15-40% de substâncias pécticas, 5-10% proteínas e traços de compostos fenólicos (AVIGAD; DEY, 1997).

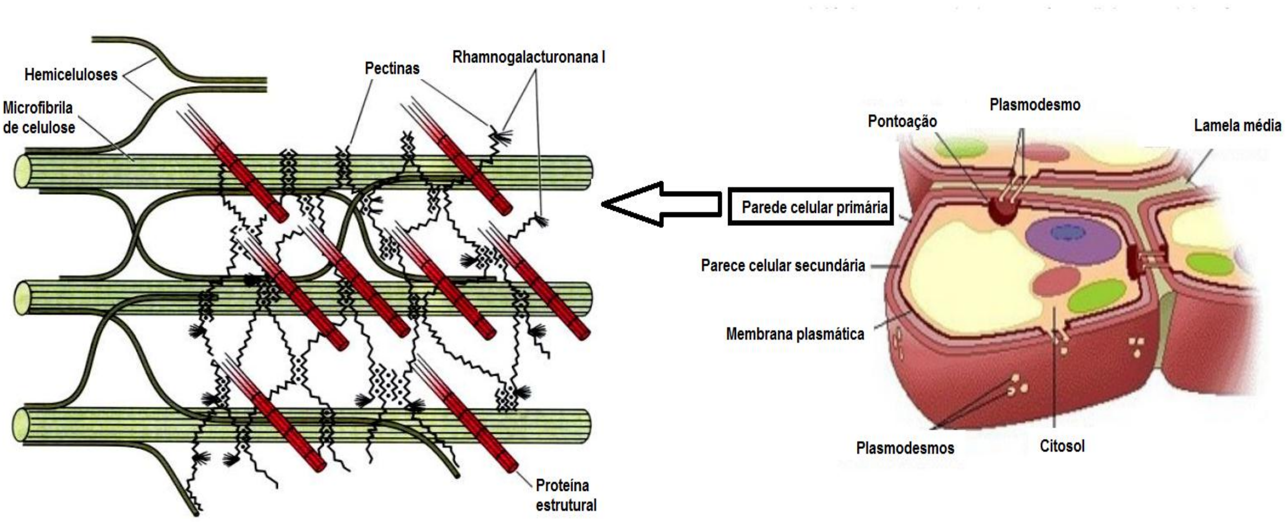

FIGURA 3: PRINCIPAIS COMPONENTES ESTRUTURAIS DA PAREDE CELULAR.
FONTE: Adaptado de BRETT e WALDRON (1996)

A parede celular secundária encontra-se entre a primária e a membrana plasmática da célula, conferindo rigidez aos tecidos vegetais (REID, 1997). A conversão da parede primária em secundária ocorre após o processo de lignificação, o qual interrompe o crescimento celular e indica, portanto, o amadurecimento celular e a diferenciação terminal da célula (ALBERSHEIM, 1996). A constituição química deste tipo de parede passa a ser de 40-45% de celulose, 15-35% de hemicelulose, 15-30% de lignina e traços de pectina (AVIGAD; DEY, 1997).

As pectinas são polissacarídeos ricos em ácido galacturônico, ramnose, arabinose e galactose. Os constituintes fundamentais das pectinas são o ácido poligalacturônico (PGA) e as ramnogalacturonanas tipo I (RGI). As ramnogalacturonanas tipo II constituem a terceira classe de polissacarídeos pécticos ricos em ácido galacturônico. Arabinanas, galactanas e arabinogalactanas são frequentemente encontradas em frações pécticas, constituindo cadeias laterais das RGI (ASPINALL, 1980; BRETT; WALDRON, 1990; CARPITA; GIBEAUT, 1993).

Os PGAs são homopolímeros helicoidais formados por unidades de ácido α-D-galacturônico (1→4)-ligadas (CARPITA e GIBEAUT, 1993). As RGI são heteropolímeros constituídos por repetições do grupo dissacarídico [→4)- α-D-Gal*p*A-(1→2)- α-L-Rha*p*-(1→] (McNEIL *et al*., 1984; CARPITA e GIBEAUT, 1993). As RGI e os PGAs formam os dois principais constituintes da matriz péctica da parede celular dos vegetais superiores (CARPITA; GIBEAUT, 1993).

As hemiceluloses são polissacarídeos que ocorrem em íntima associação com a celulose, especialmente em tecidos lignificados (ASPINALL, 1980). São hemiceluloses: xilanas, glucomananas, mananas, galactomananas, glucuronomananas, xiloglucanas, calose (β-glucanas com ligações 1→3), β-glucanas 1→3, 1→4 ligadas e arabinogalactanas tipo II (ASPINALL, 1980; BRETT; WALDRON, 1990). Este último tipo de polissacarídeo também pode estar presente em frações pécticas, associado a moléculas de RGI (CARPITA; GIBEAUT, 1993).

Sabe-se que muitas plantas utilizadas na medicina popular contêm polissacarídeos com uma variedade reconhecida de propriedades terapêuticas, incluindo atividade anti-UV, anticoagulante, anticancer, anti-HIV, hipoglicemiante e atividades antiúlcera gástrica (WANG, *et al*., 2001; ZHOU, *et al*., 2001a; XIE & NIE, 2006; SIMAS, *et al*., 2004; CIPRIANI, *et al*, 2004; CIPRIANI, *et al*, 2006; CIPRIANI, *et al*, 2008; CIPRIANI, *et al*, 2009).

Com relação à *C. sinensis*, poucos estudos foram dedicados a explicitar informações estruturais e conformacionais dos polissacarídeos encontrados. No entanto, alguns polissacarídeos foram identificados, como um polissacarídeo ácido (ATPS-2) e um polissacarídeo neutro (NTPS-1) isolados do chá verde. ATPS-2 apresentou ramnose, arabinose, galactose e ácido galacturônico em sua composição, com peso molecular médio de 4.400 Da, e suas características estruturais foram investigadas por uma combinação de análises químicas e instrumentais, como degradação de Smith, ^{1}H NMR, ^{13}C NMR e NMR bidimensional, revelando que ATPS-2 tratava-se de um polissacarídeo péctico com a cadeia principal composta por regiões de α-D-Gal*p*A (1→4) ligadas, interconectadas por resíduos de ramnose. As regiões mais ramificadas apresentaram a seguinte cadeia principal: →4)-α-D-Gal*p*A-(1→2)- α-L-Rha*p*-(1→4)- α-D-Gal*p*A-(1→, e as cadeias laterais compostas por resíduos de α-L-Ara*f* e β-D-Gal*p* (WANG *et al.,* 2009a, 2009b).

Em relação ao polissacarídeo neutro (NTPS-1), sua composição monossacarídica apresentou apenas galactose, com peso molecular médio de 21.200 Da, sendo que sua estrutura foi caracterizada por espectroscopia de infravermelho, cromatografia gasosa, degradação de Smith e espectroscopia de NMR (1D e 2D). O polissacarídeo NTPS-1 foi caracterizado como sendo uma galactana constituída de unidades galactopiranosídicas β-(1→4) ligadas (WANG *et al.,* 2009a, 2009b).

Ademais, alguns polissacáridos associados com proteínas isolados de flores, sementes e folhas de *C. sinensis* também foram identificados. Wang e colaboradores (2013) isolaram a partir das sementes da *C. sinensis*, dois heteropolissacarídeos associados com proteína, da qual 17 aminoácidos foram identificados, bem como a composição que monossacarídica apresentou principalmente arabinose, galactose, glucos, fucose, ramnose, xilose e manose. Da mesma forma, Wang e colaboradores (2010), purificaram e caracterizaram, a partir das flores do chá verde, um polissacarídeo neutro conjugado a proteína, com peso molecular de 500 kDa, com a composição monossacarídica constituída de ramnose, arabinose, manose, glucose e galactose. Os aminoácidos identificados foram alanina, treonina, glicina, valina, serina, histidina, ácido glutâmico, histidina e tirosina.

Nie e colaboradores (2006) purificaram uma glicoproteína do chá verde, sendo que a análise da composição monossacarídica e de aminoácidos resultou na identificação de ribose, ramnose, arabinose, xilose, manose, glicose e ácido galacturônico, bem como 18 diferentes aminoácidos. E, finalmente, Zhou (2009), identificou uma arabinogalactana associada à proteína em extratos de folhas de chá verde, bem como desenvolveu uma metodologia de controle de qualidade de arabinogalactana proteína (AGP), no entanto, a estrutura química fina do polissacarídeo não foi analisada.

Assim como os processos de oxidação alteram os metabólitos secundários, os polissacarídeos também podem ser afetados. Sabe-se que muitas das características estruturais de um polissacarídeo são definidas pelo seu peso molecular, composição monossacarídica, a posição das ligações glicosídicas, sequência de monossacarídeos, conformação, tamanho de partícula, solubilidade e propriedades reológicas (ZHANG; CUI, 2007; CUI, 2005). Deste modo, estudos indicam que a etapa de oxidação na produção de chás oolong e preto pode induzir a alterações da estrutura dos polissacarídeos, tais como composição monossacarídica, peso molecular e interação intermolecular, podendo alterar suas propriedades biológicas (NIE; XIE, 2011).

2.4.1.2 Proteínas

As proteínas são componentes essenciais a todas as células vivas e estão relacionadas praticamente a todas as funções fisiológicas. São utilizadas na regeneração de tecidos, funcionam como catalisadores nas reações químicas que se dão nos organismos vivos e que envolvem enzimas ou hormônios, são necessárias nas reações imunológicas e, juntamente com os ácidos nucleicos, são indispensáveis nos fenômenos de crescimento e reprodução. Quimicamente, as proteínas são polímeros de alto peso molecular (acima de 10.000 kDa), cujas unidades básicas são os aminoácidos, ligados entre si por ligações peptídicas. As propriedades de uma proteína são determinadas pelo número e espécie dos resíduos de aminoácidos, bem como pela sequência desses compostos na molécula (LEHNINGER, *et al*., 2000).

A síntese de proteínas ocorre nas células vivas sob a influência de sistemas enzimáticos, e a ligação peptídica é repetida, formando cadeias longas de resíduos de aminoácidos. A condensação de menor número de aminoácidos forma compostos de peso molecular relativamente baixo (até 10.000), chamados peptídeos (BOBBIO; BOBBIO, 2003; LEHNINGER, *et al*., 2000).

Quanto a classificação das proteínas, podem ser classificadas em três grupos principais: proteínas simples, conjugadas e derivadas, sendo que na natureza são encontrados apenas os dois primeiros grupos. As proteínas simples ou homoproteinas são constituídas, exclusivamente, por aminoácidos. Em outras palavras, fornecem exclusivamente uma mistura de aminoácidos por hidrólise, sendo classificadas, de acordo com a sua solubilidade, em albuminas, globulinas, glutelinas, prolaminas, protaminas, histonas e escleroproteínas (BOBBIO; BOBBIO, 2003).

As proteínas conjugadas São proteínas que por hidrólise liberam aminoácidos mais um radical não peptídico, denominado grupo prostético. Os grupos prostéticos podem ser orgânicos (como por exemplo, uma vitamina ou um açúcar) ou inorgânicos (por exemplo, um íon metálico) e encontram-se ligados de forma firme à cadeia polipeptídica, muitas vezes através de ligações covalentes. As proteínas conjugadas são classificadas de acordo com a natureza da parte não proteica em cromoproteínas, lipoproteínas, nucleoproteínas, glicoproteínas, fosfoproteínas e metaloproteínas (BOBBIO; BOBBIO, 2003).

As proteínas derivadas são compostos não encontrados na natureza, mas obtidos por degradação mais ou menos intensa (proteólise) de proteínas simples ou conjugadas pela ação de ácidos, bases ou enzimas. De acordo com o peso molecular, as proteínas derivadas podem ser classificadas em primárias e secundárias (BOBBIO; BOBBIO, 2003).

Quanto a sua estrutura, quatro tipos devem ser considerados para a definição da estrutura das proteínas: estrutura primária, secundária, terciária e quaternária. As proteínas sofrem mudanças nas suas estruturas com muita facilidade, o que torna bastante difícil o estudo desses compostos. Por hidrólise total, as cadeias peptídicas dão origem aos aminoácidos livres. A degradação de proteínas, seja química (por reação com ácidos ou álcalis) ou enzimática, leva à formação de polímeros menores e, finalmente, aos aminoácidos livres.

Com relação à *C. sinensis*, as folhas do chá podem conter até 30% de proteína. No chá preto, 15-23% de proteínas foram determinadas, sendo que menos de 2% é solúvel em água. Não existem muitos relatos disponíveis na literatura sobre as proteínas do chá, uma vez que elas não desempenham um papel muito significativo na bebida (ENGELHARDT, 2010).

A presença de aminoácidos livres nos chás tem sido estabelecida, sendo que estes aminoácidos podem influenciar as propriedades e o aroma do chá verde. Num estudo recente, foram identificados a alanina, arginina, asparagina, ácido aspártico, ácido glutâmico, isoleucina, histidina, leucina, fenilalanina, serina, teanina, treonina e tirosina por HPLC no chá verde, branco, preto, e pu-erh. A concentração encontrada da maioria dos aminoácidos está entre 0 e 0,3%, com a concentração no chá pu-erh sendo muito mais baixa (ALCÁZAR, *et al*, 2007).

Utilizando a análise de componentes principais (PCA), foi possível uma diferenciação dos chás branco, verde, preto, e oolong, sendo que o ácido glutâmico, asparagina, serina, alanina, leucina, isoleucina contribuíram para a maior parte desta diferenciação. Neste estudo, foram analisadas 21 amostras de chá verde, 28 do chá preto, 13 do chá branco, 13 do chá oolong, e 21 do chá pu-erh (ALCÁZAR, *et al*, 2007).

Entre os aminoácidos identificados, a teanina (ácido N-etilglutâmico) atraiu maior interesse, uma vez que ocorre quase que exclusivamente no chá, representando até 50% dos aminoácidos livres presentes, bem como devido à possibilidade de benefícios à saúde relatados, como atividade anticancerígena, anti-inflamatória e sobre o comportamento/cognição (ENGELHARDT, 2010).

A teanina ocorre naturalmente no chá quase exclusivamente como L-teanina. O teor de teanina no chá pode variar consideravelmente. Na literatura, foi relatado que há uma quantidade muito maior de teanina no chá verde quando comparado com o chá preto. Recentemente, foi observado que a quantidade de teanina foi reduzida durante a oxidação que ocorre no processamento dos chás (ENGELHARDT, 2010).

2.4.1.3 Lipídeos

Pertencem ao grupo dos lipídeos as substâncias que, em geral, são solúveis em solventes orgânicos e insolúveis ou ligeiramente solúveis em água. Contêm um grande número de diferentes tipos de substâncias, incluindo ácidos graxos, acilgliceróis, fosfolipídeos, esfingolipídeos além de compostos a estes relacionados. Os triacilgliceróis são os lipídeos mais comuns em alimentos, formados exclusivamente por produtos de condensação entre glicerol e ácidos graxos, usualmente conhecidos como óleos ou gorduras (AMABIS, 1994).

Nas plantas os lipídeos podem ocorrer em forma de óleos ou gorduras se estes forem para armazenamento. Nos tecidos fotossintetizantes são comumente encontrados glicolipídeos, tais como monogalactosildiacilglicerol (MGDG), digalactosildiacilglicerol (DGDG) e sulfoquinovosildiacilglicerol (SQDG) (CHAPMAN; BARBER, 1987; SASSAKI *et al*., 2001b).

De acordo com Mahanta (1988), a quantidade total de lipídeos na infusão de *C. sinensis* é de aproximadamente 4% e durante a manufatura do chá preto há uma mudança na sua composição. No processo oxidativo ocorre uma perda de ácido linolênico, como também dos ácidos linoléico e palmítico em menores quantidades. Além da alteração nos ácidos graxos, também foi observado alteração no conteúdo dos glicolipídeos MGDG, DGDG, cerebrosídeo e dos fosfolipídeos (fosfatidilcolina, fosfatidiletanolamina, fosfatidilglicerol), sendo observado um decréscimo de 27-90% nas folhas processadas (WRIGHT; FISHWICK,1979). Ademais, o estudo de Scoparo e colaboradores (2012) apresentou a identificação de inúmeros fosfolipídeos e glicolipídeos identificados nas folhas do chá verde e preto de *C. sinensis*.

2.4.2 Metabólitos secundários

O metabolismo secundário origina compostos que não apresentam uma distribuição universal, pois não são necessários para todas as plantas. Contudo, muito embora o metabolismo secundário nem sempre seja necessário para que uma planta complete seu ciclo de vida, ele desempenha um papel importante na interação das plantas com o meio ambiente (PERES, 2008).

Um dos principais componentes do meio externo cuja interação é mediada por compostos do metabolismo secundário são os fatores bióticos. Desse modo, produtos secundários possuem um papel contra a herbivoria, ataque de patógenos, competição entre plantas e atração de organismos benéficos como polinizadores, dispersores de semente e micro-organismos simbiontes. Contudo, produtos secundários também possuem ação protetora em relação a estresses abióticos, como aqueles associados com mudanças de temperatura, disponibilidade de água, níveis de luz, exposição à radiação UV e deficiência de nutrientes minerais (MONTANARI, 2002; PERES, 2008).

Existem três grandes grupos de metabólitos secundários: terpenos, fenólicos e alcaloides. Os terpenos são sintetizados a partir do ácido mevalônico (no citoplasma) ou do piruvato e 3-fosfoglicerato (no cloroplasto). Os compostos fenólicos são derivados do ácido chiquímico ou ácido mevalônico. Por fim, os alcaloides são derivados de aminoácidos aromáticos (triptofano, tirosina), os quais são derivados do ácido chiquímico, e também de aminoácidos alifáticos (ornitina, lisina) (PERES, 2008). A caracterização de compostos pertencentes a estas três classes será abordada juntamente com a composição química da *C. sinensis*, objeto de estudo deste trabalho.

2.4.2.1. Compostos fenólicos

Os chamados compostos fenólicos são substâncias que apresentam pelo menos um anel aromático no qual ao menos um hidrogênio é substituído por um grupamento hidroxila. Esses compostos são sintetizados a partir de duas rotas metabólicas principais: a via do ácido chiquímico e a via do ácido mevalônico, a qual é menos significativa.

Este grupo de compostos pode apresentar substituições por éteres metílicos, ácidos cinâmicos, carboidratos, entre outros. São amplamente distribuídos em plantas como produtos do metabolismo secundário. Uma grande variedade de monossacarídeos como a glucose, galactose, xilose, arabinose e ramnose podem estar ligados por meio de ligações *O*- ou *C*-glicosídicas, aumentando a variedade química dos compostos fenólicos (PICCIN, 2004; SOUZA, *et al*., 2008a)

Uma das formas de se classificar os compostos fenólicos é quanto a sua cadeia carbônica principal. Segundo esta classificação existem 4 classes principais: ácidos hidroxibenzóicos (ácido gálico), ácidos hidroxicinâmicos (ácido caféico, ferrúlico e *p*-coumárico), cumarinas (cumarina) e flavonoides (catequinas, quercetina, etc) (ESCARPA; GONZÁLES, 2001; MACHEIX *et al*., 1990).

Os flavonoides são os compostos fenólicos mais comumente encontrados no reino vegetal, e são caracterizados por apresentarem três anéis fundidos (A, B e C) (FIGURA **4**) como unidade estrutural básica.

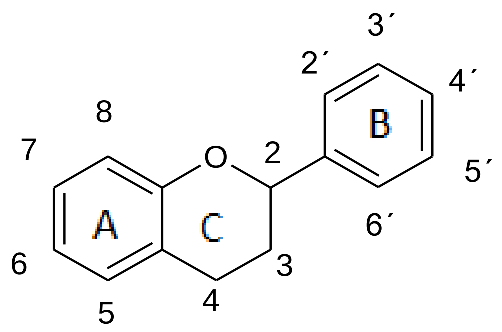

FIGURA 4: ESTRUTURA BÁSICA DE FLAVONOIDES.

Segundo a estrutura química, os flavonoides podem ainda ser subdivididos nas subclasses: Flavanol (catequina, epicatequina, etc), antocianidina (cianidina e delfinidina), flavanona (hesperetina e naringenina), flavanonol (dihidroquercetina, dihidromiricetina e dihidrocaempferol), flavona (apigenina e luteonina), isoflavona (ginisteína e gliciteína) e flavonol (quercetina, miricetina, caempferol e isoraminetina) (AHERNE; O'BRIEN, 2002).

Com relação à composição de fenólicos nos chás de *C. sinensis*, o destaque no chá verde são os flavanóis ou catequinas (Figura 5) e no chá preto as teaflavinas (FIGURA 5). Embora o conteúdo total de polifenóis dos chás verde e preto seja semelhante, os tipos de flavonoides presentes neles são diferentes, devido ao processamento desses chás, que levam à formação de estruturas provenientes da oxidação a que são submetidos (ENGELHARDT, 2010; SCOPARO, *et al*., 2012).

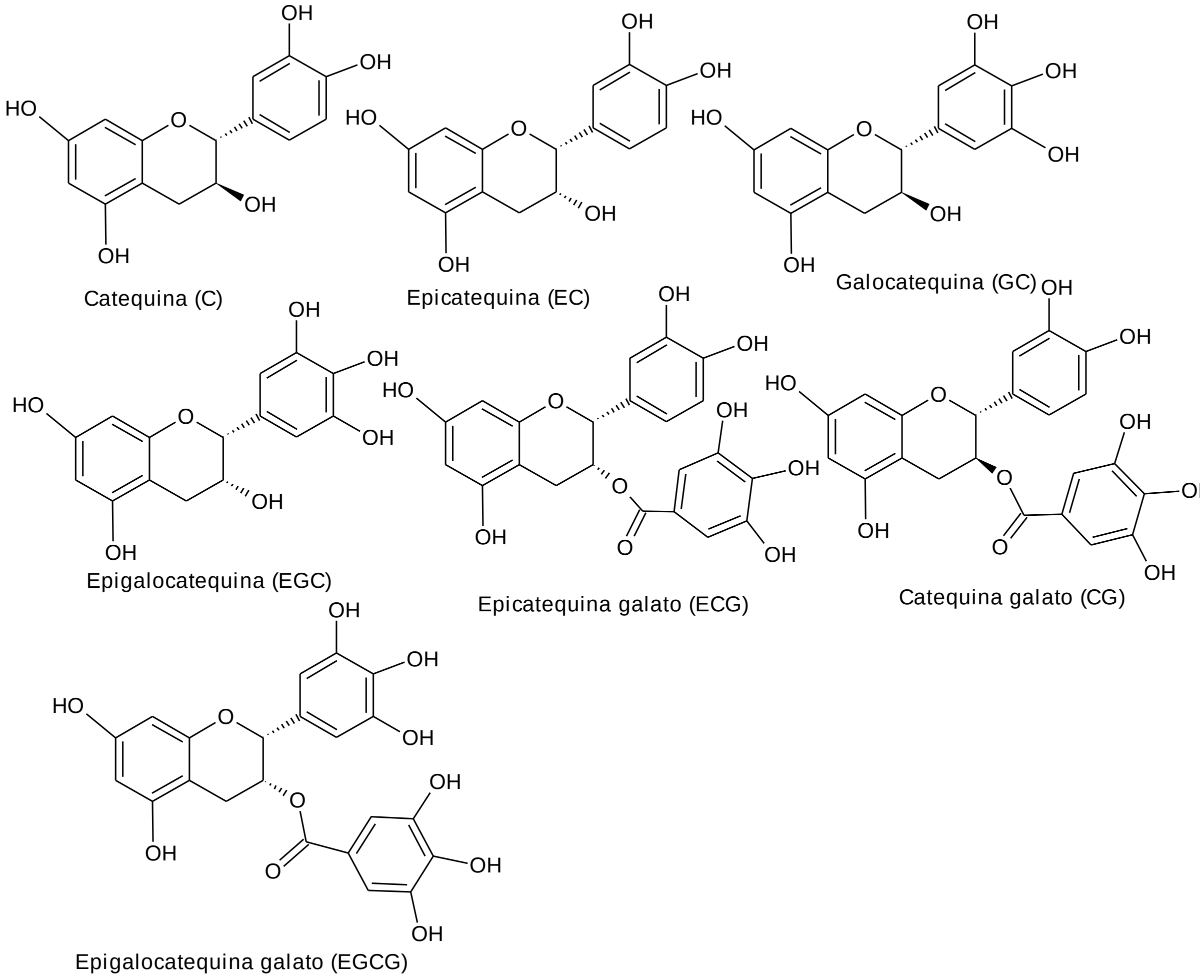

FIGURA 5: PRINCIPAIS FLAVONÓIS/CATEQUINAS PRESENTES NOS CHÁS.

Assim, sabe-se que as catequinas são modificadas durante o processo de oxidação (chá preto) dando origem às teaflavinas, bisflavanóis (teasinensinas) e ácidos epiteaflavílicos, que podem sofrer posterior oxidação levando a formação das tearubiginas. Na composição do chá preto predominam quatro teaflavinas: teaflavina simples (TF), teaflavina-3-galato (TF-3-G), teaflavina-3´-galato (TF-3`-G) e teaflavina-3,3`-digalato (TF-3,3`-dG) (FIGURA 6), além de outras teaflavinas menos abundantes (DAVIES *et al.*, 1992). As várias teaflavinas, juntamente com as tearubiginas, produzidas por dimerização oxidativa catalisadas pelas enzimas polifenol-oxidase (PFO) e peroxidase (PPO), contribuem para a diferenciação dos tipos de chás, bem como para a cor e sabor únicos encontrados no chá preto (SHARMA *et al.*, 2009; WANG *et al*., 2000).

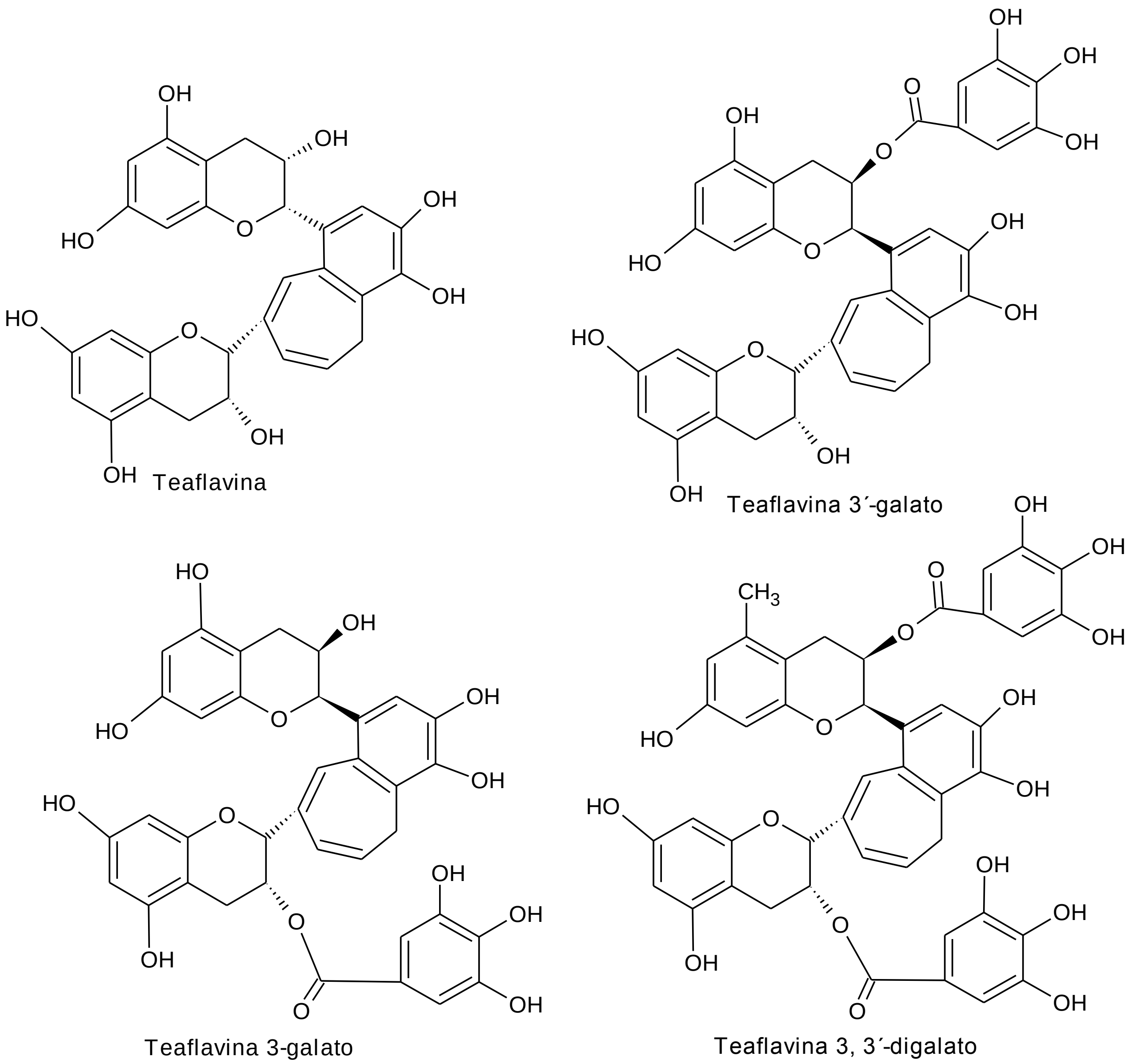

FIGURA 6: PRINCIPAIS TEAFLAVINAS PRESENTES NO CHÁ PRETO.

Ademais, outros compostos presentes no chá verde em maior quantidade do que no chá preto são os taninos (CAVALCANTI *et al.*, 2010). Classicamente, segundo sua estrutura química, os taninos são classificados em dois grupos: hidrolisáveis e condensados. Os taninos hidrolisáveis consistem de ésteres de ácidos gálicos e ácidos elágicos glicosilados, formados a partir do chiquimato (HELDT, 1997), como por exemplo, a estrictinina (FIGURA 7A). Largamente encontrados no reino vegetal, os taninos condensados ou proantocianidinas são polímeros dos flavanóis (HELDT, 1997) (FIGURA 7). Quando são ligados via C4-C8 ou C4-C6, originam-se as proantocianidinas tipo B (FIGURA 7B), se uma ligação éter extra ocorre entre C3 e C7, são denominadas tipo A (FIGURA 7C) (SOUZA *et al*., 2008b)

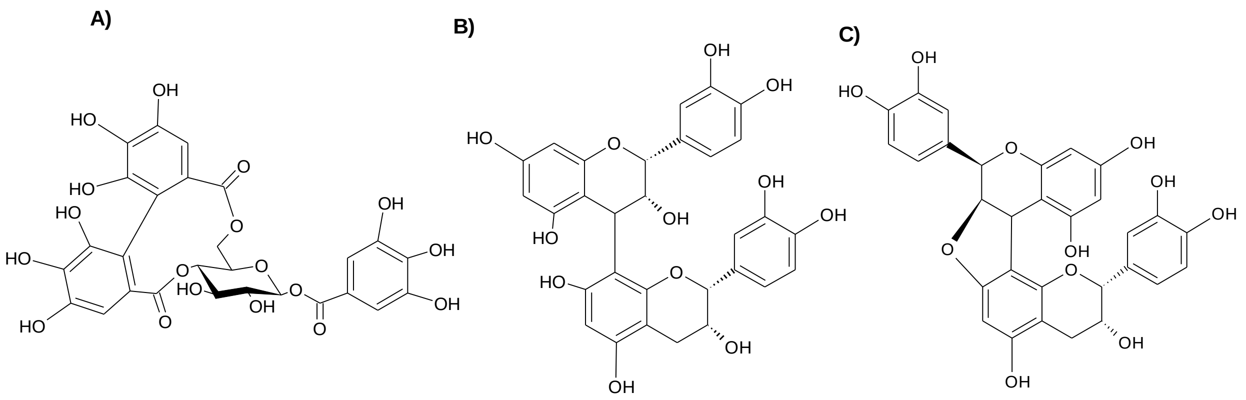

FIGURA 7: EXEMPLOS DE TANINOS HIDROSILÁVEIS: **A)** ESTRICTININA; E CONDENSADOS: **B)** PROANTOCIANIDINA TIPO B E **C)** PROANTOCIANIDINA TIPO A.

2.4.2.2 Alcaloides

Os alcaloides são compostos orgânicos cíclicos que possuem pelo menos um átomo de nitrogênio no seu anel. Na sua grande maioria os alcaloides possuem caráter alcalino, já que a presença do átomo de N representa um par de elétrons não compartilhado. Os alcaloides são sintetizados no retículo endoplasmático, concentrando-se, em seguida, nos vacúolos e, dessa forma, não aparecem em células jovens (PERES, 2008).

Essa classe de compostos do metabolismo secundário é famosa pela presença de substâncias que têm acentuado efeito no sistema nervoso, sendo muitas delas largamente utilizadas como venenos ou alucinógenos. Já na antiguidade há referência ao uso dessa classe de compostos (PERES, 2008).

Quase a totalidade dos alcaloides é derivada de aminoácidos, como a ornitina, a lisina, a tirosina e o triptofano. Enquanto a ornitina é precursora dos alcaloides pirrolidínicos (nicotina) e tropânicos (cocaína), a lisina dá origem aos alcaloides piperidínicos (coniína), a tirosina e o triptofano são formados na via do ácido chiquímico e dão origem aos alcaloides isoquinolínicos (morfina e codeína) e indólicos (estricnina) (SIMÕES, 2004).

Alguns alcaloides não são derivados diretamente de aminoácidos e sim de uma base nitrogenada. Esse é o caso da cafeína (1,3,7-trimetilxantina), uma xantina produzida a partir de uma purina (2,6-dioxipurina). Contudo, as purinas por sua vez derivam de aminoácidos tais como glicina, ácido L-aspártico e L-glutamina. A cafeína e seu precursor teobromina são produzidas por mais de 60 espécies subtropicais, incluindo o café (*Coffea arabica*), o chá (*C. sinensis*), o mate (*Ilex paraguaiensis*), o guaraná (*Paullinia cupana*), a cola (*Cola nitida*) e o cacau (*Theobroma cacao*) (ASHIHARA *et al.*, 1996; SIMÕES, 2004).

Com relação à *C. sinensis*, os principais alcaloides de purina presentes nos chás verde e preto são a teofilina (1,3-dimetilxantina), teobromina (3,7-dimetilxantina) e cafeína (1,3,7-trimetilxantina), os quais estão ilustrados na FIGURA 8 (DEL RIO *et al.*, 2004; SCOPARO, *et al*., 2012).

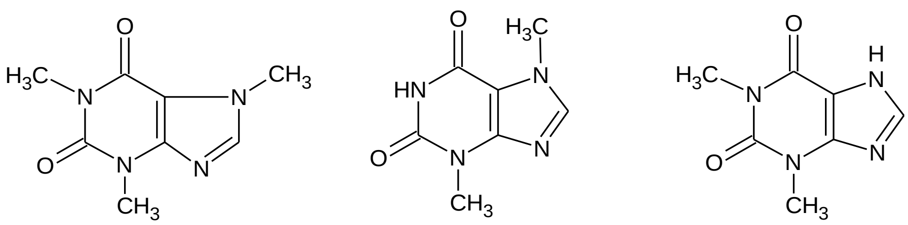

FIGURA 8: ALCALOIDES IDENTIFICADOS NO CHÁ VERDE E PRETO.

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2.4.2.3 Terpenos

Os terpenos são divididos em mono, di, tri e tetraterpenos e os poli-isoprenos (sesquiterpenos). Muitos compostos vegetais importantes são terpenos ou possuem derivados de terpenos em partes de sua molécula. Entre esses compostos encontram-se, inclusive, quatro, das seis principais classes de hormônios vegetais (BAKKALI *et al*., 2008).

Os terpenos são montados através da justaposição sucessiva de unidades de cinco carbonos denominado isopentenilpirofosfato (IPP). O IPP é derivado do ácido mevalônico ou mevalonato e dá origem a todos os outros terpenos (BAKKALI, *et al.*, 2008).

Os monoterpenos, devido ao seu baixo peso molecular, costumam ser substância voláteis, sendo, portanto, denominados óleos essenciais ou essências. A função dos óleos essenciais nas plantas pode ser tanto para atrair polinizadores (principalmente os noturnos) quanto para repelir insetos (pragas). Ademais, estudos demonstram que os compostos monoterpenóides (óleos essenciais) podem atuar como verdadeiros agentes antitumorais em diversos tipos de células (BAKKALI *et al*., 2008).

A literatura remete às análises desta classe de compostos em *C. sinensis*. Dentre estes estudos, Yanagimoto *et al.* (2003), analisaram as propriedades antioxidantes de extratos voláteis (monoterpenos) do chá verde, oolong e preto, em que os compostos voláteis que apresentaram maior atividade antioxidante foram 3,7-dimetill-1,6-octadien-3-ol para o chá verde, furfural para o chá de oolong e álcool benzílico para o chá preto. Em um outro estudo de Schuh e Schieberle (2006), foram comparados compostos voláteis presentes em infusão feita com o chá verde, com os voláteis obtidos da infusão do chá preto, resultando na redução quantitativa da concentração dos compostos voláteis durante o processamento da bebida para consumo (infusão).

Outro grupo de terpenóides encontrados nas plantas são as saponinas, constituídas de um núcleo triterpenoidal pentacíclico, ao qual encontram-se ligados mono- e/ou oligossacarídeos. Nas plantas, as saponinas desempenham um importante papel na defesa contra insetos e microorganismos. Isso pode ocorrer de diversos modos. Uma delas é a complexação das saponinas com esteroides dos fungos, tornando-os indisponíveis. As plantas também podem desenvolver saponinas como análogos de hormônios esteroides de insetos (BAKKALI *et al*., 2008).

A literatura remete à identificação de saponinas encontradas em *Camellia sinensis,* como o estudo realizado por Scoparo e colaboradores (2012) em que foram identificadas diversas saponinas presentes no chá verde, bem como seus isômeros, além de ter sido observada a ausência destes compostos no chá preto, o que possibilitou inferir que o diferente processamento a que os chás são submetidos pode estar alterando a concentração destes compostos. Outrossim, no trabalho realizado por Matsuy *et al.* (2009), foi possível observar a totalidade de saponinas já identificadas na literatura em extratos de folhas do chá verde de *C. sinensis* (FIGURA 9), as quais foram analisadas com relação a suas atividades anti-hipercolesterolêmicas.

Saponinas	**R$_1$**	**R$_2$**	**R$_3$**	**R$_4$**	**R$_5$**	**Peso Molecular**	**Fórmula Molecular**
Teasaponina B1	(E)-Cin	Ac	H	Ac	Xil	1306	$C_{65}H_{94}O_{27}$
Assamsaponina J	Ac	(E)-Cin	H	Ac	Ram	1320	$C_{66}H_{96}O_{27}$
Isoteasaponina B1	(Z)-Cin	Ac	H	Ac	Xil	1306	$C_{65}H_{94}O_{27}$
Foliateasaponina V	(E)-Cin	H	Ac	H	Xil	1264	$C_{63}H_{92}O_{26}$
Isoteasaponina B2	Ac	(E)-Cin	H	H	Xil	1264	$C_{63}H_{92}O_{26}$
Isoteasaponina B3	Ang	(E)-Cin	H	H	Xil	1304	$C_{66}H_{96}O_{26}$
Foliateasaponina I	Tig	Ac	H	Ac	Xil	1258	$C_{61}H_{94}O_{27}$
Foliateasaponina II	(E)-Cin	Ac	H	H	Xil	1264	$C_{63}H_{92}O_{26}$
Foliateasaponina III	Ang	Ac	H	Ac	Xil	1258	$C_{61}H_{94}O_{27}$
Foliateasaponina IV	(Z)-Cin	Ac	H	Ac	Xil	1306	$C_{65}H_{94}O_{27}$
Florateasaponina A	Ang	Ac	H	H	Xil	1216	$C_{59}H_{92}O_{26}$

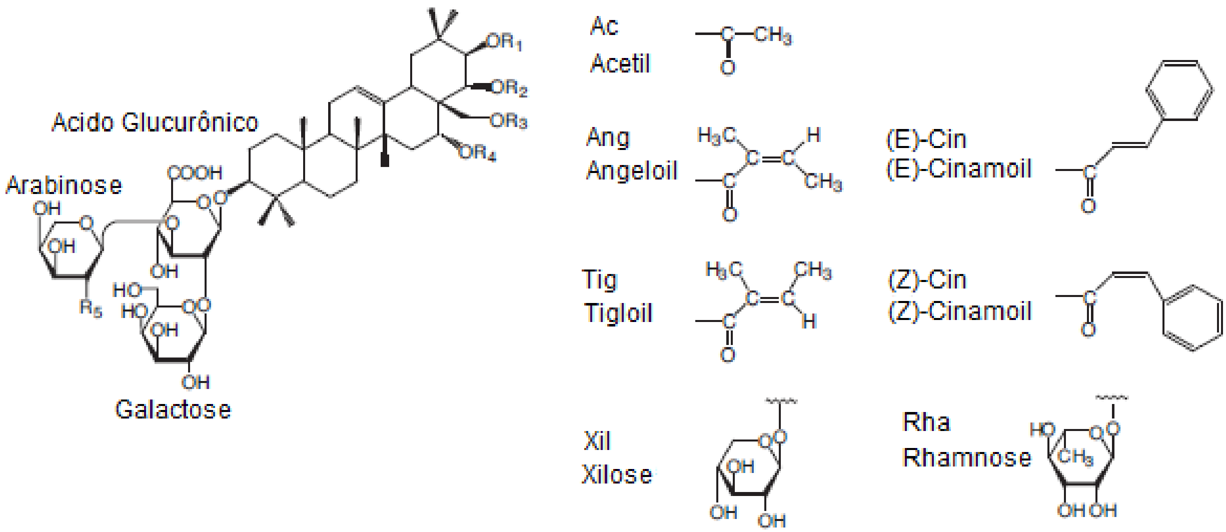

FIGURA 9: ESTRUTURAS CONHECIDAS DE SAPONINAS DE FOLHAS DE *C. sinensis.*

FONTE: Adaptado de Matsuy *et al.*, 2009.

2.5 ATIVIDADES BIOLÓGICAS DESCRITAS PARA *C. SINENSIS* E FISIOPATOLOGIA DA ÚLCERA GÁSTRICA E DA SEPSE.

A composição química das plantas é determinada por fatores genéticos, porém, outros fatores podem acarretar alterações significativas na produção de metabólitos. A *C. sinensis*, particularmente, possui uma grande variedade de compostos, uma vez que o processamento de suas folhas para a feitura dos diversos tipos de chás acarreta na diferenciação das estruturas químicas de seus compostos devido às reações químicas decorrentes do processo. Ademais, por possuir este arsenal de compostos químicos, esta planta torna-se importante para o desenvolvimento de diversos estudos que tenham por finalidade a avaliação das potencialidades terapêuticas que podem ser atreladas a ela.

As propriedades biológicas da *C. sinensis* são comprovadas por inúmeros estudos, os quais têm por objetivo avaliar, nas variadas formas de consumo desta planta, os seus extratos, bem como os compostos majoritários identificados e isolados, muitas vezes fazendo a estrita correlação entre estrutura e atividade.

Neste contexto, os compostos do metabolismo primário encontrados em *C. sinensis*, como os carboidratos, demonstram como principais atividades biológicas: imunoestimulante, antioxidante, antitumoral e antidiabética (CAO, 2013), anti-HIV, anticoagulante e antitrombótica (WANG; WANG, 1992), hipoglicemiante (ZHOU, *et al*., 1997) e antiaderente seletiva contra certas bactérias patogênicas (TAKEO; KINUGASA, 1992; LEE *et al.*, 2006),

Com referência aos compostos do metabolismo secundário, estes são os principais responsáveis pela maior parte das propriedades biológicas relatadas em plantas. Dentro deste contexto, destacam-se os compostos fenólicos, os quais são encontrados em elevadas proporções em *C. sinensis*, o que intensifica o interesse em estudos com os diversos tipos de chás, uma vez que apresentam como principais atividades biológicas: antioxidante, antimutagênica e anticancerígena (YANG, 2007; GONZALEZ*, et al*., 2009), anti-inflamatória, antiobesidade, hipocolesterêmica e proteção ultravioleta para pele (COOPER *et al*., 2005a, 2005b), antidiabética (SABU *et al*., 2002) e proteção contra doenças cardiovasculares (MINEHARU, *et al*, 2011).

Outra classe de compostos fenólicos que evidenciam diversas propriedades farmacológicas são os terpenos, onde destacam-se os monoterpenos, que apresentam atividades antioxidante, citotóxica e capacidade antimutagênica (Yanagimoto *et al.*,2003; Schuh; Schieberle, 2006) e também as saponinas que apresentam diversas atividades biológicas, entre elas: hemolítica, moluscicida, anti-inflamatória, antifúngica, antibacteriana, antiparasitica, antiviral, antitumoral e hipocolesterolêmica (SPARG *et al.*, 2004; Matsuy *et al.,* 2009).

No entanto, sabe-se que o processamento dos tipos de chás altera a estrutura de alguns compostos e esta alteração reflete em mudanças das propriedades químicas e biológicas, que são observadas principalmente nos chás verde e preto (GONZALEZ *et al.*, 2009). Isto posto, estudos que visem a determinação dos metabólitos, bem como a avaliação de suas atividades biológicas, relacionando-os com a diferenciação supracitada são de extrema relevância. Ademais, diversas patologias ainda não haviam sido avaliadas para os diversos chás de *C. sinensis*, como a atividade antiúlcera gástrica e a anticepticêmica, as quais foram objeto de estudo do presente trabalho, e, assim, serão melhor explicitadas.

2.5.1 Úlcera péptica

Úlcera péptica é o termo referido tanto para úlcera duodenal (intestinal) quanto para a úlcera gástrica (estomacais). Trata-se de uma lesão profunda na parede gástrica, que penetra através de toda a espessura da camada mucosa e pela muscular da mucosa, destruindo componentes do tecido epitelial e do tecido conjuntivo subjacente, incluindo miofibroblastos subepiteliais, células do músculo liso, vasos e nervos (MILANI e CALABRO, 2001; TARNAWSKI, 2005). Uma lesão gástrica se converte em úlcera quando há o comprometimento da camada muscular da mucosa (TARNAWSKI, 2000, 2013).

Os mecanismos que desencadeiam as úlceras pépticas são considerados um processo multifatorial, que pode ser atribuído ao desequilíbrio entre fatores agressores (como o ácido clorídrico, a pepsina, infecção por *Helicobacter pylori* além do uso de anti-inflamatórios não esteroidais, fumo e álcool) e defesas locais da mucosa (como a secreção de bicarbonato, muco, prostaglandinas, fluxo sanguíneo e óxido nítrico). O tratamento pode ser conduzido para a redução dos fatores agressores, ou ser dirigido para o fortalecimento das defesas da mucosa do estômago e duodeno (VENKATARANGANNA *et al.,* 1998; JAIN *et al.,* 2006; TARNAWSKI, 2013) (FIGURA 10).

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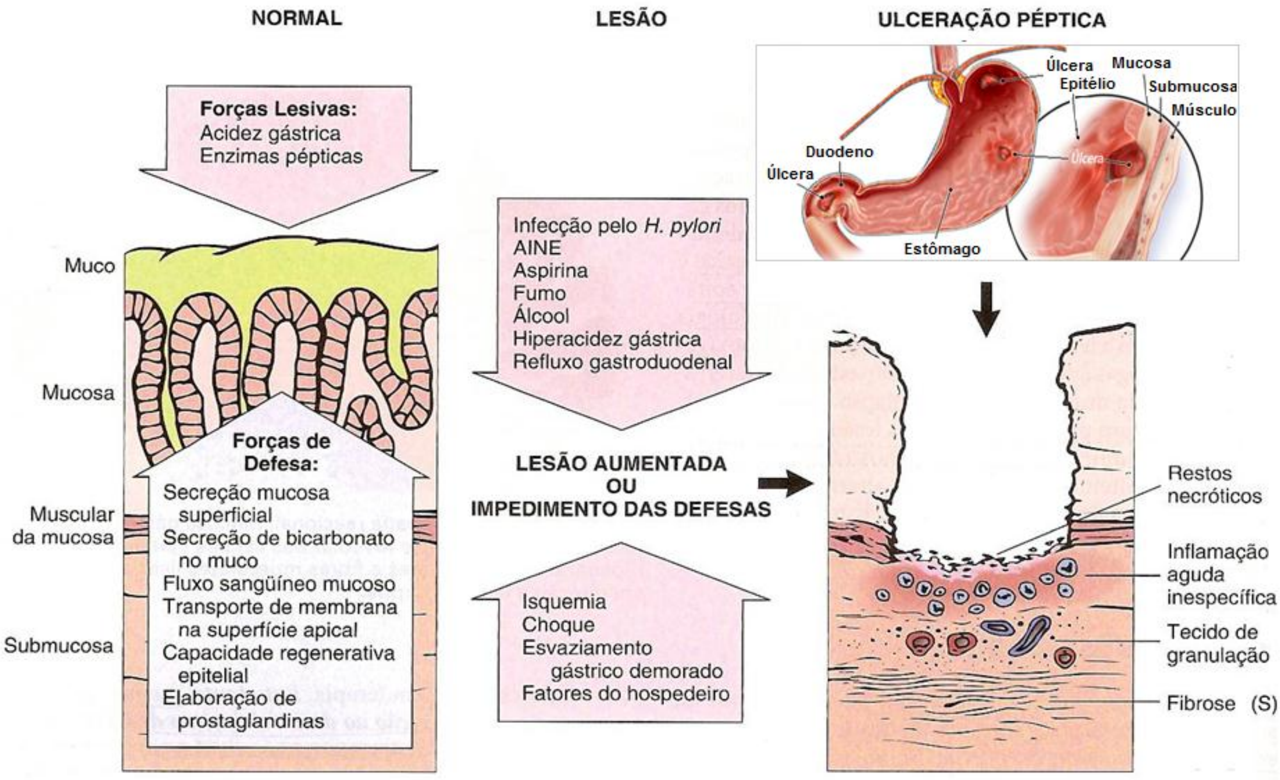

FIGURA 10: ILUSTRAÇÃO REPRESENTATIVA DA ULCERA PÉPTICA

Fonte: Adaptada de Milani e Calabro, 2001.

A úlcera geralmente é uma doença de evolução crônica e recorrente, e, quando ocorre no estômago, acomete principalmente o antro pilórico (65%) e a junção do antro com o corpo estomacal na pequena curvatura (25%) (BRUNTON *et al*., 2006; BOGLIOLO, *et al*., 2011). A sua incidência durante a vida é superior a 10%, com um pico que ocorre entre 65 e 74 anos (JOSHUA, 2000), acometendo indivíduos de qualquer sexo ou idade, mas ocorrendo com maior frequência em mulheres acima dos 60 anos de idade (RAMAKRISHNAN *et al.,* 2007, THORSEN *et al.,* 2011). LIN e colaboradores (2011) mostram que ainda são altas as taxas de incidência de úlcera péptica na população, sendo, aproximadamente, 1 caso por 1000 pessoas/ano.

A patogênese da doença ulcerosa péptica está relacionada, principalmente, a fatores genéticos, fatores endógenos (distúrbios fisiopatológicos com aumento da produção de suco gástrico) e fatores exógenos (físicos, biológicos e químicos) como o estresse, dieta alimentar, uso de AINEs (anti-inflamatório não esteroidal) e infecção por *Heliobacter pylori* (CARVALHO, 2000). A utilização contínua e exacerbada de AINEs, bem como a infecção pelo *H. pylori* são as causas mais frequentes para o desenvolvimento da úlcera. Eles atuam de forma similar sobre a mucosa ao desarmarem os mecanismos de defesa, remanescendo o epitélio suceptível a ação do fluido gástrico - altamente ácido (pH ~ 1-2) - promovendo a lesão epitelial (YEOMANS, 2011; OKABE *et al.,* 2012).

Neste contexto, observa-se que, constantemente, agentes nocivos causam lesões na mucosa gástrica. No entanto, devido aos inúmeros mecanismos de defesa presentes na mucosa, a mesma apresenta-se capaz de manter sua integridade estrutural e funcional (TARNAWSKI, 2013). Os mecanismos que promovem a proteção da mucosa ocorrem por meio de três níveis (barreiras), quer sejam, pré-epitelial, epitelial e sub-epitelial (DONG e KAUNITZ, 2006).

A defesa pré-epitelial consiste na camada de muco e bicarbonato. O trato gastrointestinal, desde o estômago até o cólon, produz o muco pelas células epiteliais. Este mecanismo é estimulado por hormônios gastrointestinais como a gastrina, secretina, ou pelas prostaglandinas (PGs) e agentes colinérgicos (LAINE *et al*., 2008). O muco é composto principalmente por água (95%), lipídeos e glicoproteínas (denominadas mucinas).

Quimicamente, a unidade de mucina tem 80% de seu peso composto por carboidratos, sendo um tetrâmero ligado por pontes dissulfeto. O gel viscoso, que se adere à mucosa, é originado a partir da polimerização da mucina (WALLACE, 2001), e, tem por finalidade, prevenir a colonização bacteriana, reter íons bicarbonato secretados, além de impedir a ação proteolítica da pepsina no epitélio gástrico (LAINE *et al.,* 2008). No entanto, vários agentes (álcool, pepsina) podem clivar as ligações próximas aos centros dos tetrâmeros, sendo que a consequência desta clivagem a dissolução da camada de muco protetora. Após este mecanismo, para que ocorra o restabelecimento da integridade da camada da mucosa, são sintetizadas novas mucinas (BERNE *et al.,* 2004).

Por sua vez, o bicarbonato também é produzido pelas células epiteliais superficiais da mucosa gastroduodenal. Isto ocorre quando as células são estimuladas pelo cálcio, prostaglandinas, estímulos colinérgicos e acidificação do lúmen gástrico, proporcionando um gradiente de pH 6-7 ao longo da superfície das células epiteliais, em contrapartida com o pH 1-2 encontrado na superfície luminal do estômago (LAINE *et al.,* 2008).

Neste contexto, se a barreira pré-epitelial for lesada, outros mecanismos de proteção são recrutados, como o componente epitelial, cuja proteção da mucosa gástrica é conferida pelos aspectos anatômicos e bioquímicos das células epiteliais gástricas. O tecido epitelial é formado por uma camada contínua de células superficiais que controlam a passagem de substâncias do lúmen para a mucosa e submucosa gástrica. Outra característica é que a superfície epitelial é formada por fosfolipídeos, que por sua vez conferem hidrofobicidade ao tecido epitelial funcionando como uma barreira contra a ação da secreção ácida (TARNAWSKI, 2013). O epitélio gástrico é, ainda, responsável pela produção de bicarbonato, muco, fosfolipídios, peptídeos, prostaglandinas (PGs) e proteínas de choque térmico (LAINE *et al.,* 2008), essenciais para a citoproteção.

Relativamente ao sistema de defesa sub-epitelial, a formação de novos vasos sanguíneos é um importante mecanismo, visto que atua tanto na manutenção quanto na reparação de danos causados à mucosa gástrica (TARNAWSKI *et al.,* 2005). A extensa rede vascular além de fornecer nutrientes e oxigênio para o tecido gástrico, aumenta o aporte de bicarbonato, que neutraliza a acidez gástrica gerada através da secreção de ácido clorídrico pelas células parietais, elimina toxinas e é responsável pela produção de potentes vasodilatadores, como o óxido nítrico (NO) e as prostaciclinas (PGI_2) (TARNAWSKI *et al.,* 2013).

Os mecanismos de defesa gástrica são, em sua grande maioria, modulados pelas PGs (TARNAWSKI *et al.,* 2013), as quais, por sua vez, no tecido gástrico, estão envolvidas na inibição da secreção ácida, na liberação de bicarbonato e muco, na inibição sobre as funções de neutrófilos e adesão de leucócitos ao endotélio vascular, na manutenção de um fluxo sanguíneo adequado da mucosa e na restituição das células epiteliais (LAINE *et al.,* 2008).

A formação da úlcera ocorre quando as barreiras gástricas são destruídas, assim, a renovação contínua das células epiteliais é fundamental no processo de cicatrização, ao repor as células gástricas necróticas pela proliferação e migração de células epiteliais sobreviventes da margem da lesão (LAINE *et al*., 2008; TARNAWSKI e AHLUWALIA, 2012).

As falhas nos níveis superficiais de defesa, resultantes de uma contribuição inadequada de nutrientes à mucosa, pode gerar isquemia e morte tecidual, que por sua vez, associada ao aumento de substâncias quimiotáticas, ativa a liberação de agentes pró-inflamatórios (TNF-α, IL-1α e IL-1β) pelos leucócitos que são atraídos até o local da lesão (TARNAWSKI, 2005). Assim, inicia-se a fase inflamatória da úlcera.

O desenvolvimento do processo inflamatório inclui, principalmente, alterações vasculares, acarretando no aumento do fluxo sanguíneo e permitindo que proteínas plasmáticas e leucócitos polimorfonucleados, as primeiras células a serem recrutadas pelo sistema imune, deixem a circulação, migrando até o foco da lesão, dirigidos por gradientes quimiotáticos, onde, então, são ativados para fagocitar produtos da destruição tecidual e para eliminar o agente nocivo através de um acúmulo de enzimas hidrolíticas e proteínas bactericidas (ROBBINS *et al.,* 2013).

A cicatrização da úlcera pode ter início alguns dias após a lesão e inclui a proliferação e migração celular, angiogênese e remodelação do tecido (TARNAWSKI, 2005; TARNAWSKI e AHLUWALIA, 2012). O processo inicial de cicatrização das úlceras é acompanhado pelo aumento do fluxo sanguíneo na área ulcerada, dos níveis plasmáticos de gastrina e de citocinas pró-inflamatórias como TNF-α e IL-1β, mas que têm seus níveis reduzidos ao longo do processo de cicatrização. Todos estes processos são controlados basicamente por fatores de crescimento, além das citocinas, hormônios e fatores de transcrição, capazes de estimular a proliferação e migração celular (TARNAWSKI *et al.,* 2013).

Desta forma, as células necróticas vão sendo substituídas da margem da úlcera para o tecido de granulação pela migração de células sobreviventes. Este processo possibilita a ocorrência da reepitelização, tendo como principal finalidade, a proteção do tecido contra infecções e danos mecânicos e químicos (LAINE *et al.,* 2008). Assim, é restabelecida a integridade física da mucosa gástrica, sendo que a cicatrização, pela consequente reepitelização, é um processo fundamental, visto que, o epitélio é uma barreira contínua do tecido de granulação (tecido ricamente vascularizado) que protege a mucosa.

Tem sido contínua e importante a investigação de novas drogas para o tratamento de doenças relacionadas ao trato gastrintestinal. A supressão da secreção ácida gástrica, que constitui o recurso terapêutico mais utilizado, mesmo que em doses recomendadas, às vezes pode conduzir a uma acloridria (ausência de ácido clorídrico) podendo desencadear infecções entéricas como a febre tifóide, cólera e disenteria (JAIN *et al*., 2007). Assim, estudos mais recentes já evidenciam a procura de fármacos que atuem em conjunto com os tratamentos convencionais para melhorar a qualidade de cicatrização e evitar a tendência à recidiva da úlcera (KANGWAN *et al.,* 2014).

Dentro deste contexto, diversos metabólitos têm sido avaliados em estudos pré-clínicos quanto a sua ação protetora na úlcera gástrica, os quais demonstraram uma eficiência significativa no combate à lesão, como, por exemplo, polissacarídeos como arabinogalactanas tipo I e tipo II de *Cereus peruvianus* (TANAKA *et al*., 2010), *Cochlospermum tinctorium (*NERGARD, *et al., 2005)* e *Maytenus ilicifolia* (CIPRIANI, *et al*., 2006), bem como polissacarídeos pécticos de *Bupleurum falcatum* (YAMADA *et al., 1993*), heteroxilanas ácidas de *M. ilicifolia* e *Phyllanthus niruri* (CIPRIANI, *et al*., 2008), heteroxilanas de goma de exsudato da *Scheelea phalerata* (SIMAS *et al*., 2004), arabinoxilanas do bagaço de cana (MELLINGER *et al*., 2011), e, por fim, uma ramnogalacturonana isolada de *Acmella oleracea* (NASCIMENTO *et al.*, 2013).

Metabólitos secundários também têm apresentado atividade protetora contra úlcera gástrica, como os flavonoides (MOTA, *et al*, 2012; HAMAISHI *et al., 2006;* ADHIKARY *et al*., 2011a, 2011b, 2011c; ROZZA *et al*., 2012), bem como coumarinas, alcaloides, ácidos fenólicos, taninos, terpenos e saponinas (MOTA *et al*., 2012; CECHINEL *et al*, 1998; MORIKAWA *et al*., 2006; YOSHIKAWA *et al.,* 2005).

Assim posto, emerge como uma área promissora ao desenvolvimento de novos compostos que possam atuar sobre esta importante patologia clínica. Desta forma, a *C. sinensis* revela-se propícia à avaliação de sua atividade frente à úlcera gástrica, devido ao abrangente arsenal de compostos químicos relatados para esta planta, os quais podem atuar eficientemente na gastroproteção.

2.5.2 Sepse

Sepse é um conjunto de manifestações graves sistêmicas, definida como uma síndrome da resposta inflamatória sistêmica (SRIS), sendo decorrente de infecção, principalmente por bactérias, não obstante também possa ser consequência de infecção por fungos, helmintos e vírus (BENJAMIM, 2001). Pode decorrer, também, devido a queimaduras, complicações cirúrgicas, infecções hospitalares, apendicite, dentre outras patologias (QUEZADO; NATANSON, 1992; CARVALHO *et al*, 2003).

A SRIS inclui, além da sepse, doenças de origem não infecciosas, tais como a isquemia, hemorragia, trauma e pancreatite (BENJAMIM, 2001; BLOCH, 2007). Do ponto de vista clínico, a apresentação da sepse se relaciona às múltiplas possibilidades de interação entre homem e microrganismos, distinguindo-se assim as situações como infecção, SRIS, sepse, sepse grave, choque séptico e disfunção de múltiplos órgãos e sistemas (Quadro 1).

A consequência mais comum da SRIS ou sepse é a síndrome da disfunção de múltiplos órgãos (SDMO), a qual chega a acometer aproximadamente 1/3 dos pacientes que apresentam o quadro de sepse, sendo que, praticamente todos acabam por desenvolver uma disfunção de ao menos um órgão. Relativamente ao choque séptico, este é proveniente de um quadro de agravamento acentuado da sepse, cuja principal característica são as alterações hemodinâmicas severas (BENJAMIM, 2001).

Termo	Conceito
Colonização	Refere-se à presença de microrganismos em um determinado local, sem que esteja ocorrendo dano ao hospedeiro.
Infecção	Presença de um determinado agente que esteja causando dano ao hospedeiro (está presente resposta inflamatória ao microrganismo).
Bacteremia	Ocorrência de bactérias viáveis no sangue, podendo ser transitória; por extensão, é possível caracterizar-se viremia, fungemia e parasitemia.
Síndrome de resposta inflamatória sistêmica (SIRS)	Caracterizada por ser uma resposta inespecífica do organismo a uma variedade de situações que geram inflamação - infecção, queimaduras, pancreatite aguda, trauma, e outras. Para sua detecção, são necessárias duas das seguintes condições: Temperatura > 38,0 ºC ou < 36,0 ºC Freqüência cardíaca > 90 bpm Freqüência respiratória > 20 irpm ou $PaCO_2$ < 32 mmHg Leucócitos > 12.000/mm³ ou < 4.000/mm³ ou > 10% de bastões
Sepse	SIRS desencadeada por infecção bacteriana, viral, fúngica ou parasitária.
Hipotensão	Pressão arterial sistólica < 90mmHg ou uma redução de 40mmHg da pressão "basal".
Sepse grave	Aquela associada com disfunção orgânica, hipoperfusão tissular (caracterizada, entre outros aspectos, por oligúria, distúrbio mental agudo e/ou acidose láctica) ou hipotensão arterial.
Choque séptico	Hipotensão (não atribuível a outra causa) com hipoperfusão tecidual ocasionada por sepse. Pode ser *precoce*, quando dura menos de uma hora (em resposta à infusão de solução cristalóide, 0,5-1 litro), ou *tardio*, com duração maior que uma hora e/ou necessidade de uso de aminas vasoativas.
Disfunção de múltiplos órgãos e sistemas (DMOS)	Alterações da função de órgãos de um enfermo grave, de modo que a homeostase não pode ser mantida sem intervenção terapêutica. É *primária* se conseqüente à própria injúria (p. ex., insuficiência respiratória secundária à pneumonia comunitária grave) e *secundária*, se oriunda não da injúria, mas da resposta orgânica do hospedeiro à condição mórbida (p. ex., síndrome do desconforto respiratório agudo em enfermo com pancreatite aguda necrótica).

Quadro 1: DEFINIÇÕES IMPORTANTES PARA O ENTENDIMENTO DA SEPSE
FONTE: Adaptado de SIQUEIRA-BATISTA *et al*. (2011)

No Brasil, foi identificada uma incidência de sepse de 57,9 por 1000 pacientes/dia, bem como a taxa de letalidade de pacientes com SRIS (independente se devido à sepse ou outra causa), sepse, sepse grave e choque séptico foi 24,2%, 33,9%, 46,9%, e 52,2%, respectivamente. Da mesma forma, estudos revelam que as taxas de letalidade por sepse nas UTIs no Brasil foram maiores (56%) da que aquelas de outros países em desenvolvimento (45%) e de países desenvolvidos (30%), apesar de não existirem diferenças nas idades medianas de cada grupo, nem nos escores de prognóstico e de disfunção orgânica (SIQUEIRA-BATISTA *et al*., 2011).

Apesar do arsenal terapêutico disponível na clínica, a sepse continua sendo a principal causa de mortalidade nas unidades de terapia intensiva em todo o mundo (COHEN, 2002; SIQUEIRA-BATISTA *et al*., 2011).

2.5.2.1 Fisiopatologia e mediadores inflamatórios envolvidos na sepse

O sistema imune é constituído por uma complexa rede de células e substâncias que tem por finalidade manter a homeostase do organismo, combatendo as agressões em geral, como componentes estranhos, independentemente das consequências fisiológicas ou patológicas dessa reação (OPAL; ESMON, 2003).

Entretanto, estudos recentes sugerem uma complexa interação entre as cascatas de inflamação, coagulação e fibrinólise que ocorrem em resposta a um disparo imune, por exemplo, a infecção bacteriana. O início simultâneo desses três processos tem papel central na fisiopatologia da sepse e a continuidade destes pode desencadear disfunção endotelial e falência múltipla de órgãos culminando, se não houver tratamento, com óbito (OPAL; ESMON, 2003).

Durante a sepse, devem ser avaliados e combatidos três processos distintos, porém interligados, que acontecem concomitantemente: o foco infeccioso, as alterações hemodinâmicas e a resposta inflamatória local e generalizada. Em geral o tratamento de pacientes com sepse e choque séptico são utilizados antibióticos e drogas que interferem nas alterações cardiovasculares consequentes da sepse (hipotensão), não se intervindo na resposta inflamatória, podendo ser esse um dos motivos da elevada mortalidade de pacientes com choque séptico (BENJAMIN, 2001).

Um dos eventos que desencadeiam a sepse é o acesso das bactérias à corrente sanguínea e a consequente liberação de suas endotoxinas. Essas endotoxinas ativam células do sistema imune, especialmente os macrófagos, iniciando a resposta inflamatória por meio da liberação de mediadores como o óxido nítrico, os eicosanóides e as citocinas pró-inflamatórias, com destaque para as citocinas inicializadoras TNF-α (fator de necrose tumoral–α) e as IL-1β (interleucina – 1β), IL-2, IL-6 , IL-8 e IL-12, que são consideradas cruciais para o desenvolvimento de sepse. No entanto, interleucinas anti-inflamatórias, IL-4, IL-5, IL-10, IL-11 e IL-13 são também produzidas, especialmente em condições em que o paciente sobrevive ao desenvolvimento de uma desordem relacionada com inflamação sistêmica, como a sepse (SIQUEIRA-BATISTA et al., 2011). A bacteremia resulta na ativação e migração de leucócitos polimorfonucleares (neutrófilos) para vários tecidos do organismo, mesmo aqueles distantes da origem da infecção (WANG *et al.*, 1999; OHLSSON *et al.*, 1990; NEVIERE et al., 1999).

Deste modo, os neutrófilos são as primeiras células a chegarem ao sítio de infecção. No interior dos neutrófilos são produzidos grânulos azurófilos, os quais são liberados em resposta ao processo infeccioso. Nestes grânulos são estocadas enzimas proteolíticas como a mieloperoxidase (MPO), importante na produção de espécies reativas de oxigênio. A atividade de MPO é usada como um importante marcador para a infiltração de neutrófilos nos tecidos, uma vez que, no organismo com sepse, os pulmões são um dos primeiros órgãos a sofrer as consequências da migração e ativação de neutrófilos, resultando em danos nos tecidos e na função do órgão (ALVES-FILHO et al., 2010).

Além desta enzima, os neutrófilos produzem e liberam espécies reativas de oxigênio (ROS) como o óxido nítrico (NO), peróxido de hidrogênio (H_2O_2) e ânion superóxido (O_2^-). Todos estes mediadores favorecem a fagocitose dos patógenos. Entretanto também podem participar da lesão tecidual e intensificar os efeitos da sepse (DOWNEY *et al.*, 1993).

Além disso, ativação de células endoteliais e neutrófilos por citocinas e endotoxinas liberadas pelos agentes patogênicos induzem a expressão das enzimas óxido nítrico sintase induzível (iNOS) e ciclo-oxigenase-2 (COX-2). A iNOS está envolvida na produção de óxido nítrico (NO), o qual é um importante mensageiro intercelular nos mamíferos superiores, potente vasodilatador (pode estar associado à hipotensão associada a sepse) e, em concentrações mais elevadas, microbicida (PECK, 2007; ALVES-FILHO et al., 2010). A expressão da iNOS também é induzida em resposta a estímulos inflamatórios, tais como o lipopolissacarídeo bacteriano (LPS) e citocinas pró-inflamatórias (por exemplo, IL-1, TNF-α). Quando expressa, iNOS produz grandes quantidades de NO por períodos longos, o que também provoca danos celulares (TINKER; WALLACE, 2006).

A COX-2 é outra enzima importante no processo inflamatório, pois, catalisa a formação de eicosanóides inflamatórios, tais como prostaglandinas e tromboxanos, metabólitos oxigenados do ácido araquidônico, que são pequenas moléculas que regulam as respostas fisiopatológicas durante a sepse (HAYASHI *et al.*, 2011). A associação destes inúmeros fatores podem acabar agravando significativamente a sepse, culminando no óbito do portador desta patologia.

As disfunções em muitos órgãos e sistemas são causadas pela progressão da sepse por um provável mecanismo de lesão endotelial disseminada, com extravasamento de fluidos e, em consequência, edema intersticial e hipovolemia, além dos distúrbios da coagulação (com formação de microtrombos, que diminuem o aporte de oxigênio e nutrientes para os tecidos atingidos). Ademais, é possível observar um incremento na liberação de hormônios contra-insulínicos (glucagon, corticosteróides, catecolaminas e hormônio do crescimento), resultando em hipermetabolismo, com aumento da glicogenólise e da gliconeogênese hepática, aumento da lipólise e do catabolismo proteico muscular, intestinal e do tecido conjuntivo. Todos esses mecanismos, em conjunto, levam à hipóxia tissular, acidose láctica, sendo que a hiperlactatemia associa-se com a gravidade da doença e, por fim, a morte celular (SIQUEIRA-BATISTA *et al*., 2011).

Na atualidade, mesmo na presença de grande desenvolvimento científico e tecnológico, o tratamento terapêutico do enfermo com diagnóstico de sepse continua a ser um desafio para os profissionais da saúde. O tratamento da sepse, da sepse grave, do choque séptico e da disfunção de múltiplos órgãos e sistemas (DMOS) incluem as manobras de reposição volêmica, a abordagem da infecção, o emprego de corticosteroides, a terapia anticoagulante, o controle glicêmico, o suporte ventilatório e medidas terapêuticas adicionais (SIQUEIRA-BATISTA *et al*., 2011).

Considerando que a disfunção de órgãos é uma grande preocupação na fisiopatologia da sepse e contribui para a sua elevada taxa de mortalidade, recentemente, armadilhas extracelulares de neutrófilos, conhecidas como NETs (neutrofil extracellular traps), têm sido implicadas na lesão endotelial, as quais participam na patogênese da disfunção orgânica em várias condições. Este mecanismo envolve a liberação do DNA e de proteínas com propriedades microbicidas, denimonadas histonas (que podem estar nos grânulos ou dispersas no citoplasma). O DNA e as histonas formam uma rede, a qual se atribui a denominação de NETs. A rede serve como armadilha para prender e confinar os agentes patológicos, evitando a sua disseminação pelo organismo e permitindo, ao mesmo tempo, a ação conjunta e localizada das proteínas microbicidas. Assim, trabalhos tem sido realizados para avaliar a formação de NETs e sua participação como agentes terapêuticos na sepse (CZAIKOSKI, *et al*., 2016; ISOBE, *et al*., 2016).

Ademais, dentro deste contexto, inúmeros compostos químicos isolados de plantas também têm sido testados em modelos experimentais de sepse em camundongos, e tem demonstrado benefícios no controle do processo inflamatório na sepse, como por exemplo, a fração butanólica obtida das folhas de *Eugenia uniflora*, a qual é rica em flavonoides (RATTMANN, 2012), fucogalactanas isoladas dos fungos *Agaricus bisporus* e *Lactarius rufus* (RUTHES *et al.,* 2012), a fração metanólica de *Pterodon emarginatus*, contendo D-pinitol como componente majoritário (KIATKOSKI, 2011), entre outros.

Desta forma, pode-se inferir, a partir dos resultados da literatura, que produtos naturais têm sido alvos de intensa investigação quanto aos efeitos na patologia da sepse. Assim, a *C. sinensis*, a qual apresenta inúmeras propriedades farmacológicas, uma vez que dispõe de uma vasta gama de compostos químicos, torna-se um importante objeto de estudo para uma completa e ampla investigação quanto a sua atividade na sepse.

3. JUSTIFICATIVA

O uso de fitoterápicos acompanha a evolução da humanidade e continua a expandir-se rapidamente em todo o mundo. Além do seu potencial terapêutico amplamente considerado na medicina popular, as plantas constituem a maior fonte de matéria-prima para a descoberta de novas moléculas.

Neste contexto, os metabólitos de plantas apresentam-se como promissores para desenvolvimento de análises químicas que permitam a completa elucidação de sua estrutura, bem como para a avaliação de suas potencialidades farmacológicas. Ademais, considerando a complexidade dos extratos vegetais, emerge uma área da pesquisa voltada para desenvolvimento de análises que permitam a identificação desses compostos presentes nas plantas medicinais.

A *Camellia sinensis,* em particular, apresenta uma elevada complexidade quanto a sua composição química, uma vez que inúmeros compostos têm, continuamente, sido relatados para esta planta, o que contribui significativamente para o interesse cada vez maior na elucidação de todo este arsenal químico. Além disso, uma vasta gama de potencialidades farmacológicas tem sido relatada, o que acarreta em um incremento do interesse científico, cultural e econômico sobre a *C. sinensis*.

Ademais, esta planta tem uma diversificação em seu processamento pós-colheita para obtenção dos diversos tipos de chás atualmente disponíveis no mercado (verde, preto, branco, oolong, pu-ehr), sendo grande o interesse de um estudo comparativo dos seus metabólitos. Além disso, pela planta apresentar inúmeros compostos químicos, torna-se imprescindível o estudo de diferentes técnicas de extração a fim de isolar compostos ainda não identificados, bem como prover uma melhor separação e caracterização dos mesmos.

Considerando que não há na literatura uma detalhada caracterização dos polissacarídeos presentes em extratos de *C. sinensis*, a determinação da química estrutural, associada à avaliação de suas propriedades biológicas, torna-se um campo de pesquisa promissor. Além disso, com relação aos compostos de baixa massa molecular, devido à complexidade estrutural desta planta, esta pode ser utilizada no desenvolvimento de novas metodologias de análises fitoquímicas, correlacionado sua estrutura e atividade.

Há de se ressaltar que esta tese atende a relevantes etapas para o desenvolvimento de produtos fitoterápicos, uma vez que tem como propósito a ampla investigação da constituição química de uma planta de interesse nacional, atendendo à portaria nº 971, de 3 de maio de 2006, que aprova a Política Nacional de Práticas Integrativas e Complementares no Sistema Único de Saúde e à Resolução RDC nº 14, de 31 de março de 2010 (Diário Oficial da União, nº 63, 5 de abril de 2010), que dispõe sobre o registro de medicamentos e fitoterápicos, bem como designa que a autorização oficial do uso de fitomedicamentos e seus derivados prescinde de comprovações experimentais, as quais são fornecidas por meio de testes biológicos. Este estudo, atendendo à importância da interdisciplinariedade entre os diversos ramos da ciência, se destaca pela integração das áreas de Bioquímica, Química, Farmacologia e Ciências Farmacêuticas.

4. OBJETIVOS

4.1 OBJETIVO GERAL

Este estudo teve como objetivo a obtenção, caracterização e comparação dos diferentes polissacarídeos presentes em dois tipos de chá da C. sinensis, o chá verde e o chá preto, bem como a avaliação de suas propriedades farmacológicas relacionadas à ação anti-inflamatória em modelo de septicemia e ulcera péptica, bem como o desenvolvimento de metodologias para separação cromatográfica e elucidação estrutural dos compostos de baixa massa molecular do chá verde e preto, associado a um estudo bio-guiado para a avaliação de atividades anti-inflamatórias na úlcera e na sepse dos extratos, frações e dos compostos identificados.

4.2 OBJETIVOS ESPECÍFICOS

- ✓ Submeter as folhas do chá verde e chá preto a extrações hidroalcoólica, aquosas (decocção e infusão) e alcalina;
- ✓ Identificar os polissacarídeos presentes no extrato aquoso (infusão) e no extrato alcalino por técnicas de espectroscopia de NMR de ^{1}H e ^{13}C e de cromatografia gasosa acoplada ao espectrômetro de massas (GC-MS)
- ✓ Avaliar a atividade dos polissacarídeos fracionados por congelamento e degelo, após extração aquosa, na sepse, e, após extração alcalina, na úlcera gástrica;
- ✓ Isolar e identificar os compostos de baixa massa molecular após particionamento líquido/líquido dos extratos obtidos por meio da extração hidroalcoólica e aquosa (infusão), por meio do acoplamento da cromatografia líquida à espectrometria de massas;
- ✓ Avaliar a atividade antiúlcera gástrica dos extratos hidroalcoólicos, frações particionadas e compostos isolados;
- ✓ Avaliar a atividade dos extratos obtidos por meio da infusão, bem como das frações particionadas e compostos identificados, na sepse.

6. CONCLUSÕES

Após a análise dos resultados obtidos nesta pesquisa, pode-se concluir que:

- A partir da extração aquosa (decocção) das folhas dos chás verde e preto, de *Camellia sinensis,* foram caracterizadas ramnogalacturonanas do tipo I, ligadas a arabinogalactanas do tipo II. Estes polissacarídeos dos chás verde e preto culminaram na diminuição da letalidade causada por sepse polimicrobiana em camundongos em 40% e 25%, respectivamente. Este efeito benéfico parece ser, pelo menos em parte, devido a uma redução na migração de neutrófilos. A diferença entre os polissacarídeos de ambos os chás parece resultar do processamento das folhas para a produção dos diferentes tipos de chás, bem como a diferente capacidade para prevenir a letalidade dos camundongos pode estar relacionada aos diferentes teores de ácido urônico obtidos para os polissacarídeos de ambos os chás.

- A partir da extração alcalina dos chás verde e preto obtidos das folhas de *C. sinensis,* foram identificadas uma arabinogalactana proteína (AGP) nas frações solúveis no congelamento e degelo, e uma heteroxilana nas frações insolúveis do mesmo fracionamento, ambos identificados para os dois tipos de chás. Foi demonstrado que a AGP do chá verde e do chá preto inibiram as lesões gástricas induzidas por etanol em 71% e 66%, assim como as heteroxilanas em 76% e 62%, respectivamente. Este efeito positivo pode ser devido à restauração observada nos ensaios dos níveis de GSH e de níveis de muco gástrico, os quais são dois importantes fatores na gastroproteção.

- Com relação à análise química dos metabólitos de baixa massa, uma ampla gama de compostos pôde ser identificada nas frações hidroalcoólicas do chá verde e do chá preto, com destaque para as catequinas e seus derivados galatos e de oxidação, os flavonoides glicosilados, ácido gálico e hidroxicinâmicos e também ésteres de ácido quínico, bem como saponinas, lipídeos e alcaloides. A partir de um estudo bioguiado, os extratos hidroalcoólicos dos chás verde e preto apresentaram ação gastroprotetora, sendo o chá verde 2,8 vezes mais potente que o chá preto, e a fração acetato de etila do chá verde e fração aquosa do chá preto foram, respectivamente, 6 e 10 vezes mais potente do que os seus extratos brutos. Além disso, a epigalocatequina galato (EGCG), sendo o principal composto encontrado no chá verde, parece ser responsável pelo efeito biológico observado pra este chá. No entanto, o mesmo não foi observado para a rutina, um dos principais compostos do chá preto. Estes efeitos podem ser associados com a manutenção de níveis de muco e GSH na mucosa gástrica. A diferença na composição química de ambos os chás pode ser resultado do processamento a que a folhas são submetidas durante a produção dos chás, e a distinta capacidade para evitar lesão gástrica pode estar relacionada com os diferentes compostos presentes em cada fração analisada.

- Na análise dos compostos de baixa massa presentes na infusão dos chás verde e preto, várias classes de compostos puderam ser identificadas nos extratos e nas frações acetato de etila obtidos, como as catequinas e os seus derivados galatos e produtos de oxidação (teaflavinas e teasinensinas), além de proantocianidina, flavonoides glicosilados, alcaloides, ácidos hidroxibenzóicos e hidroxicinâmicos, e também saponinas. Além disso, os extratos da infusão, as frações de acetato de etila e os compostos majoritários identificados a partir de ambos os chás (EGCG, teaflavinas e cafeína) foram analisados quanto à sua atividade na sepse murina. Os tratamentos com o chá verde e preto resultaram num decréscimo de mortalidade na sepse de 43% e 40%, respectivamente, e, o tratamento com as frações acetato de etila reduziram a mortalidade em 50% e 66,6%, respectivamente. Os tratamentos com a EGCG, teaflavinas ou cafeína mostraram uma sobrevivência de 50%, 40% e 20%, respectivamente. Além disso, parâmetros inflamatórios importantes foram avaliados, demonstrando que a inibição da MPO, a redução de citocinas pró-inflamatórias, um aumento de citocinas anti-inflamatórias e uma diminuição dos níveis de expressão das enzimas iNOS e COX-2 estão diretamente relacionados com o efeito benéfico observado para as frações e os compostos analisados.

Isto posto, os resultados obtidos neste trabalho corroboram para a obtenção de princípios biologicamente ativos de *Camellia sinensis*, os quais podem ter uma aplicação terapêutica importante para o auxílio no tratamento de importantes patologias que envolvem processos inflamatórios e são causas acentuadas de acometimento e mortalidade da população.

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ANEXO I

- Artigos puplicados como autora e co-autora durante o período de doutorado:

SCOPARO, C. T.; DE SOUZA, L. M.; DARTORA, N.; SASSAKI, G. L.; SANTANA-FILHO, P.M.; WERNER, M. F. P.; BORATO, D. G.; BAGGIO, C. H.; IACOMINI, M. Chemical characterization of heteropolysaccharides from green and black teas (Camellia sinensis) and their anti-ulcer effect, International Journal of Biological Macromolecules, v. 86, p. 772-781, 2016.

SCOPARO, C. T., DE SOUZA, L.M., RATTMANN, Y.D., KIATKOSKI, E.C., DARTORA, N. & IACOMINI,M., The protective effect of green and black teas (*Camellia sinensis*) and their identified compounds against murine sepsis, Food Research International, v. 83, p. 102-111, 2016.

SCOPARO, C. T.; BORATO, D. G.; SOUZA, L. M.; DARTORA, N.; SILVA, L. M.; FERREIRA, D. M.; SASSAKI, G. L.; BAGGIO, C. H.; IACOMINI, M. Gastroprotective bio-guiding fractionation of hydro-alcoholic extracts from green- and black-teas (*Camellia sinensis*). Food Research International, v. 64, p.577-586, 2014.

SCOPARO, C. T.; SOUZA, L. M.; RATTMANN, Y. D.; DARTORA, N.; MALQUEVICZ-PAIVA, S. M.; SASSAKI, SASSAKI, G. L.; GORIN, P. A. J.; IACOMINI, M. Polysaccharides from green and black teas and their protective effect against murine sepsis. Food Research International, v. 53, p. 780-785, 2012.

BORATO, D. G.; **SCOPARO, C. T**.; MARIA-FERREIRA, D.; DA SILVA, L. M.; SOUZA, L. M.; IACOMINI, M.; WERNER, M. F. P.; BAGGIO, C. H. Healing mechanisms of the hydroalcoholic extract and ethyl acetate fraction of green tea (Camellia sinensis (L.) Kuntze) on chronic gastric ulcers. Naunyn-Schmiedeberg's Archives of Pharmacology, 2015. DOI 10.1007/s00210-015-1200-8

DARTORA, N.; SOUZA, L. M.; PAIVA, S. M. M.; **SCOPARO, C. T**.; IACOMINI, M.; GORIN, P. A. J.; RATTMANN, Y. D.; SASSAKI, G. L. Rhamnogalacturonan from *Ilex paraguariensis*: A potential adjuvant in sepsis treatment. Carbohydrate Polymers, v. 92, p. 1776-1782, 2013.

SOUZA, L. M., DARTORA, N., **SCOPARO, C. T**., CIPRIANI, T. R., GORIN, P. A. J., IACOMINI M., SASSAKI, G. L. Comprehensive analysis of maté (*Ilex paraguariensis*) compounds: Development of chemical strategies for matesaponin analysis by mass spectrometry. *Journal of Chromatography A*, v. 1218, p. 7307-7315, 2012.

ANEXO II: ARTIGO PUBLICADO NA FOOD RESEARCH INTERNATIONAL 53 (2013) 780–785.

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Polysaccharides from green and black teas and their protective effect against murine sepsis

Camila T. Scoparo [a], Lauro M. de Souza [a,*], Yanna D. Rattmann [a,b], Nessana Dartora [a], Simone M.M. Paiva [a], Guilherme L. Sassaki [a], Philip A.J. Gorin [a], Marcello Iacomini [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980, Curitiba-PR, Brazil*
[b] *Departamento de Saúde Comunitária, Universidade Federal do Paraná, Rua Padre Camargo, 280, CEP: 80060-240, Alto da Glória, Curitiba-PR, Brazil*

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A B S T R A C T

Polysaccharides from green and black teas were obtained via hot aqueous extraction, and after fractionation, chemical analysis showed that they consisted mainly of a rhamnogalacturonan containing a long sequence of →4)-6-O-Me-α-D-GalpA-(1→, interrupted by α-L-Rhap residues, which were substituted by type II arabinogalactan chains. The polysaccharides were similar, except that black tea contained lesser galacturonic acid residues (35%), when compared with green tea (65%). Starch was present in both teas. The polysaccharides were tested, via oral administration in mice against induced-polymicrobial sepsis, at doses of 30, 50 and 100 mg/kg. Green and black tea polysaccharides were capable of reducing mortality rate by 40% and 25% respectively, compared to sham-controls. The influx of neutrophils was also affected, decreasing its accumulation in lungs and tissue injury. The difference between the green and black tea polysaccharides seems to result from the processing that the leaves have undergone and the different abilities to prevent mice death can be related to the different uronic acid contents.

ANEXO III: TERMO DE LICENÇA DO ARTIGO PUBLICADO NA FOOD RESEARCH INTERNATIONAL 53 (2013) 780–785.

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Title: Polysaccharides from green and black teas and their protective effect against murine sepsis

Author: Camila T. Scoparo,Lauro M. de Souza,Yanna D. Rattmann,Nessana Dartora,Simone M.M. Paiva,Guilherme L. Sassaki,Philip A.J. Gorin,Marcello Iacomini

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Gastroprotective bio-guiding fractionation of hydro-alcoholic extracts from green- and black-teas (*Camellia sinensis*)

Camila T. Scoparo [a], Débora G. Borato [b], Lauro M. Souza [a], Nessana Dartora [a], Luísa M. Silva [b], Daniele Maria-Ferreira [b], Guilherme L. Sassaki [a], Philip A.J. Gorin [a], Cristiane H. Baggio [b,**], Marcello Iacomini [a,*]

[a] *Department of Biochemistry and Molecular Biology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil*
[b] *Department of Pharmacology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil*

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Epigallocatechin gallate
Rutin gastroprotection

ABSTRACT

Hydro-alcoholic extracts from leaves of *Camellia sinensis* (green- and black-tea leaves) were submitted to a fractionation, promoting the compound separation according to their polarity, and analyzed by ultra-high performance liquid chromatography–mass spectrometry. A wide range of compounds could be identified, such as catechins and their gallate (esters) or oxidation derivatives (theaflavins), glycosylated flavonoids and other phenolics, as well as lipids, saponins and alkaloids. Also have been developed, via bio-guided examination, the gastroprotective property of the compounds identified. The samples were assayed using the model of acute gastric lesions induced in rats by ethanol. Hydro-alcoholic extracts of green-tea and black-tea protected the gastric mucosa with $ED_{50} = 3.6$ and 10.2 mg/kg, respectively, with participation of gastric mucus and reduced glutathione (GSH). The ethyl acetate fraction from green-tea and aqueous fraction from black-tea were, respectively, 6 and 10 times more effectiveness than the initial extracts. Moreover, the epigallocatechin gallate (EGCG, 0.204 mg/kg), a main component of ethyl acetate fraction from green tea, reduced the gastric lesion by 56% and restored the mucus levels, however the rutin (0.0133 mg/kg), a flavonoid found in the most active fraction of black-tea, was less significant at the natural concentrations. These results have confirmed that the different compounds present in green- and black-tea hydro-alcoholic extracts and partitioned fractions produce relevant gastroprotection mainly via maintenance of the protective factors, mucus and GSH.

ANEXO V: TERMO DE LICENÇA DO ARTIGO PUBLICADO NA FOOD RESEARCH INTERNATIONAL 64 (2014) 577–586

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Title: Gastroprotective bio-guiding fractionation of hydro-alcoholic extracts from green- and black-teas (Camellia sinensis)

Author: Camila T. Scoparo,Débora G. Borato,Lauro M. Souza,Nessana Dartora,Luísa M. Silva,Daniele Maria-Ferreira,Guilherme L. Sassaki,Philip A.J. Gorin,Cristiane H. Baggio,Marcello Iacomini

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Chemical characterization of heteropolysaccharides from green and black teas (*Camellia sinensis*) and their anti-ulcer effect

Camila T. Scoparo [a], Lauro M. Souza [d], Nessana Dartora [b], Guilherme L. Sassaki [a], Arquimedes P. Santana-Filho [a], Maria Fernanda P. Werner [c], Débora G. Borato [c], Cristiane H. Baggio [c,*], Marcello Iacomini [a,*]

[a] *Department of Biochemistry and Molecular Biology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil*
[b] *Department of Biology, Sector of Biological Sciences, State University of Centro-Oeste, Guarapuava, PR, Brazil*
[c] *Department of Pharmacology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil*
[d] *Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdade Pequeno Príncipe, Curitiba, PR, Brazil*

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ABSTRACT

In order to obtain polysaccharides from green and black teas (*Camellia sinensis*), commercial leaves were submitted to infusion and then to alkaline extraction. The extracts were fractionated by freeze-thawing process, giving insoluble and soluble fractions. Complex arabinogalactan protein from the soluble fractions of both teas (GTPS and BTPS) were determined by methylation analysis and $^{1}H/^{13}C$-HSQC spectroscopy, showing a main chain of (1→3)-β-Gal*p*, substituted at *O*-6 by (1→6)-linked β-Gal*p* with side chains of α-Ara*f* and terminal units of α-Ara*f*, α-Fuc*p* and α-Rha*p*. A highly branched heteroxylan from the insoluble fractions (GTPI and BTPI) showed in methylation analysis and $^{1}H/^{13}C$-HSQC spectroscopy the main chain of (1→4)-β-Xyl*p*, substituted in *O*-3 by α-Ara*f*, β-Gal*p* and α-Glc*p* units. Evaluating their gastroprotective activity, the fractions containing the soluble heteropolysaccharides from green (GTPS) and black teas (BTPS) reduced the gastric lesions induced by ethanol. Furthermore, the fraction of insoluble heteropolysaccharides of green (GTPI) and black (BTPI) teas also protected the gastric mucosa. In addition, the maintenance of gastric mucus and reduced glutathione (GSH) levels was involved in the polysaccharides gastroprotection.

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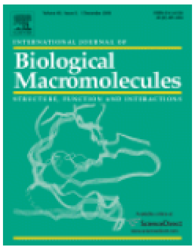

Title: Chemical characterization of heteropolysaccharides from green and black teas (Camellia sinensis) and their anti-ulcer effect

Author: Camila T. Scoparo,Lauro M. Souza,Nessana Dartora,Guilherme L. Sassaki,Arquimedes P. Santana-Filho,Maria Fernanda P. Werner,Débora G. Borato,Cristiane H. Baggio,Marcello Iacomini

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The protective effect of green and black teas (*Camellia sinensis*) and their identified compounds against murine sepsis

Camila T. Scoparo [a], Lauro M. de Souza [a,b], Yanna D. Rattmann [c], Elaine C. Kiatkoski [a], Nessada Dartora [d,*], Marcello Iacomini [a,**]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, 81531-980 Curitiba, PR, Brazil*
[b] *Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdade Pequeno Príncipe, 80250-200 Curitiba, PR, Brazil*
[c] *Departamento de Saúde Comunitária, Universidade Federal do Paraná, 80060-240 Curitiba, PR, Brazil*
[d] *Departamento de Biologia, Universidade Estadual do Centro-Oeste, 85040-080 Guarapuava, PR, Brazil*

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Several compound classes could be identified in the infusion of green tea (GTI) and black tea (BTI) from *Camellia sinensis*, mainly catechins and their gallate derivatives in GTI and their oxidation products, theaflavins and theasinensins, in BTI. We also managed to identify other components, such as proanthocyanidins, flavonoid glycosides, alkaloids, hydroxybenzoic and hydroxycinnamic acids and saponins. The infusions were fractionated and the ethyl acetate fractions [green tea ethyl acetate fraction (GEAF) and black tea ethyl acetate fraction (BEAF)] have concentrated the catechins, theaflavins and caffeine. So, the infusions, fractions and major compounds identified from both teas were assayed against murine sepsis, in order to assess whether they are effectively protecting from this pathology. The infusions at 70 mg/kg resulted in a decrease of sepsis mortality in 43% (GTI) and 40% (BTI), respectively. The ethyl acetate fractions were also efficient, allowing a survival rate of 50% (GEAF) and 66.6% (BEAF) at concentrations of 30 and 70 mg/kg, respectively. The main components from GEAF and BEAF were also assayed; epigallocatechin gallate (10 mg/kg), theaflavins (9 mg/kg) and caffeine (18 mg/kg) showed a survival rate of 50%, 40% and 20%, respectively. This protective effect can be explained, at least in part, by some of these compounds' ability to modulate some important inflammatory parameters, such as neutrophil influx, avoiding its accumulation in lungs, depletion of the pro-inflammatory cytokines (TNF-α and IL-6), increase the anti-inflammatory cytokine (IL-10) and, also, a significant decrease in tissue expression of iNOS and COX-2.

ANEXO IX: TERMO DE LICENÇA DO ARTIGO PUBLICADO NA FOOD RESEARCH INTERNATIONAL 83 (2016) 102-111

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Title: The protective effect of green and black teas (Camellia sinensis) and their identified compounds against murine sepsis

Author: Camila T. Scoparo,Lauro M. de Souza,Yanna D. Rattmann,Elaine C. Kiatkoski,Nessada Dartora,Marcello Iacomini

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Polysaccharides from green and black teas and their protective effect against murine sepsis

Camila T. Scoparo [a], Lauro M. de Souza [a,*], Yanna D. Rattmann [a,b], Nessana Dartora [a], Simone M.M. Paiva [a], Guilherme L. Sassaki [a], Philip A.J. Gorin [a], Marcello Iacomini [a,*]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980, Curitiba-PR, Brazil
[b] Departamento de Saúde Comunitária, Universidade Federal do Paraná, Rua Padre Camargo, 280, CEP: 80060-240, Alto da Glória, Curitiba-PR, Brazil

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ABSTRACT

Polysaccharides from green and black teas were obtained via hot aqueous extraction, and after fractionation, chemical analysis showed that they consisted mainly of a rhamnogalacturonan containing a long sequence of →4)-6-*O*-Me-α-D-Gal*p*A-(1→, interrupted by α-L-Rha*p* residues, which were substituted by type II arabinogalactan chains. The polysaccharides were similar, except that black tea contained lesser galacturonic acid residues (35%), when compared with green tea (65%). Starch was present in both teas. The polysaccharides were tested, via oral administration in mice against induced-polymicrobial sepsis, at doses of 30, 50 and 100 mg/kg. Green and black tea polysaccharides were capable of reducing mortality rate by 40% and 25% respectively, compared to sham-controls. The influx of neutrophils was also affected, decreasing its accumulation in lungs and tissue injury. The difference between the green and black tea polysaccharides seems to result from the processing that the leaves have undergone and the different abilities to prevent mice death can be related to the different uronic acid contents.

1. Introduction

After water, teas from *Camellia sinensis* L. (mainly green and black teas) are considered to be the most consumed beverage worldwide. The main difference among the teas is based on the process of auto-oxidation catalyzed by the enzymes polyphenol oxidase (PPO) and peroxidase. Basically, oxidation is a process to which the leaves of *C. sinensis* interact with oxygen, after being processed (e.g. being minced, crushed or dried) that results in darkening. Those from *C. sinensis* are thus classified as white, green, oolong, black and Pu'erh teas which undergo unfermented, semi-fermented, and fermented processes (Engelhardt, 2010; Ho, Lin, & Shahidi, 2008).

Products of secondary metabolism, especially flavonoids from *C. sinensis*, have been widely studied for their chemical composition and biological effects (Scoparo et al., 2012; Sharangi, 2009), although the polysaccharides have received less attention. However, in infusions, many compound classes are extracted from the leaves, including polysaccharides which are then ingested. It is known that many plants used in folk medicine contain polysaccharides with a recognized variety of properties, including immunological, anti-radiation, anticoagulation, anti-cancer, anti-HIV, hypoglycemic and gastric protection activities (Cipriani et al., 2006; Wang, Wang, Li, & Zhao, 2001; Xie & Nie, 2006; Zhou, Xie, & Fu, 2001).

Recent studies have shown that the structures of polysaccharides are closely related to their biological activities (Cooper, Morré, & Morré, 2005; Harold & Graham, 1992), so elucidation of their structures is fundamental for understanding structure–activity relationships. Some structural studies have been carried out on tea polysaccharides, being described a polysaccharide composed of rhamnose, glucose, galactose, arabinose and xylose (Zhou, Xie, & Nie, 2004). Also, other polysaccharides were identified as neutral (1→4)-β-galactan and pectin-type polysaccharide formed by blocks of (1→4)-α-D-galactopyranosyluronic acids interconnected by (1→2)-linked rhamnose residues (Wang, Wei, & Jin, 2009a, 2009b).

Several pharmacological properties have been attributed to polysaccharides from different sources, although few investigations have correlated their structure with sepsis treatment. Sepsis is a considerable health problem and a leading cause of morbidity and mortality in many intensive care units. It represents a state of overproduction of pro-inflammatory mediators which frequently occurs after various noxious injuries, especially bacterial infection arising from abdominal surgery, appendicitis, perforated ulcers, or an ischemic bowel (Angus, Linde-Zwirble, Lidicker, & Clermont, 2001; Cohen, 2002). We now

Abbreviations: AcOH, acetic acid; Ac_2O, acetic anhydride; CLP, cecal ligation and puncture; EtOH, ethanol; $CHCL_3$, chloroform; HPSEC, high-performance size-exclusion chromatography; GC–MS, gas chromatography–mass spectrometry; MPO, myeloperoxidase; TMSP-d_4, 2,2,3,3-tetradeuterium-3-trimethylilsilylpropionate; GT, green tea; BT, black tea; GSP, green soluble polysaccharide; BSP, black soluble polysaccharide.

* Corresponding authors. Tel.: +55 41 3361 1577; fax: +55 41 3266 2042.
E-mail addresses: lauro.bioquimica@ufpr.br (L.M. de Souza), iacomini@ufpr.br (M. Iacomini).

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evaluate differences in the chemical structure of the polysaccharides isolated from both green and black teas and their protection against sepsis, in a murine model of cecal ligation and puncture (CLP), and their effects on neutrophil migration.

2. Experimental methods

2.1. Plant material

Green and black teas were purchased in a local market (Curitiba, State of Paraná, Brazil), as commercial processed leaves.

2.2. Polysaccharide extraction and fractionation

Green and black teas (50 g of each) were submitted to aqueous extraction (100 °C, 500 mL, ×3). The extracts were combined and evaporated to 250 mL. High molecular weight components, mainly polysaccharides, were precipitated by addition of cold EtOH (3 vol.), and centrifuged (8,000 rpm at 4 °C, 20 min). The sediment was then dissolved in H_2O and dialyzed, in 12,000 Da cutoff membrane, against tap water for 24 h to remove the remaining low-molecular weight compounds, giving rise to crude polysaccharide fractions. These were frozen and then allowed to thaw at room temperature (Gorin & Iacomini, 1984), resulting in green (GSP) and black soluble polysaccharide (BSP) fractions and insoluble fractions which were separated by centrifugation as described above. The insoluble fractions were not analyzed.

2.3. Monosaccharide analysis

GSP and BSP (1 mg) were each hydrolyzed with 0.5 mL of 2 M TFA at 100 °C for 8 h, then the solution was evaporated, and the residue was dissolved in water (1 mL). The resulting monosaccharides were submitted to thin layer chromatography (TLC) on silica gel-60 (Merck), the eluant being ethyl acetate:acetic acid:*n*-propanol:water (4:2:2:1 v/v), followed by development with orcinol-sulfuric acid at 100 °C (Skipski, 1975). The monosaccharides were reduced with 2 mg $NaBH_4$ yielding alditols, which were acetylated in Ac_2O-pyridine (1:1 v/v, 0.5 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were extracted with $CHCl_3$, and analyzed by GC–MS (Varian, Saturn 2000R-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225-MS column (30 m × 0.25 mm × 0.25 μm) programmed from 50 to 220 °C at 40 °C/min, with He as carrier gas. Monosaccharides were identified by their relative retention times and typical electron ionization spectra (EI — 70 eV). The uronic acid contents of the soluble fractions were determined using the colorimetric *m*-hydroxybiphenyl method of Filisetti-Cozzi and Carpita (1991).

GSP and BSP (10 mg) were each submitted to carboxy-reduction through the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving the reduced products (GSP-CR, BSP-CR), and releasing their neutral equivalent monosaccharide.

2.4. Methylation analysis

The fractions GSP, BSP, GSP-CR and BSP-CR (5 mg) were each per-*O*-methylated in DMSO (0.5 mL), powdered NaOH (30 mg) and iodomethane (0.5 mL), adapted from the method of Ciucanu and Kerek (1984). The suspension was neutralized with acetic acid and dialyzed (12,000 Da cutoff) to eliminate reagents. The samples were lyophilized and alkylated polysaccharides were then hydrolyzed with 45% aq. v/v formic acid (1 mL) for 12 h at 100 °C, filtered and evaporated to dryness. The partially *O*-methylated monosaccharides were reduced with $NaBD_4$ and acetylated as described above, giving rise to a mixture of partially *O*-methylated alditol acetates, which were analyzed by GC–MS similar to what was described above, except that the temperature program was 50 to 210 °C in 30 min. Identification of the partially *O*-methylated alditol acetates was based on the *m*/*z* spectra (EI — 70 eV), and by comparison with the standard library (Sassaki, Gorin, Souza, & Czelusniak, 2005), the results being expressed as a relative percentage of each component.

2.5. Nuclear magnetic resonance (NMR) analysis

Mono- (^{13}C, ^{1}H and DEPT-135) and bi-dimensional $^{1}H/^{13}C$ (HSQC) NMR spectra were obtained by employing a Bruker Avance III 400 MHz spectrometer. Samples were dissolved in D_2O at ~40 mg in 400 μL and analysis was carried out at 70 °C. The chemical shifts were expressed in δ (ppm), standardized with TMSP-d_4 (sodium 2,2,3,3-tetradeuterio-3-trimethylsilyl-propionate) δ = 0 for ^{13}C and ^{1}H.

2.6. Animals

Male albino Swiss mice (3 months old, weighing 25–30 g), from the University of Paraná colony, were used for biological tests. They were maintained under standard laboratory conditions, with a constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C), and standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available ad libitum. All experimental procedures were previously approved by the Institutional Ethics Committee of the university (authorization number 430).

2.7. Sepsis induction by cecal ligation and puncture (CLP)

Mice were randomly grouped into five clusters of 10 mice: sham-operation, CLP plus vehicle (water p.o.), and CLP plus GSP or BSP (30, 50 and 100 mg/kg, p.o.). According to the body weight (~30 g), they were administered with 50 μL of each tea polysaccharide solution. Ketamine (80 mg/kg) and xylazine (20 mg/kg) were injected intraperitoneally to anesthetize the mice prior to surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). A midline incision of ~1.5 cm was performed on the abdomen and the cecum was carefully exposed and 50% of the distal portion was ligated. The cecum was then punctured three times with a sterile 16-gauge needle and squeezed to extrude fecal material from the wounds. The cecum was replaced and the abdomen was stitched surgically. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. Each mouse received subcutaneous sterile saline injection (1 mL) for fluid resuscitation after surgery. The mice were then kept on a heating pad (35 °C) until they recovered from the anesthesia. Food and water, ad libitum, were provided throughout the experiment. The survival rate was monitored for 7 days, 12 h each. During this period, vehicle (water) and treatments were orally administered daily.

In another set of experiment, 1 h prior to the surgery, mice were orally treated with vehicle, GSP, or BSP (30 and 50 mg/kg, p.o.). After 6 h post-operation, mice were sacrificed. Their lungs were collected and frozen for further use to determine the myeloperoxidase (MPO) activity.

2.8. Lung MPO activity

MPO activity was measured in order to determine neutrophil influx, according to established protocols (Bradley, Priebat, Christensen, & Rothstein, 1982). Briefly, the lung tissue was homogenized in 0.5 mL of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatants were then assayed at a 1:20 dilution in reaction buffer (9.6 mM 3,3,5,5-tetramethylbenzine, 150 nM H_2O_2 in 50 mM potassium phosphate buffer), and read at 620 nm. Results were expressed as change in optical density per milligram of protein (measured by Bradford assay).

2.9. Statistical analysis

Data were expressed as means ± SEM of, at least, 10 mice examined in each group. Statistical error was determined by one-way ANOVA; the post hoc test was Bonferroni's. Calculations were performed with Graphpad Prism 5.0, and the p values < 0.05 were considered significant.

3. Results and discussion

3.1. Structural characterization of green and black tea polysaccharides

Currently, polysaccharides have received special attention due to their pharmacological activities, so that structural analysis is required for understanding their actions. Thus, like in hot infusions, green and black teas were extracted with water at 100 °C. Their polysaccharides were obtained by precipitation with excess of ethanol, being recovered by centrifugation and dialysis, yielding crude polysaccharide fractions, for the green (12%) and black teas (10%), which were freeze-dried, then fractionated by a freeze–thawing procedure, resulting in cold water-soluble fractions, for green (GSP) and black tea (BSP), at 83% and 64% respectively, and the insoluble fractions being discarded.

The monosaccharide composition of GSP and BSP was obtained by GC–MS analysis and a colorimetric method was employed for the uronic acids, resulting in galacturonic acid (65%), arabinose (19%), galactose (7%), glucose (7%) and rhamnose (2%) for green tea (GSP); and galacturonic acid (35%), arabinose (30%), galactose (16%), rhamnose (3%) and glucose (16%) for black tea (BSP). The galacturonic acid content was confirmed by TLC (data not shown) and carboxy-reduction and further analysis of neutral monosaccharide by GC–MS. The polysaccharide from green tea had a greater concentration of galacturonic acid when compared to black tea which, in contrast, had higher amounts of arabinose, galactose and rhamnose, suggesting that the processing that the leaves undergo in black tea preparation may be interfering with its monosaccharide composition.

3.1.1. NMR analysis

The $^{1}H/^{13}C$ HSQC spectra of GSP and BSP (Fig. 1A and B, respectively) suggest the presence of a similar pectic structure, with units of α-D-galactopyranosyluronic acid, with signals at δ 100.6/4.9 attributed to C-1/H-1 of Gal*p*A units. The signal at δ 79.2/4.4 corresponded to C4/H4 from (1→4)-linked α-D-galacturonic acid residues, and those at δ 71.0/5.0, 69.3/3.94 and 68.4/3.84 to C5/H5, C-3/H-3 and C-2/H2 of Gal*p*A units, respectively (Cipriani, Mellinger, Gorin, & Iacomini, 2004; Ovodova, Golovchenko, Popov, & Popova, 2009; Popov, Ovodova, Golovchenko, & Popova, 2011). The ^{13}C signals at δ 171.4, and δ 171.2 were from C-6, confirming the presence of Gal*p*A residues. The chemical shifts of C-6 from Gal*p*A residues are usually close to δ 176, although many of the carboxyl groups from Gal*p*A are substituted by methyl ester, and these signals would shift to a higher magnetic field. Thus, the signals at δ ~ 171 suggest the occurrence of methyl esterification in Gal*p*A residues. This was confirmed by the HSQC-signals at δ 53.4/3.75, from the methyl ester group (Wang et al., 2009a, 2009b).

Type I rhamnogalacturonan (RGI) is a family of pectic polysaccharides that contain a backbone with repeating disaccharide (1→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p* (Lau, McNeil, Darvill, & Albersheim, 1985), but the rhamnosyl units may also interrupt long runs of polygalacturonic acid (Jarvis, 1984). Side chains of arabinans, galactans, and highly branched arabinogalactans (AGs) with many configurations and sizes can be attached to the *O*-4 of many of the rhamnosyl residues (Carpita & Gibeaut, 1993). The HSQC signal at δ 100.3/5.2 (C1/H1), those at δ 76.1/3.9 (C2/H2) and δ 82.8/4.13 (C4/H4) were attributed to linked α-L-Rha*p* units. HQSC-signals at δ 17.4/1.28 and 17.28/1.24 (Fig. 1A, B upper insertions), confirmed the presence of rhamnose, (1→2)- and (1→2, 1→4)-linked, as expected from a rhamnogalacturonan (Renard, Lahaye, Mutter, Voragen, & Thibault, 1998).

Type II arabinogalactans are usually found in association with rhamnogalacturonans, being classified according to their main chain of (1→3)-β-D-Gal*p*, substituted by (1→6)-β-D-Gal*p* side-chains, differing from type I arabinogalactans which are (1→4)-β-D-Gal*p* linked (Renard et al., 1998). The HSQC spectra of GSP and BSP (Fig. 1A and B) contained signals consistent with (1→3) and (1→6)-linked β-D-Gal*p* units, appearing at δ 104.8/4.55 and 103.4/4.4 (C1/H1), 82.2/4.19 (C3/H3) and δ 68.6/3.68 (C6/H6). Arabinose was found attached to C-3 of the β-D-Gal*p* units, forming (1→3) and (1→5)-linked side chains. The HSQC spectra from both GSP and BSP contained typical signals of (1→3)- and (1→5)-linked α-L-Ara*f* units, appearing at δ 109.6/5.2 and δ 108.1/5.01 (C1/H1), in addition to signals at δ 74.0/3.87 (C3/H3) and δ 63.9/3.61 (C5/H5) of the substituted α-L-Ara*f* (Delgobo, Gorin, Jones, & Iacomini, 1998; Wang et al., 2009a, 2009b).

The presence of starch was also identified for both teas, in the anomeric region (Fig. 1A, B) that signals at δ 100.3/5.27 corresponding to C1/H1 of the α-D-Glc*p* units, and those at δ 75.4/3.64 (C-4/H-4) from the *O*-substituted α-D-Glc*p* units of amylose and amylopectin, and δ 69.7/3.85 (C-6/H-6) from the *O*-substituted α-D-Glc*p* units of amylopectin. Also present was a signal at δ 60.3/3.69 which is consistent with the C6/H6 non-substituted units. Starch is the main storage carbohydrate of higher plants, and after cellulose is the most abundant polysaccharide. It is commonly found in leaves, seeds, fruits, roots, rhizomes, stems and tubers, accumulated in the form of granules in the plant cell cytoplasm (Agrawal, 1992; BeMiller, 2001).

3.1.2. Methylation analysis

Methylation analysis of native and carboxy-reduced GSP and BSP (Table 1) showed that both were similar. For green tea, its GSP contained 2-*O*- (1%) and 2,4-di-*O*-substituted Rha*p* units (0.5%). Also present were nonreducing end-units of Ara*f* (7%), and Gal*p* (3.1%). The galactopyranosyl units were mainly 3-*O*- (5%), 6-*O*- (1.2%) and 3,6-di-*O*-substituted (0.5%). Arabinose appeared as 3-*O*- (3.0%), 5-*O*- (3.5%), and 3,5-di-*O*-substituted (1%) units. Methylation makes the differences between green (GSP) and black (BSP) tea polysaccharides more evident, considering that, although same derivatives were found, their ratios were quite different. In BSP, the Rha*p* units were also 2-*O*-substituted (1.5%) and 2,4-di-*O*-substituted (1%), with greater amounts of nonreducing end-units of Ara*f* (11%) and Gal*p* (7.2%). In the side chains, the Gal*p* units are substituted in *O*-3, *O*-6 or both, thus giving the partially methylated derivatives of 2,4,6-Me_3-Gal (10.6%), 2,3,4-Me_3-Gal (3.0%) and 2,4-Me_2-Gal (1.2%). The partially methylated derivatives from arabinose residues confirmed linkages at 5-*O*- (5.8%), 3-*O*- (6.1%) and 3,5-di-*O*- (1.2%) positions. The percentages of each methyl derivative are presented relative to neutral monosaccharide contents, i.e. 35% in green tea and 65% in black tea.

In order to confirm the polygalacturonic acid chain structure, both GSP and BSP were carboxy-reduced prior to methylation analysis. As expected, this gave an increase in 2,3,6-Me_3-galactitol acetate for GSP (62%) and BSP (33%), indicating that (1→4)-linked galacturonic acid residues were present in the native GSP and BSP (Table 1).

In regard to starch, the presence of 2,3,4,6-Me_4-, 2,3,6-Me_3-, and 2,3-Me_2-glucitol acetate was also found for green tea (GSP), the amount being 3.7%, 3.3%, and 0.3%, and for black tea, 5.9%, 6.0%, and 1.0% respectively corresponding to terminal, (1→4)- and (1→4,→6)-linked α-D-Glc*p*.

Based on methylation and NMR analysis of GSP and BSP, it was possible to infer the presence of a type I rhamnogalacturonan

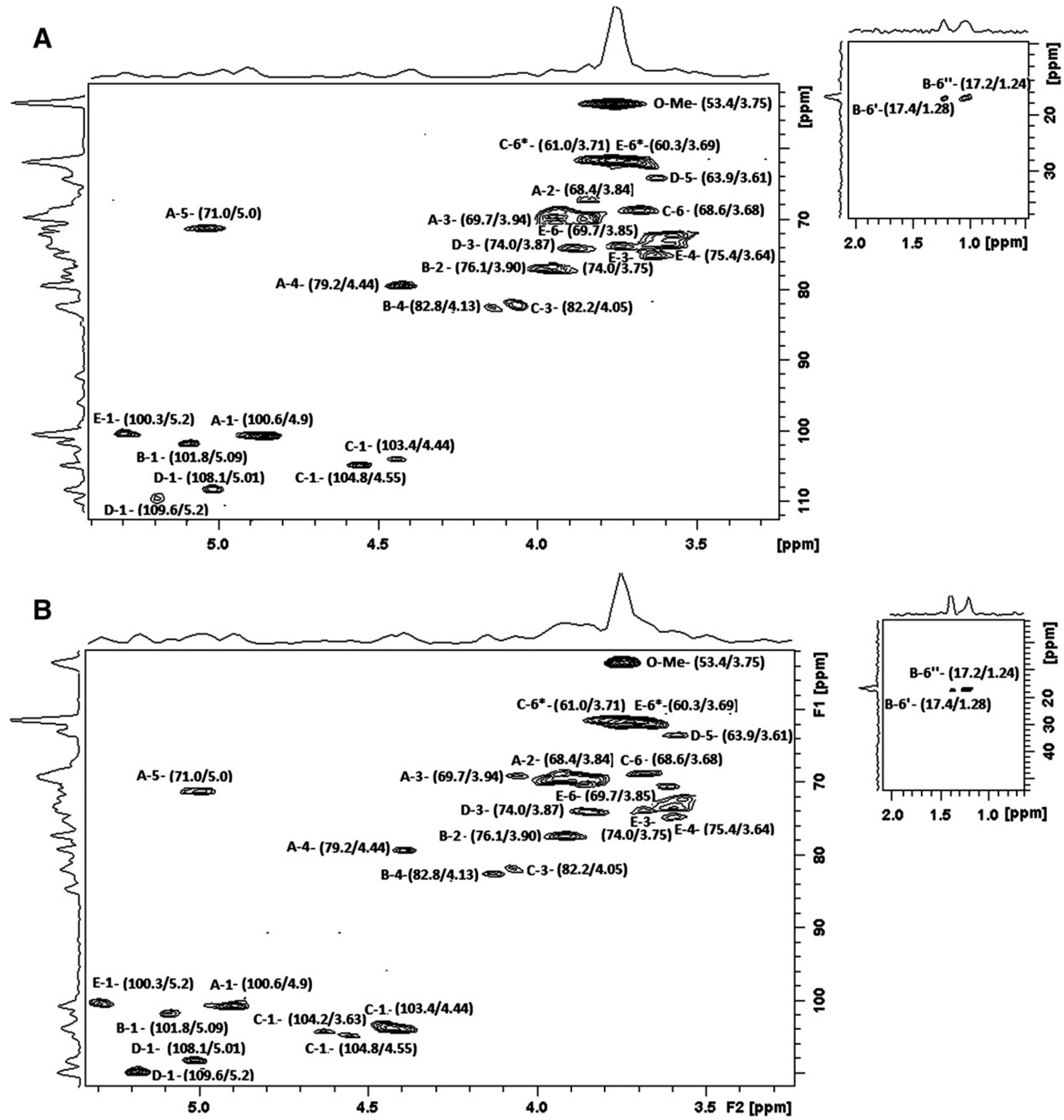

Fig. 1. $^{1}H/^{13}C$ HSQC NMR spectrum of GSP (A) and BSP (B) in D_2O at 70 °C; numerical values are in δ ppm. The inserted letter A indicates chemical shifts from 6-OMe-α-D-Gal*p*A, B from α-L-Rha*p*, C from β-D-Gal*p*, D from α-L-Ara*f* and E from α-D-Glc*p*. The letters are followed by the number of carbons of the monosaccharide unit and their chemical shifts. The C-6 region of the Rha*p* units is also illustrated in the insets.

attached by type II arabinogalactan, but a low amount of starch was also observed in both experiments.

GSP and BSP were similar, although in the BSP polysaccharide there was a considerable decrease in the galacturonic acid content in the rhamnogalacturonan. If this was a result of the processing of the leaves to produce black tea, it is possible that oxidation leads to uronic acid degradation and to a corresponding increase in the neutral monosaccharide ratio.

3.2. Effects of green and black tea polysaccharides on the survival rate of septic mice

It is known that polysaccharides have biological activities, such as antitumor, anti-inflammatory, antiviral and immunomodulatory effects (Paulsen, 2001). Thus, in order to determine the anti-inflammatory effects of polysaccharides from green (GSP) and black (BSP) teas, the samples were tested against the induced-polymicrobial sepsis. The polysaccharides were tested at doses of 30, 50 and 100 mg/kg, and their effects on the survival rate of infected mice and neutrophil influx were evaluated.

The mice treated only with vehicle died between 12 and 24 h after sepsis induction (CLP), with a mortality rate reaching 35% and 65% in 36 and 96 h respectively, exhibiting an area under the curve of 6.056 (arbitrary units) (Fig. 2A and B). The overall mortality in this group, at the end of the observation period (7 days), was 100%. The mortality rate was significantly reduced by treatment with polysaccharides from green tea (GSP) with doses of 30, 50 and 100 mg/kg, with areas under the curve increasing to 7.050, 9.720 and 10.200,

Table 1
Partially *O*-methylated alditol acetates (PMAA) of the GSP, BSP, GSP-CR and BSP-CR polysaccharides.

PMAA	T_r[a]	GSP[b] %	BSP[c] %	GSP-CR[d] %	BSP-CR[e] %	Structure
2,3,5-Me_3-Ara	0.805	7.0	11	7.3	11.5	Ara*f*-(1→
2,5-Me_2-Ara	0.860	3.0	6.1	3.1	6.3	→3)-Ara*f*-(1→
3,4-Me_2-Rha	0.957	1.0	1.5	1.3	1.9	→2)-Rha*p*-(1→
2,3,4,6-Me_4-Glc	1	3.7	5.9	3.9	5.8	Glc*p*-(1→
2,3-Me_2Ara	1.023	3.5	5.8	3.7	6.1	→5)-Ara*f*-(1→
2,3,4,6-Me_4-Gal	1.044	3.1	7.2	3.3	7.5	Gal*p*-(1→
3-Me-Rha	1.192	0.5	1.0	0.7	1.2	→2,4)-Rha*p*-(1→
2-Me-Ara	1.240	1.0	1.2	1.2	1.4	→3,5)-Ara*f*-(1→
2,3,4-Me_3-Glc	1.272	1.0	2.0	1.2	1.9	→6)-Glc*p*-(1→
2,3,6-Me_3-Gal	1.312	0.5	0.9	62	33	→4)-Gal*p*-(1→
2,4,6-Me_3-Gal	1.360	5.0	10.6	5.5	11	→3-Gal*p*-(1→
2,3,6-Me_3Glc	1.366	3.3	6.0	3.5	6.1	→4)-Glc*p*-(1→
2,3,4-Me_3-Gal	1.504	1.2	3.0	1.5	3.2	→6)-Gal*p*-(1→
2,3-Me_2-Glc	1.759	0.3	1.0	0.6	0.9	→4,6)-Glc*p*-(1→
2,3-Me_2-Gal	1.865	0.4	0.6	0.5	0.8	→4,6)-Gal*p*-(1→
2,4-Me_2-Gal	2.026	0.5	1.2	0.7	1.4	→3,6)-Gal*p*-(1→

[a] T_r = retention time relative to 2,3,4,6-Me_4Glc acetate.
[b] Galacturonic acid content of GSP was 65%.
[c] Galacturonic acid content of BSP was 35%.
[d] Carboxy-reduced GSP.
[e] Carboxy-reduced BSP.

respectively. At the end of the observation, the survival of these groups corresponded to 25% at a dose of 30 mg/kg and 45% for both doses of 50 and 100 mg/kg (Fig. 2A).

Similar results, but with less protection against lethality, were found in the groups treated with black tea polysaccharide (BSP) (Fig. 2B). At doses of 30, 50 and 100 mg/kg their areas under the curve increased to 7.200, 8.550 and 7.680, respectively, compared with the control (6.056). At the end of the observation period, the rate of survival corresponded to 20%, 25% and 20%, relative to the doses above. Considering that no deaths were observed in the sham-operated group, with an area under the lethality curve corresponding to 16.800

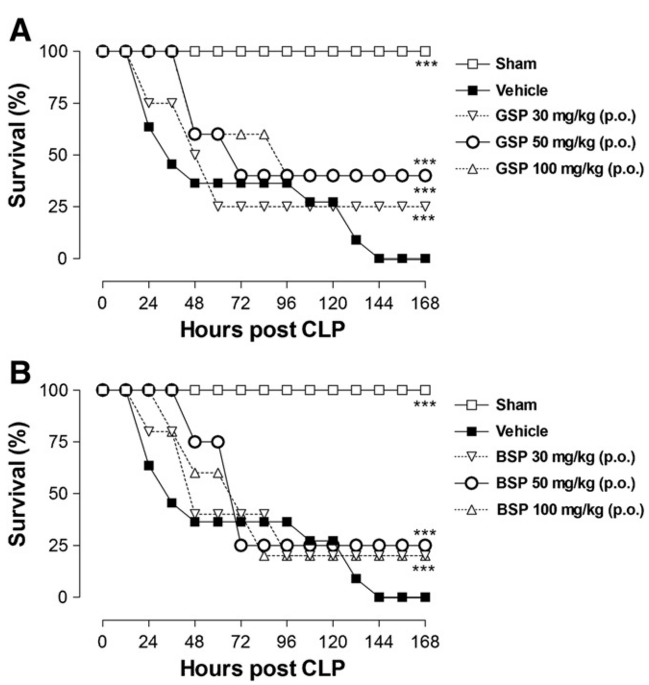

Fig. 2. Effect of polysaccharides GSP (A) and BSP (B) on survival rate in sepsis. GSP and BSP protect against sepsis-induced lethality. Mice (10 animals/group) were orally administered various doses of GSP (30, 50 or 100 mg/kg), vehicle (water) and dexamethasone (0.5 mg/kg s.c). Survival analyses were compared by a log-rank test. These calculations were performed with Graphpad Prism 5.0. The null hypothesis was rejected when $p<0.05$. ***$p<0.001$ versus vehicle.

(arbitrary units), this confirms that surgical intervention, without perforation, was not sufficient to cause death.

3.3. Neutrophil influx is significantly inhibited by GSP and BSP

Sepsis is associated to an aberrant inflammatory response, causing cell injury and organ dysfunction. During sepsis, the generation of inflammatory mediators induced recruitment of a large quantity of immune cells. Neutrophil infiltration is an important pathophysiological alteration associated with the severity of sepsis, which amplifies the systemic inflammatory response and causes multiple organ failure (Landry & Oliver, 2001; Mainous, Ertel, Chaudry, & Deitch, 1995).

When compared with the sham group, CLP surgery markedly increased lung infiltration of neutrophils (50%), indirectly assessed by MPO activity (Fig. 3). It confirms that the neutrophil migration occurs after the experimental sepsis outcome. This increase was significantly prevented by both polysaccharides from green and black teas (GSP and BSP) at a dose of 50 mg/kg, corresponding to 33% and 28% inhibition respectively (Fig. 3). At a dose of 30 mg/kg, there was no significant difference in comparison to the control group (CLP). Dexamethasone, used as an anti-inflammatory control, also inhibited infiltration of neutrophils (45%). GSP and BSP prevented elevation of MPO activity, indirectly indicating reduction in neutrophil recruitment to the lung and oxidative tissue damage, as well. This is of particular relevance because oxidative stress is known as a probable mechanism for organ dysfunction during sepsis, amplifying and perpetuating the initial systemic inflammatory responses (Pastores, Katz, & Kvetan, 1996).

Recently, polysaccharides have demonstrated beneficial effects in sepsis treatment, as reported for an acidic polysaccharide isolated from *Phellinus linteus* which as able to decrease the septic shock induced by high dose lipopolysaccharide injection in mice (Kim et al., 2003). Fucogalactans from the mushrooms *Agaricus bisporus* (RFP-Ab) and *Lactarius rufus* (RFP-Lr) had a potent anti-inflammatory action in a sepsis model (Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2011). Also, a polysaccharide obtained from *Panax ginseng* had demonstrated a potent anti-septicemic activity, via macrophage activation, acting as an immunomodulator against sepsis induced by *Staphylococcus aureus* (Lim et al., 2002).

Based on the results now obtained, the polysaccharide fractions from green and black teas showed a significant protective effect on sepsis, but

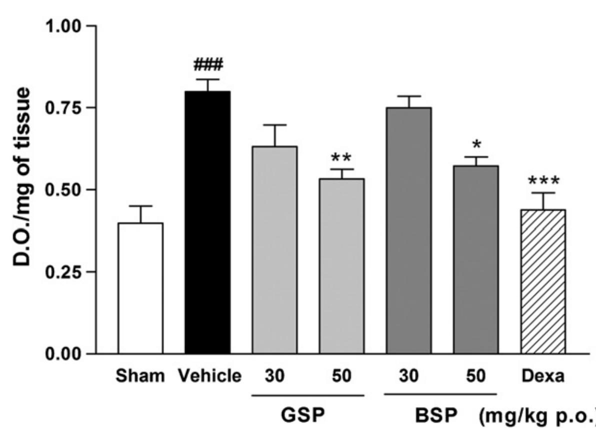

Fig. 3. Effect of polysaccharides on MPO activity in sepsis. Mice subjected to sepsis by CLP were treated with saline, GSP and BSP (30 and 50 mg/kg) or dexamethasone (0.5 mg/kg s.c). Myeloperoxidase activity (MPO, indicator of neutrophil accumulation) was measured spectrophotometrically in lung samples harvested 18 h after induction of CLP. Results are expressed as change in optical density per milligram of protein. Values represent means ± SEM. ###$p<0.001$ versus the sham group; ***$p<0.001$, **$p<0.01$ and *$p<0.05$ indicated values versus the vehicle group. ANOVA followed by Bonferroni's test.

those from green tea were better for this model. Recent studies suggest that the beneficial effects of pectins are closely related to their structural characteristics, for example, pectins isolated from fresh sweet pepper and celery stalks, had high anti-inflammatory activity (Popov et al., 2011; Ovodova et al., 2009). Also, it was suggested that pectins that have high amounts of methyl esters, present the higher positive effects on inflammation (Chien-Ho et al., 2006).

As can be seen from the above results on the characterization of both pectins, green tea shows a greater content of galacturonic acid (65%) than black tea (35%), both with high amounts of esterified units. Accordingly, the green tea polysaccharide fraction showed a greater protection on mortality caused by sepsis than those of the black tea fraction, suggesting that this is related to the difference in uronic content.

4. Conclusion

On the basis of chemical analysis, the polysaccharides from green and black teas obtained by aqueous extraction of tea followed by purification by freezing/thawing were mainly a type I rhamnogalacturonan, formed by a long sequence of →4)-6-OMe-α-D-Gal*p*A-(1→ units, interspersed by some α-L-Rha*p* residues, and substituted by side chains of type II arabinogalactans; the presence of starch was also observed. We also clearly demonstrated that GSP and BSP were able to prevent lethality caused by polymicrobial sepsis in mice at different rates. This beneficial effect seems to be, at least in part, due to a reduction in neutrophil migration, as observed using the MPO assay and consequent protection against tissue damage, The green tea polysaccharide had greater activity than black tea, which may be related to the lower amounts of acid structures in the BSP composition, which may result from the processing in the manufacture of black tea.

Furthermore, it is important to consider that the polysaccharides of *C. sinensis* also have pharmacological properties, as now demonstrated, and that the therapeutic properties usually attributed exclusively to the polyphenol content from green and black teas could be, at least in part, shared with the polysaccharide molecules.

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Gastroprotective bio-guiding fractionation of hydro-alcoholic extracts from green- and black-teas (*Camellia sinensis*)

Camila T. Scoparo [a], Débora G. Borato [b], Lauro M. Souza [a], Nessana Dartora [a], Luísa M. Silva [b], Daniele Maria-Ferreira [b], Guilherme L. Sassaki [a], Philip A.J. Gorin [a], Cristiane H. Baggio [b,**], Marcello Iacomini [a,*]

[a] Department of Biochemistry and Molecular Biology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil
[b] Department of Pharmacology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil

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ABSTRACT

Hydro-alcoholic extracts from leaves of *Camellia sinensis* (green- and black-tea leaves) were submitted to a fractionation, promoting the compound separation according to their polarity, and analyzed by ultra-high performance liquid chromatography–mass spectrometry. A wide range of compounds could be identified, such as catechins and their gallate (esters) or oxidation derivatives (theaflavins), glycosylated flavonoids and other phenolics, as well as lipids, saponins and alkaloids. Also have been developed, via bio-guided examination, the gastroprotective property of the compounds identified. The samples were assayed using the model of acute gastric lesions induced in rats by ethanol. Hydro-alcoholic extracts of green-tea and black-tea protected the gastric mucosa with $ED_{50} = 3.6$ and 10.2 mg/kg, respectively, with participation of gastric mucus and reduced glutathione (GSH). The ethyl acetate fraction from green-tea and aqueous fraction from black-tea were, respectively, 6 and 10 times more effectiveness than the initial extracts. Moreover, the epigallocatechin gallate (EGCG, 0.204 mg/kg), a main component of ethyl acetate fraction from green tea, reduced the gastric lesion by 56% and restored the mucus levels, however the rutin (0.0133 mg/kg), a flavonoid found in the most active fraction of black-tea, was less significant at the natural concentrations. These results have confirmed that the different compounds present in green- and black-tea hydro-alcoholic extracts and partitioned fractions produce relevant gastroprotection mainly via maintenance of the protective factors, mucus and GSH.

1. Introduction

Camellia sinensis (L.) Kuntze (Theaceae) is a species of plant used extensively in infusions, popularly known as “tea”, that are made from their dried fresh (white and green teas), enzymatically oxidized (oolong and black teas) or microorganism fermented (pu-erh tea) leaves (Jiang et al., 2011; Sharangi, 2009; Ho, Lin, & Shahidi, 2008). Currently the products from primary and secondary metabolism of *C. sinensis* are the focus of several chemical investigations. In this field, a comprehensive metabolite profile has been characterized (Del Rio et al., 2004; Lin, Chen, & Harnly, 2008; Miketova et al., 2000; Neilson, Green, Wood, & Ferruzzi, 2006; Scoparo et al., 2012) and many pharmacological activities has also been evaluated, showing potential application for human health (Cooper, Morré, & Morré, 2005a, 2005b; Nie & Xie, 2011; Pinto, 2013; Scoparo et al., 2013; Sharangi, 2009).

Previous studies showed that leaves of *C. sinensis* have several chemical components such as polyphenols (catechins, flavonoids, proanthocyanidins), alkaloids (caffeine, theobromine, theophylline), terpenoids, amino acids, and polysaccharides, among others (Del Rio et al., 2004; Engelhardt, 2010; Xie & Nie, 2006). Despite this large variety of compounds, the polyphenols have been considered the most important for health benefits (Chen et al., 2008; Cooper et al., 2005a, 2005b). The catechins (flavan-3-ol class of flavonoids) are the most abundant tea polyphenols, and green-tea is rich in a variety of these compounds namely catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin-gallate and epigallocatechin-gallate (EGCG). As know, the oxidative process to produce black-tea may lead to a rearrangement of a series of compounds, as well as lead to compound decomposition. Thus, black-tea contain the oxidation/condensation products of catechins, such as theaflavins and

Abbreviations: EtOH, ethanol; BuOH, *n*-butanol; $CHCl_3$, chloroform; LC–MS, liquid chromatography–mass spectrometry; UHPLC, ultra high performance liquid-chromatography; GEt, green-tea hydro-alcoholic extract; BEt, black-tea hydro-alcoholic extract; GCl, green-tea chloroform fraction; GEAc, green-tea ethyl acetate fraction; GBu, green-tea butanolic fraction,; GAq, green-tea aqueous fraction; BCl, black-tea chloroform fraction; BEAc, black-tea ethyl acetate fraction; BBu, black-tea butanolic fraction; BAq, black-tea aqueous fraction.

* Correspondence to: M. Iacomini, Department of Biochemistry and Molecular Biology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil.
** Correspondence to: C.H. Baggio, Department of Pharmacology, Sector of Biological Sciences, Federal University of Parana, P.O. Box 19031, Curitiba, PR 81531-980, Brazil. Tel.: +55 41 3361 1655.
E-mail addresses: crisbaggio@gmail.com (C.H. Baggio), iacomini@ufpr.br (M. Iacomini).

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polymeric thearubigins (Del Rio et al., 2004; Engelhardt, 2010; Lin et al., 2008; Zhou et al., 2005; Zhu et al., 2004).

It is well established that these changes in the chemical composition of different teas may reflect in their biological properties and taking into consideration that the gastrointestinal tract is the first to come into contact with the compounds present in tea, usually in high concentrations, the aim of this study was to determine the compounds or compound class, present in hydro-alcoholic extracts and fractions from liquid/liquid partition from green- and black-tea leaves and, via bio-guided examination, investigate in which kind of compounds the gastroprotective activity remains, using the model of acute gastric lesions induced by ethanol in rats.

2. Materials and methods

2.1. Plant material and chemicals

HPLC-grade methanol, formic acid, ethyl acetate, chloroform and *n*-butanol were purchased from Tedia, water was Milliq (Millipore). The samples (green- and black-teas) were purchased in a local market (Curitiba, Brazil) as commercially processed leaves. Standards of (−)-epigallocatechin-3-*O*-gallate (EGCG) and rutin were purchased from Sigma-Aldrich.

2.2. Extraction

Green- and black-tea leaves (100 g of each) from *C. sinensis* were submitted to extraction by refluxing in 500 ml of a hydro-alcoholic solution (ethanol 70%, v/v) for 1 h, repeated thrice. The solvent was evaporated under reduced pressure and the resulting hydro-alcoholic extracts were named GEt for green-tea and BEt for black-tea, which were stored in freezer.

2.3. Liquid/liquid partition

Green- (GEt) and black-tea (BEt) extracts (10 g) were subjected to liquid/liquid partitioning. The samples were dissolved in H_2O (200 ml) and $CHCl_3$ (200 ml) was added. The mixture was vigorously stirred and allowed to stand until the layers separation. The organic layer was removed to yield the green-tea chloroform fraction (GCl) and black-tea chloroform fraction (BCl). In the remaining aqueous layer, ethyl acetate (200 ml) was added and stirred. Similarly, the organic layer was removed to yield green-tea ethyl acetate fraction (GEAc) and black-tea ethyl acetate fraction (BEAc). Subsequently, *n*-butanol (200 ml) was added to the aqueous layer, stirred and separated to give rise to green-tea butanolic fraction (GBu), black-tea butanolic fraction (BBu), green-tea aqueous fraction (GAq) and black-tea aqueous fraction (BAq). The fractions were dried under reduced pressure lyophilized, stored in freezer (Fig. 1).

2.4. Ultra-high performance liquid chromatography (UHPLC)

An Acquity-UPLC™ system (Waters, Milford, MA) equipped with a binary solvent pump, column oven, auto-sampler and a photodiode array detector (PDA) was used for chromatographic analysis.

The hydro-alcoholic extracts and fractions from green- and black-teas were prepared in MeOH–H_2O (1:1, v/v) at 1 mg/ml. The chromatography was performed on Acquity phenyl BEH column, with 50 × 2.1 mm i.d. and 1.7 μm particle size (Waters). The samples were held at room temperature (22 °C) and the separation has occurred at 60 °C (column temperature) using water (solvent A) and MeOH (solvent B), both containing 0.1% formic acid, with a linear gradient of solvent B from 0 to 50% over 8 min, then to 80% at 10 min and 100% at 11 min, held to 12 min at flow rate of 400 μl/min. After returning to initial conditions (0% B, in 13 min) the system was re-equilibrated for 2 min. The injection volume of the samples was 10 μl and detection was provided by PDA (200–400 nm) and liquid chromatography–mass spectrometry (*m/z* 100–1500).

EGCG and Rutin were quantified on the basis of their calibration curves (R^2 > 0.989) with authentic standards, each prepared at 50, 100, 250, and 500 μg/ml. Detection was at PDA (210–400 nm), then processed at 280 nm (EGCG) and 355 nm (rutin). Quantification was carried out in triplicate.

2.5. LC–MS conditions

The mass spectrometer was directly connected to the UHPLC effluent, and positive (+)/negative (−) ESI-MS were carried out at atmospheric pressure ionization (API) with a LTQ-XL (Thermo-Scientific), using N_2 for sample desolvation with sheath gas at flow rate of 60 arbitrary units (abu) and auxiliary gas at 20 abu, and the capillary temperature at 380 °C. The ionization was following the operational parameters: for positive polarity, electrospray voltage at 3.6 kV capillary voltage 47 V, tube lens offset 200 V; for negative polarity electrospray at 3.5 kV, capillary at −18 V and tube lens at −200 V. Instrument calibration was performed externally prior to each sequence with a calibration solution.

2.6. Animals

Female Wistar rats (180–200 g) provided by the Federal University of Paraná colony were used for experiments. The animals were submitted to standard laboratory conditions, with a cycle of 12 h at light and at dark, at temperature of 22 ± 2 °C and free access to food and water. Over a period of 15 to 18 h prior to the experiment food was withdrawn. All experimental procedures were previously approved by the local Animal Ethics Committee of Federal University of Paraná (CEUA/BIO-UFPR; approval number 689).

2.7. Induction of acute gastric lesion

The acute gastric lesion was induced by Ethanol P.A. (Robert, Nezamis, Lancaster, & Hanchar, 1979). All animals were orally pretreated (gavage) with vehicle (Control: water, 1 ml/kg), omeprazole (40 mg/kg), extracts [GEt (1, 3, 10 and 30 mg/kg) and BEt (3, 10, 30 and 100 mg/kg)], fractions [GCl (1.2 mg/kg), GEAc (0.6 mg/kg), GBu (1 mg/kg), GAq (1 mg/kg), BCl (3.2 mg/kg), BEAc (2 mg/kg), BBu (3.2 mg/kg) and BAq (1 mg/kg)], rutin (0.0133 mg/kg) or epigallocatechin gallate (EGCG, 0.204 mg/kg), 1 h before oral administration of ethanol P.A. (0.5 ml/200 g). After 1 h of ethanol administration the animals were sacrificed, their stomachs were removed to measure the area of lesions (mm^2) by the software Image Tool 3.0® as previously described (Potrich et al., 2010).

2.8. Determination of gastric wall mucus

The glandular gastric mucosa segment was weighed and incubated (2 h) at room temperature in Alcian Blue solution (0.1%). The excess of Alcian Blue was removed by two successively washes with 0.25 M sucrose, the first for 15 min and the second for 45 min. Then, the gastric wall mucus complexed with Alcian Blue dye was extracted with a magnesium chloride solution (0.5 M) and the segments of glandular gastric mucosa were shaken, in intervals of 2 h, for 1 min at 30 min. An equal volume of diethyl ether was added and mixed with the extract, and the solution was centrifuged (3600 rpm) by 10 min in order to separate the aqueous phase for spectrophotometric determination of Alcian Blue amounts at 598 nm. The quantification of mucus levels were performed using standard curve of Alcian Blue (6.25–100 μg) and the result was expressed in μg of Alcian Blue/g of tissue (Corne, Morrissey, & Woods, 1974).

2.9. Determination of glutathione content

The glutathione levels in gastric mucosa were determined following Sedlak & Lindsay's (1968) method. In this experiment, the glandular segment of gastric mucosa were weighed and homogenized, on ice bath, with potassium phosphate buffer (200 mM) (pH 6.5) using a homogenizer. Trichloroacetic acid (12.5%) was mixed with the homogenates and the suspension was vigorously shaken and centrifuged (15 min, 3000 rpm). A buffer Tris–HCl (0.4 M) (pH 8.9) and 0.01 M DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] were mixed with the supernatant aliquots in 96-well plates. The absorbance was measured with a microplate reader by spectrophotometry (415 nm). The individual values interpolated into a standard curve of GSH (0.375–3 μg) and results was expressed as μg of GSH/g of tissue.

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) with 6–8 animals examined in each group. Statistical error was determined by one-way ANOVA, the post hoc test was Bonferroni's. Differences in *P*-values < 0.05 were considered significant. The ED_{50} values (effective dose capable of inhibiting the gastric lesions formation by 50% relative to the control value) were determined by nonlinear regression analysis and reported as geometric mean. Calculations were performed with Graph-pad software (GraphPad software, San Diego, CA, USA).

3. Results and discussion

3.1. Analysis of green- and black-tea hydro-alcoholic extracts and fractions

It is well known that growth/harvest conditions, as well as leaf processing could change the components in many tea preparations. Here, we focused to analyze the most popular types of the manufacturing process of *C. sinensis*: non-oxidized (green-tea) and oxidized (black-tea). This plant is known to have a great variety of metabolites and can be considered as a model for the development of analytical procedures for phytochemical mixtures, which can be observed in several publications (Del Rio et al., 2004; Engelhardt, 2010; Ho et al., 2008; Neilson et al., 2006; Scoparo et al., 2012; Zhu et al., 2004). So, in a previous study, we performed a comprehensive analysis of the compounds present in green- and black-teas from *C. sinensis* from the development of a two-dimensional chromatographic technique (2D-LC).

Likewise, in the present study a wide variety of compounds were identified, ranging from primary metabolites, such as lipids and carbohydrates, to the products of secondary metabolism, as phenolic compounds, alkaloids and saponins from green and black-tea hydro-alcoholic extracts (Fig. 2A and F, respectively). The choice for using hydro-alcoholic extracts was due to the fact that, although the compounds identified in aqueous and hydro-alcoholic extracts were very similar (data not shown), in the aqueous extract a considerable amount of high molecular weight compounds such as polysaccharides, are extracted, which are out of the scope of the present work, that aims to study mainly the low molecular weight compounds, such as flavonoids, (See Fig. 1.)

It can be seen that many peak overlapping have occurred, making their analysis difficult, thus, in order to reduce the complexity, the hydro-alcoholic extracts (GEt and BEt) were submitted to liquid/liquid partition in order to separate the components accordingly to their polarity. The fractionation was bio-monitored via gastroprotective activity.

The sequence of fractionation is depicted in a flowchart with the yields of each fractionation step (Fig. 1), and the fractions were analyzed by UHPLC–MS. The chloroform fraction from both teas (GCl and BCl) retained mainly theobromine (peak 7), caffeine (peak 22) and phospho- and glycolipids (peaks 90–103) [Fig. 2B and G, Table 1].

In the ethyl acetate fraction from green-tea (GEAc), the catechins were identified, as gallocatechin (peak 9), epigallocatechin (peak 10), catechin (peak 17), gallocatechin-3-*O*-gallate (peak 23), gallocatechin dimer (26), catechin-gallate (peak 32) and the epigallocatechin-3-*O*-gallate (peak 20), which is the major compound related in the green-tea extracts (Fig. 2C, Table 1). On the other hand, in the ethyl acetate fraction from black-tea (BEAc), it was observed a considerable decrease in flavan-3-ol content in comparison to green-tea (GEAc), as the absence of peaks corresponding to catechins (peaks 9, 17 and 23), as well as significantly reduce of the peak identified as EGCG (peak 20) in black-tea chromatograms. Such reduction of the compounds observed is resulting from oxidation promoted by enzyme activation (i.e., polyphenoloxidase), because the catechins are structurally modified to give theaflavins, theasinensins (bisflavonols) and epiteaflavic acids, so, in this fraction it was found four predominant theaflavins: simple theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-digallate (peaks 55, 57, 59 and 60).

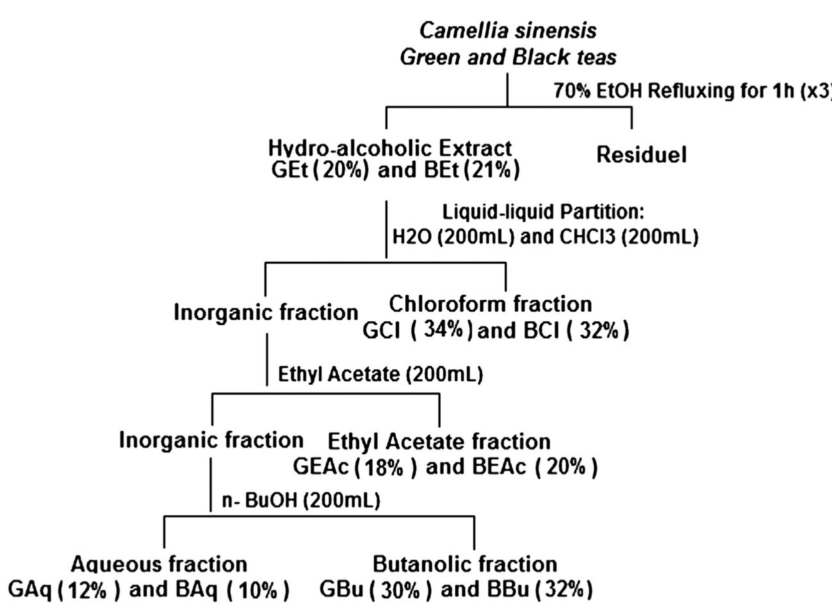

Fig. 1. Scheme of extraction and purification of green- and black-teas from *Camellia sinensis* leaves and their respective yields.

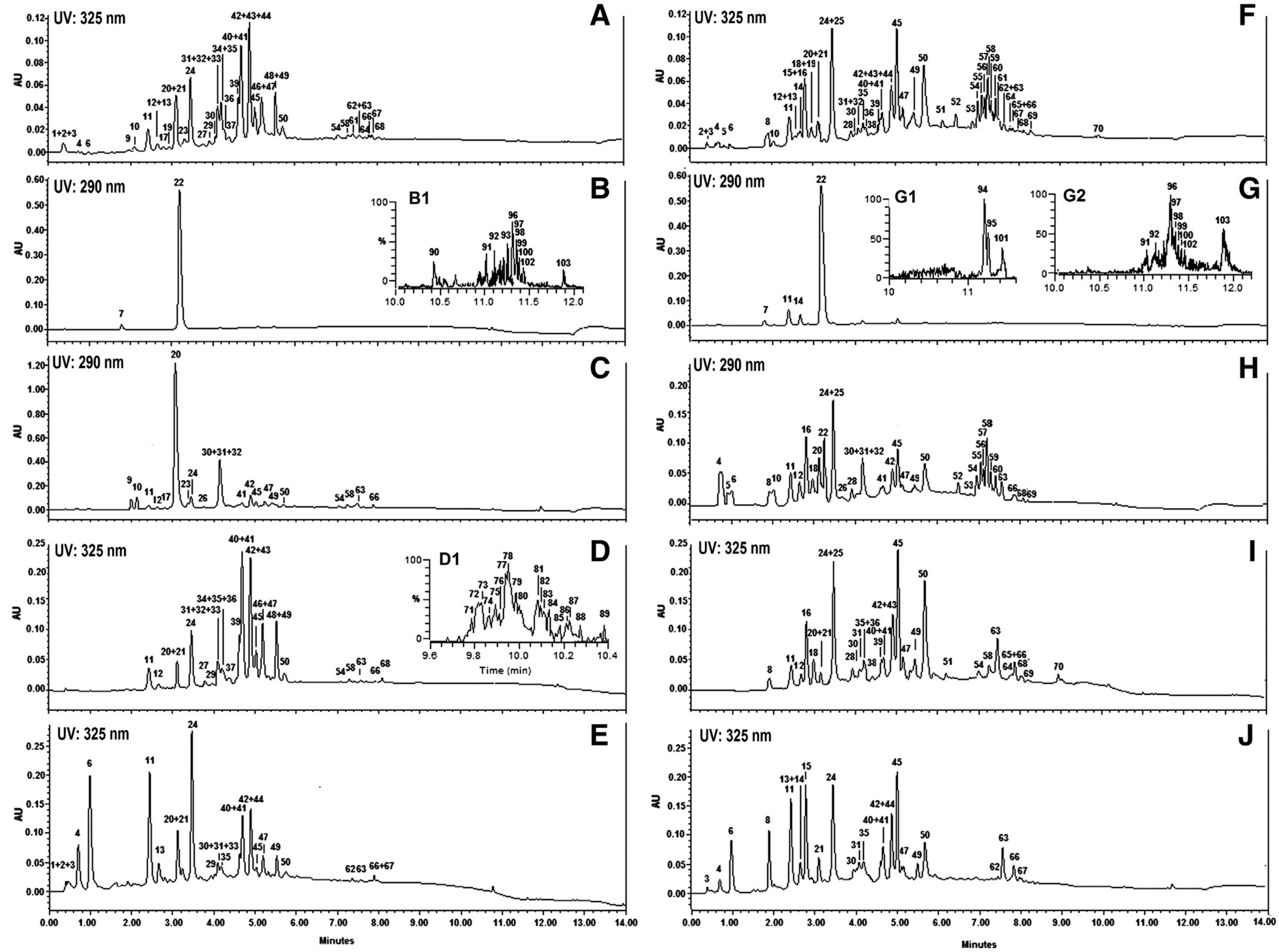

Fig. 2. UHPLC chromatogram from green- and black-tea hydro-alcoholic extract and fractions. (A) Green-tea hydro-alcoholic extract, (B) green-tea chloroform fraction. (B1) Positive ESI-MS of lipids. (C) Green-tea ethyl acetate fraction, (D) green-tea butanolic fraction. (D1 and D2) Negative ESI-MS of extracted ions from saponins. (E) Green-tea aqueous fraction, (F) black-tea hydro-alcoholic extract, (G) black-tea chloroform fraction. (G1 and G2) Positive and negative ESI-MS of lipids, respectively. (H) Black-tea ethyl acetate fraction, (I) black-tea butanolic fraction, (J) black-tea aqueous fraction.

Furthermore, it is known that the accumulation of epigallocatechin quinone dimers, during tea oxidation, results in the formation of theasinensins (bisflavonols), so these products were found in the BEAc fraction, since only black tea is subjected to oxidative process, and identified as theasinensin C, theasinensin B, theasinensin A and theasinensin D (peaks 5, 8, 16 and 18 respectively). Also, proanthocyanidin dimer (peak 12), proanthocyanidin trimer (peak 25) and theaflavate A and B (peaks 53 and 56) were found. Flavonoids glycosides were observed in these fractions (GEAc and BEAc), composed of quercetin or kaempferol attached to different oligosaccharides (peaks 30, 31, 41, 42, 45, 47 and 49) and those acylated with a *p*-coumaroyl group (peaks 50, 54, 58, 63, 66) (Fig. 2C and H, Table 1).

The flavonoid glycosides were concentrated in the butanolic fraction obtained from green- (GBu) and black-teas (BBu) (Fig. 2D and I, Table 1). In these fractions, it were found quercetin-3-*O*-hexoside (peak 21), quercetin-3-*O*-rhamnosylglucoside (rutin) (peak 45), kaempferol-3-*O*-*p*-coumaroylglucoside (peak 50), and the isomers: apigenin-6-*C*-glucosyl-8-*C*-araboniside and apigenin-6-*C*-arabinosyl-8-*C*-glucoside (peaks 39 and 40); quercetin-3-*O*-galactosylrutinoside and quercetin-3-*O*-glucosylrutinoside (peaks 41 and 42); and kaempferol-3-*O*-galactosylrutinoside and kaempferol-3-*O*-glucosylrutinoside (peaks 47 and 49). Other conjugates of quercetin, kaempferol, myricetin and apigenin were also found (peaks 31, 35, 36, 43, 54, 56, 58, 63, 65 and 66, Table 1). However, also many peak overlapping have occurred. Another class of glycosides, the saponins, was present in a considerable proportion in fraction GBu (peaks 71–89) and in contrast, in the BBu fraction no saponins were found (Fig. 2I, Table 1).

The aqueous fraction from green- (GAq) and black- (BAq) teas (Fig. 2E and J, respectively) retained sucrose (peak 1), some acidic compounds, as quinic acid (peak 3), and its ester 3-, and 5-galloylquinic acid (peaks 4 and 6), and 3- and 5-*p*-coumaroyl acid (peaks 11 and 24). In addition, 3-, and 5-caffeoylquinic acid (peaks 14 and 15) and quercetin-3-*O*-rhamnosylglucoside (rutin) (peak 45) appeared in significant amount in this fraction from black-tea (BAq) and it was also observed that this fraction, EGCG was absent (Fig. 2J, Table 1). Flavonoid glycosides were also found in the aqueous fraction from both teas (peaks 40, 41, 42, 45, 47 and 49) as well some flavonoids glycosides acylated with *p*-coumaroyl group (peaks 50, 62, 63, 66 and 67).

3.2. Quantification of EGCG and rutin

EGCG and rutin are important compounds found with a significant proportion in the main active fraction in green- and black-teas, respectively. Thus, they were quantified in the extracts and fractions. EGCG

Table 1
UHPLC–MS of the compounds from hydro-alcoholic extracts and fractions partitioned from green- and black- tea and their presence in each tea type.

Peak	R_t*	[M + Li]+ (m/z)	[M–H]− (m/z)	Structure**	Green tea	Black tea
1	0.37	349	341	Saccharose	x	x
2	0.40		533	Unknown	x	x
3	0.42		191	Quinic acid	x	x
4	0.71	351	343	3-Galloylquinic acid	x	x
5	0.89	617	609	Theasinensin C		x
6	0.98	351	343	5-Galloylquinic acid	x	x
7	1.82	187		Teobromine	x	x
8	1.98	769	761	Theasinensin B		x
9	2.03		305	(+)-Gallocatechin	x	
10	2.10		305	(−)-Gallocatechin	x	x
11	2.43	345	337	3-*p*-coumaroylquinic acid	x	x
12	2.65	345	577	Proanthocyanidin dimer	x	x
13	2.66		633	Strictinin	x	x
14	2.68		353	3-Caffeoylquinic acid		x
15	2.78		353	4-Caffeoylquinic acid		x
16	2.80	921	913	Theasinensin A		x
17	2.81		289	(+)-Catechin	x	
18	2.94	921	913	Theasinensin D		x
19	2.96		577	Proanthocyanidin dimer	x	x
20	3.11	465	457	(−)-Gallocatechin-3-*O*-gallate	x	x
21	3.12	471	463	Quercetin-3-*O*-hexoside	x	x
22	3.24	202		Caffeine	x	x
23	3.35	465	457	(+)-Gallocatechin-3-*O*-gallate	x	
24	3.45	345	337	5-*p*-coumaroylquinic acid	x	x
25	3.47	893	885	Proanthocyanidin trimer		x
26	3.66		609	Gallocatechin dimer	x	x
27	3.78	639	631	Myricetin-galloyl-hexoside	x	
28	3.92		635	Trigalloylglucose		x
29	3.93	795	787	Myricetin-3-*O*-hexosylrutinoside	x	x
30	4.10	601	593	Kaempferol 3-*O*-rutinoside	x	x
31	4.14	487	479	Myricetin-3-*O*-galactoside	x	x
32	4.15	449	441	(+)-(−)-Catechingallate	x	x
33	4.15	795	787	Myricetin-3-*O*-hexosylrutinoside	x	
34	4.16	455	447	Kaempferol-3-*O*-hexoside	x	
35	4.19	487	479	Myricetin-3-*O*-glucoside	x	x
36	4.26	639	631	Myricetin-galloyl-hexoside	x	x
37	4.40		431	Apigenin-C-glucoside	x	
38	4.41		609	(−)-Gallocatechin-3–3′-di-*O*-gallate		x
39	4.62		563	Apigenin 6-*C* glucosyl-8-*C*-arabinoside	x	x
40	4.67		563	Apigenin 6-*C* garabinosyl-8-*C*-glucoside	x	x
41	4.68	779	771	Quercetin-3-*O*-galactosylrutinoside	x	x
42	4.87	779	771	Quercetin-3-*O*-glucosylrutinoside	x	x
43	4.88	623	615	Quercetin-galloyl-hexoside	x	x
44	4.89	439	431	Kaempferol-3-*O*-rhamnoside	x	x
45	5.02	617	609	Quercetin-3-*O*-rhamnosylglucoside	x	x
46	5.17	607	599	Kaempferol-galloyl-hexoside	x	
47	5.19	763	755	Kaempferol-3-*O*-galactosylrutinoside	x	x
48	5.50	607	599	Kaempferol-galloyl-hexoside	x	
49	5.51	763	755	Kaempferol-3-*O*-glucosylrutinoside	x	x
50	5.69	601	593	Kaempferol-3-*O*-*p*-coumaroylglucoside	x	x
51	6.20	789	781	Kaempferol-3-*O*-acetyldirhamnosylhexoside		x
52	6.50		551	Epitheaflagalline-3-gallate		x
53	6.95		699	Theaflavate B		x
54	7.01	925	917	Kaempferol-3-*O*-*p*-coumaroylglucosylrhamnosylgalactoside	x	x
55	7.08	571	563	Theaflavin		x
56	7.13		851	Theaflavate A		x
57	7.22		715	Theaflavin-3-gallate		x
58	7.25	925	917	Kaempferol-3-*O*-*p*-coumaroylglucosylrhamnosylglucoside	x	x
59	7.29		715	Theaflavin-3′-gallate		x
60	7.39	875	867	Theaflavin-3–3′-digalate		x
61	7.41	601	593	Caempferol-3-*O*-6″-*p* coumaroilglucosídeo	x	x
62	7.50	909	901	Quercetin 3-*O*-*p*-coumaroyldirhamnosylhexoside	x	x
63	7.54	1057	1049	Querceetin-3-*O*-glucosylrhamnosyl-(*p*-coumaroylhexosyl)galactoside	x	x
64	7.76	1041	1033	Kaempferol-3-*O*-*p*-coumaroylarabinosylglucosylrhamnosylgalactoside		x
65	7.81	893	885	Kaempferol-3-*O*-*p*-coumaroyldirhamnosylhexoside		x
66	7.84	1057	1049	Quercetin-3-*O*nglucosylrhamnosyl-(*p*-coumaroyl-hexosyl)glucoside	x	x
67	7.90	909	901	Quercetin 3-*O*-*p*-coumaroyldirhamnosylhexoside	x	x
68	8.08	1041	1033	Kaempferol-3-*O*-*p*-coumaroylarabinosylglucosylrhamnosylglucoside		x
69	8.20	893	885	Kaempferol-3-*O*-*p*-coumaroyldirhamnosylhexoside		x
70	8.94	747	739	Kaempferol-3-*O*-*p*-coumaroylglucosylrhamnoside		x
71	9.78		1215	Floratheasaponin A isomer	x	
72	9.82		1257	Foliatheasaponin I isomer	x	
73	9.83		1215	Floratheasaponin A isomer	x	

(continued on next page)

Table 1 *(continued)*

Peak	R_t*	[M + Li]+ (m/z)	[M–H]− (m/z)	Structure**	Green tea	Black tea
74	9.86		1215	Floratheasaponin A isomer	x	
75	9.89		1303	Isotheasaponin B3 isomer	x	
76	9.90		1257	Foliatheasaponin I isomer	x	
77	9.94		1257	Foliatheasaponin I isomer	x	
78	9.95		1303	Isotheasaponin B3 isomer	x	
79	9.99		1263	Isotheasaponin B1/B2 isomer	x	
80	10.00		1305	Foliatheasaponin IV isomer	x	
81	10.07		1319	Assamsaponin J isomer	x	
82	10.08		1303	Isotheasaponin B3 isomer	x	
83	10.08		1263	Isotheasaponin B1/B2 isomer	x	
84	10.08		1305	Foliatheasaponin IV isomer	x	
85	10.18		1319	Assamsaponin J isomer	x	
86	10.20		1305	Foliatheasaponin IV isomer	x	
87	10.23		1263	Isotheasaponin B1/B2 isomer	x	
88	10.25		1319	Assamsaponin J isomer	x	
89	10.39		1303	Isotheasaponin B3 isomer	x	
90	10.40	779		Unknown Lipid	x	x
91	11.05	813		Monogalactosyldiacylglycerol (C20:2/C18:2)		x
92	11.13	797		Phosphatidylcholine (C18:3/C19)	x	x
93	11.26	813		Monogalactosyldiacylglycerol (C20:1/C18:3)	x	x
94	11.26		819	Sulfoquinovosyldiacylglycerol (C18:1/C16)		x
95	11.28	749		Phosphatidylglycerol (C18/C16)		x
96	11.30	944		Digalactosyldiacylglycerol (C18:3/C18:3)	x	x
97	11.32	922		Digalactosyldiacylglycerol (C18:3/C16)	x	x
98	11.35	782		Phosphatidylcholine (C18:2/C18:2)	x	x
99	11.36	781		Monogalactosyldiacylglycerol (C18:3/C18:3)	x	x
100	11.38	787		Phosphatidylcholine (C18:2/C18)	x	x
101	11.41		837	Sulfoquinovosyldiacylglycerol (C18:3/C18:3)		x
102	11.41	789		Phosphatidylcholine (C18:1/C18)	x	x
103	11.90	871		Unknown lipid	x	x

* R_t: retention time.
** For references see Scoparo et al. (2012) and Lin et al. (2008).

was found at higher amounts in green-tea (GEt) with 180.98 ± 0.97 μg/mg. The liquid/liquid partition has concentrated the EGCG in the GEAc increasing to 340.55 ± 0.42 μg/mg, whereas GBu had 27.88 ± 0.29 μg/mg and GAq 5.46 ± 0.47 μg/mg. As a result of auto-oxidation, the amounts of EGCG were significantly reduced in the extract and fractions from black-tea, that showed 20.11 ± 0.74 in BEt, 77.89 ± 0.70 μg/mg in BEAc and 16.35 ± 0.55 μg/mg in BBu, while in BAq EGCG was not detected (Table 2).

The rutin was one of the major compounds of black-tea, having 10.74 ± 0.58 μg/mg in BEt, 13.06 ± 0.36 μg/mg in BEAc, 13.35 ± 0.61 in BAq and 29.58 ± 0.57 μg/mg in BBu. Nevertheless, in green-tea, this metabolite appeared in smaller quantities, 0.78 ± 0.21 μg/mg in GEAc, 2.18 ± 0.43 μg/mg in GAq and 5.28 ± 0.53 μg/mg in GBu (Table 2).

The flavonoids glycosides can change during manufacturing process of tea and the quantification of these compounds shows that their proportion in black-teas can be higher than that of catechins (Engelhardt, Finger, Herzig, & Kuhr, 1992).

3.3. Effect of green- and black-tea hydro-alcoholic extracts on acute gastric lesions induced by ethanol

The gastroprotective activity of several natural products is well known, and has been discussed in previous studies, such as from phenolic compounds (Baggio et al., 2007; Im, Nam, Park, & Sohn, 2013) and polysaccharides from *Maytenus ilicifolia* and *Ilex paraguariensis* (Cipriani et al., 2006; Maria-Ferreira et al., 2013). Teas from *C. sinensis* are highly consumed worldwide because of their pleasing taste and, moreover, due to their digestive benefit, causing more people to choose this plant (Sharangi, 2009). Also, green- and black-teas have presented anti-ulcer effect (Adhikary, Yadav, Bandyopadhyay, & Chattopadhyay, 2011; Hamaishi, Kojima, & Ito, 2006; Koo & Cho, 2004; Morikawa et al., 2006; Maity, Vedasiromoni, & Ganguly, 1995). Because the major differences in the composition of teas are related to post-harvest processes (oxidation), in order to investigate whether the processing have also altered the gastroprotective activity, hydro-alcoholic extracts from green- and black-teas (GEt and BEt) were examined in an experimental model of gastric lesion induced by ethanol.

With the model of ethanol induced gastric lesions, it is observed a reduction of the protective factors of the mucosa such as the mucus barrier, secretion of bicarbonate and non-proteic sulphydrilic groups (NP-SH). This is due to penetration of this necrotizing agent in the mucous during the procedure causing membrane damage, erosion and ulcer formation (Szabo, Trier, Brown, & Schnoor, 1985). The hydro-alcoholic extracts were orally administered in the animals and this pretreatment inhibited the gastric lesions in a dose-dependent manner. GEt reduced lesions by 46%, 65% and 87% at doses of 3, 10 and 30 mg/kg, with ED_{50} value of 3.6 mg/kg and BEt reduced the lesions by 51 and 73% at doses of 10 and 30 mg/kg, with ED_{50} value of 10.2 mg/kg (Fig. 3A and B, respectively). These ED50 values showed that GEt was 2.8 times more

Table 2
Concentration of major phenolics in green- and black- tea hydro-alcoholic extracts and partitioned fractions.

Extract	Epigallocatechin-3-*O*-gallate[a]	Rutin[a]
GEt	180.98 ± 0.97	5.98 ± 0.78
GCl	0 ± 0	0 ± 0
GEAc	340.55 ± 0.42	0.78 ± 0.21
GBu	27.88 ± 0.29	5.28 ± 0.53
GAq	5.46 ± 0.47	2.18 ± 0.43
BEt	20.11 ± 0.74	10.74 ± 0.58
BCl	0 ± 0	0 ± 0
BEAc	77.89 ± 0.70	13.06 ± 0.36
BBu	16.35 ± 0.55	29.58 ± 0.57
BAq	0 ± 0	13.35 ± 0.61

GEt, green tea hydro-alcoholic extract; GCl, green tea chloroform fraction; GEAc, green tea ethyl acetate fraction; GBu, green tea butanolic fraction, GAq, green tea aqueous fraction; BEt, black tea hydro-alcoholic extract; BCl, black tea chloroform fraction; BEAc, black tea ethyl acetate fraction; BBu, black tea butanolic fraction, BAq, black tea aqueous fraction.
[a] The yields are presented as μg/mg.

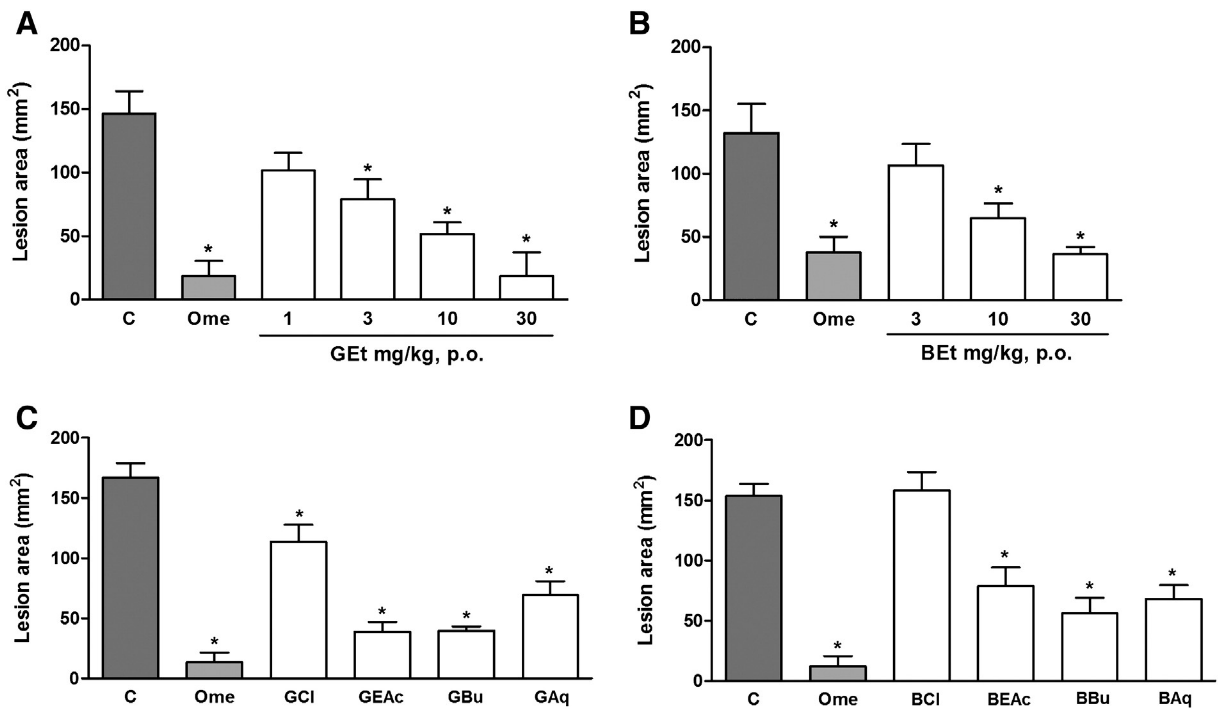

Fig. 3. Effect of (A) green- and (B) black-tea hydro-alcoholic extracts and (C) green- and (D) black-tea hydro-alcoholic fractions on acute gastric lesions induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 ml/kg), omeprazole (Ome: 40 mg/kg), GEt (1, 3, 10 and 30 mg/kg), BEt (3, 10 and 30 mg/kg) GCl (1.2 mg/kg), GEAc (0.6 mg/kg), GBu (1 mg/kg) and GAq (1 mg/kg) or BCl (3.2 mg/kg), BEAc (2 mg/kg), BBu (3.2 mg/kg) and BAq (1 mg/kg), 60 min before oral administration of ethanol P.A. (0.5 ml/200 g). Results are expressed as mean ± S.E.M. ($n = 6$–8) and statistical comparison was performed using one-way ANOVA followed by post hoc Bonferroni's test. *$P < 0.05$ compared to the control group.

efficient than BEt. Omeprazole, used for positive control at 40 mg/kg (p.o.), reduced the gastric lesions up to 87% and 74%, for green- and black-tea experiments, respectively (Fig. 3A and B).

Both extracts (GEt and BEt) are rich in polyphenols which have antioxidant effects (Matsuzaki & Hara, 1985; Tanizawa et al., 1984; Thawonsuwan, et al., 2010). However, the polyphenols content in green-tea varies from 30% to 40% whereas only 3% to 10% in black-tea (Sharangi, 2009). Instead catechins, black-tea presents their oxidative products, such as theaflavins, as well as the products of accumulation of quinone dimmers, the theasinensins (Engelhardt, 2010). This difference in polyphenols and their amounts could be responsible for the lower activity of BEt, suggesting the possible involv
ement of non-oxidized catechins on the gastroprotective effect.

3.4. Effect of fractions from liquid/liquid partition of green- and black-teas on acute gastric lesions induced by ethanol

In order to investigate which compound classes were responsible for its gastroprotective activity, a bioguided liquid/liquid partition was developed on the hydro-alcoholic extracts. The fractions were tested at doses calculated on the basis of the ED_{50} values, obtained from the precursor extracts (GEt and BEt) and their relative fractions yielding.

The chloroform fraction [GCl, 1.2 mg/kg, per os (p.o.)], ethyl acetate fraction (GEAc, 0.6 mg/kg, p.o.), butanolic fraction (GBu, 1 mg/kg, p.o.) and aqueous fraction (GAq, 1 mg/kg, p.o.) from green-tea extract (GEt) inhibited the gastric lesions area by 54, 77, 76 and 58%, respectively, compared to control group (C: 166.9 ± 12.1 mm^2) (Fig. 3C). For the black-tea, the ethyl acetate fraction (BEAc, 2 mg/kg), butanolic fraction (BBu, 3.2 mg/kg) and aqueous fraction (BAq, 1 mg/kg) reduced the ethanol-induced gastric lesions in 49, 63 and 56%, respectively, when compared to control group (C: 153.6 ± 10.0 mm^2) (Fig. 3D). However, the chloroform fraction (BCl, 3.2 mg/kg, p.o.) was not able to protect the gastric mucosa (Fig. 3D). Omeprazole (40 mg/kg, p.o.) inhibited the gastric lesions up to 93% (Fig. 3C and D).

Based on the relative abundances and theoretical ED_{50}, the ethyl acetate fraction from green-tea (GEAc) was 6 times more potent than the precursor extract (GEt) and the aqueous fraction of black-tea (BAq) was 10 times more potent than the precursor extract (BEt). The ethyl acetate fraction of green tea showed a significant amount of catechins, and the main one is the EGCG (Fig. 3C, Tables 1 and 2). It is known that EGCG is efficient in the treatment of stomach ulcer (Adhikary et al., 2011), so this compound must contribute to the gastroprotective effect of GEAc fraction observed. However, in black tea, the amount of EGCG is reduced (Table 2), and in the most active fraction (BAq), absent. Then some other compound may be assisting the gastroprotective properties. On the chemical analysis, rutin (Fig. 3J, Tables 1 and 2) was presented in significant amounts in the aqueous fraction of black tea, and this flavonoid glycoside was also reported to have gastroprotective activity (La Casa et al., 2000).

3.5. Effect of EGCG and rutin on acute gastric lesions induced by ethanol

EGCG and rutin were the major components observed on the more potent fractions (GEAc and BAq, respectively). The authentic standard of EGCG and rutin were prepared accordingly to their relative abundances in their respectively fractions (GEAc and BAq). Thus, EGCG (at 0.204 mg/kg, p.o.) reduced the gastric lesions area by 56%, compared to control group (C: 167.0 ± 20.5 mm^2) (Fig. 4), whereas, rutin (at 0.0133 mg/kg) did not inhibit the formation of lesions in rats. The omeprazole (40 mg/kg, p.o.), positive control, reduced the lesions by 88% (Fig. 4).

Previous studies have demonstrated that EGCG and rutin can protect the gastric mucosa against lesion formation induced by indomethacin and ethanol in rodents (Abdel-Raheem, 2010; Adhikary et al., 2011; La Casa et al., 2000). However, the rutin concentrations tested in that experiments were higher than the theoretical ED_{50} calculated here. So, these findings suggest that EGCG are involved on the gastroprotection of green-tea, but rutin was less significant at the natural concentrations in black-tea. It is probably that other compounds are also involved this protection, perhaps via synergistic effect.

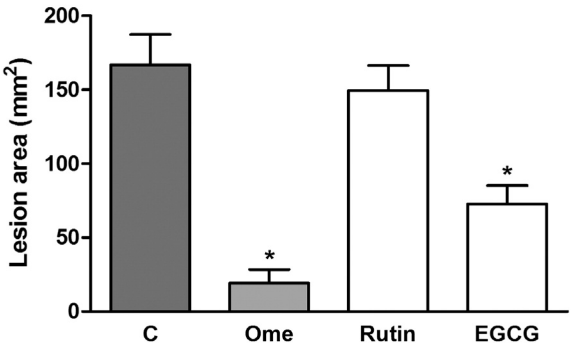

Fig. 4. Effect of EGCG and rutin on acute gastric lesions induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 ml/kg), omeprazole (Ome: 40 mg/kg), epigallocatechin gallate EGCG (0.204 mg/kg) or rutin (0.0133 mg/kg) 60 min before oral administration of ethanol P.A. (0.5 ml/200 g). Results are expressed as mean ± S.E.M. ($n = 6$–8) and statistical comparison was performed using one-way ANOVA followed by post hoc Bonferroni's test. *$P < 0.05$ compared to the control group.

3.5.1. Effect of green and black-tea hydro-alcoholic extracts and fractions on gastric wall mucus

The mucus constitutes the first line of mucosal protection against acid secretion due to formation of a viscous, elastic, adherent and transparent gel (Laine, Takeuchi, & Tarnawski, 2008; Phillipson et al., 2008). In order to investigate if this protective mechanisms of gastric mucosa are involved in the gastroprotection promoted by the hydro-alcoholic extracts, partitioned fractions and standards compounds, the gastric wall mucus was determined.

The administration of ethanol P.A. decreased the levels of gastric mucus up to 60% when compared to non-lesioned group (NL) (Table 3). Oral treatment of animals with GEt, at doses of 3 and 10 mg/kg, restored the mucus content to 600.8 ± 62.9 and 554.5 ± 63.9 μg of Alcian Blue/g of tissue compared to control group (C: 241.0 ± 49.5 μg of Alcian Blue/g of tissue) (Table 3). Omeprazole (40 mg/kg, p.o.) prevented the decrease of gastric mucus levels (469.8 ± 52.7 μg of Alcian Blue/g of tissue) when compared with the control group (Table 3). In other set of experiments, administration of BEt prevented the decrease of mucus levels (657.9 ± 63.8 μg of Alcian Blue/g of tissue) only at dose of 30 mg/kg when compared to control group (C: 336.1 ± 30.2 μg of Alcian Blue/g of tissue) (Table 3).

When testing the fractions from GEt and BEt, the pretreatment of animal with BAq (1 mg/kg, p.o.) was able to prevent the depletion of mucus content (743.7 ± 94.8 μg of Alcian Blue/g of tissue), when compared to control group (C: 347.9 ± 36.3 μg of Alcian Blue/g of tissue) (Table 4). However, the treatment with GCl (1.2 mg/kg), GBu (1 mg/kg) and GAq (1 mg/kg) obtained from green-tea; BCl (3.2 mg/kg), BEAc (2 mg/kg) and BBu (3.2 mg/kg) obtained from black-tea did not alter the amount of gastric mucus (Table 4). Despite the fact that ethyl acetate fraction had been the main active fraction on the reduction of the lesions area, GEAc at 0.6 mg/kg, had no statistically significant difference in the amount of gastric mucus compared to the control group (607.9 ± 25.4 μg of Alcian Blue/g of tissue; C: 430.3 ± 58.8 μg of Alcian Blue/g of tissue) suggesting that the gastroprotection observed in this fraction possibly acts by another cytoprotective mechanism.

Together, the results with green- and black-tea extracts (GEt and BEt) and aqueous fraction from black-tea (BAq) restore the gastric mucus levels to basal levels. GEt and BEt contain several classes of compounds such as phenolic compounds, alkaloids, carbohydrates and lipids. BAq contain mainly quinic acids and their esters, as galloyl and caffeoyl quinic acid, and mainly flavonoids glycosides of quercetin, kaempferol, apigenin and myricetin glycosilated in higher amounts than the equivalent fraction from green-tea (GAq), which may be contributing to increase the protective factor of the gastric mucosa (Alarcón de la Lastra, Martín, & Motilva, 1994; Yan et al., 2011).

3.5.2. Effect of green and black-tea hydro-alcoholic extracts and fractions on GSH levels

Another important cytoprotective mechanisms against gastric lesion formation is the reduced glutathione (GSH), which acts as an antioxidant, providing cellular protection against oxidative damage (Grant, 2001). Similar to gastric mucus, the administration of ethanol P.A. decreased the levels of GSH up to 56% when compared to non-lesioned group (Table 3).

The treatment with GEt, at doses of 10 and 30 mg/kg, restored the GSH levels to 419.8 ± 38.0 and 423.8 ± 58.5 μg/g of tissue, when compared to control group (C: 199.4 ± 20.5 μg/g of tissue) (Table 3). Moreover, BEt in all tested doses (3, 10 and 30 mg/kg) was able to maintain the GSH levels in 570.6 ± 121.8, 585.0 ± 73.1 and 655.3 ± 72.5 μg/g of tissue, respectively, compared to control group (C: 223.1 ± 35.4 μg/g of tissue) (Table 3).

The fractions BBu and BAq restored the GSH levels to 316.2 ± 32.0 and 300.2 ± 18.8 μg/g of tissue, respectively, compared to control

Table 3
Effect of oral administration of green-tea hydro-alcoholic extract (GEt) or black-tea hydro-alcoholic extract (BEt) on mucus and GSH levels in acute gastric lesions induced by ethanol P.A. in rats.

Extract	Treatment	Mucus (μg of Alcian Blue/g of tissue)	GSH (μg/g of tissue)
Green-tea hydro-alcoholic extract	NL	611.3 ± 42.7	333.3 ± 30.1
	Control (1 ml/kg)	241.0 ± 49.5#	199.4 ± 20.5#
	Omeprazole (40 mg/kg)	469.8 ± 52.7*	364.7 ± 30.3*
	GEt (1 mg/kg)	440.4 ± 39.0	276.1 ± 20.1
	GEt (3 mg/kg)	600.8 ± 62.9*	293.7 ± 20.1
	GEt (10 mg/kg)	554.5 ± 63.9*	419.8 ± 38.0*
	GEt (30 mg/kg)	296.9 ± 32.4	423.8 ± 58.5*
Black-tea hydro-alcoholic extract	NL	665.4 ± 83.9	513.8 ± 67.6
	Control (1 ml/kg)	336.1 ± 30.2#	223.1 ± 35.4#
	Omeprazole (40 mg/kg)	492.0 ± 74.0	447.8 ± 69.1
	BEt (3 mg/kg)	376.9 ± 31.6	570.6 ± 121.8*
	BEt (10 mg/kg)	450.0 ± 35.5	585.0 ± 73.1*
	BEt (30 mg/kg)	657.9 ± 63.8*	655.3 ± 72.5*

The results were expressed as mean ± S.E.M. and statistical comparison was performed using ANOVA followed by Bonferroni's test.
# $P < 0.05$ when compared to non-lesionated group (NL).
* $P < 0.05$ when compared with the control group.

Table 4
Effect of oral administration of green-tea hydro-alcoholic fractions or black-tea hydro-alcoholic fractions on mucus and GSH levels in acute gastric lesions induced by ethanol P.A. in rats.

Fractions	Treatment	Mucus (μg of Alcian Blue/g of tissue)	GSH (μg/g of tissue)
Green tea hydro-alcoholic fractions	NL	700.0 ± 62.7	386.3 ± 23.9
	Control (1 ml/kg)	430.3 ± 58.8#	228.5 ± 30.0#
	Omeprazole (40 mg/kg)	676.8 ± 77.2	305.2 ± 39.6
	GCl (1.2 mg/kg)	646.9 ± 35.3	323.1 ± 37.3
	GEAc (0.6 mg/kg)	607.9 ± 25.4	344.4 ± 41.1
	GBu (1 mg/kg)	679.8 ± 92.7	310.7 ± 21.6
	GAq (1 mg/kg)	619.1 ± 52.8	336.7 ± 20.0
Black tea hydro-alcoholic fractions	NL	665.4 ± 83.9	394.3 ± 24.2
	Control (1 ml/kg)	347.9 ± 36.3#	168.3 ± 32.1#
	Omeprazole (40 mg/kg)	540.7 ± 64.4	263.0 ± 27.3
	BCl (3.2 mg/kg)	416.8 ± 42.6	131.3 ± 40.2
	BEAc (2 mg/kg)	435.5 ± 41.4	223.9 ± 29.8
	BBu (3.2 mg/kg)	626.6 ± 80.7	316.2 ± 32.0*
	BAq (1 mg/kg)	743.7 ± 94.8*	300.2 ± 18.8*

The results were expressed as mean ± S.E.M. and statistical comparison was performed using ANOVA followed by Bonferroni's test.
Green- tea hydro-alcoholic fractions: chloroform fraction, GC; ethyl acetate fraction, GEAc; butanolic fraction, GBu; and aqueous fraction, GAq. Black tea hydro-alcoholic fractions: chloroform fraction, BC; ethyl acetate fraction, BEAc; butanolic fraction, BBu; and aqueous fraction BAq.
# $P < 0.05$ when compared to non-lesionated group (NL).
* $P < 0.05$ when compared with the control group.

group (C: 168.3 ± 32.1 μg/g of tissue), whereas, BCl, BEAc, and all fractions from green-tea (GEt) did not significantly restored the GSH levels (Table 4). Flavonoids glycosides and quinic acids were presented in BBu and BAq at larger amounts than in fractions from green-tea, and these compounds were related to have effect on GSH levels (Im et al., 2013). Thus they may be responsible for the gastroprotection observed on active fractions.

3.5.3. Effect of EGCG and rutin on gastric wall mucus and GSH levels

EGCG and rutin were evaluated toward their gastric protection barriers, being observed that only EGCG (at 0.204 mg/kg, p.o.) restored the levels of gastric mucus (847.8 ± 119.0 μg of Alcian Blue/g of tissue) when compared to control group (C: 469.2 ± 85.0 μg of Alcian Blue/g of tissue). However, rutin (at 0.0133 mg/kg, p.o.) did not revert the mucus wall depletion (Table 5). Indeed, Hamaishi et al. (2006) also observed an increase in the rate of gastric mucus in animals treated with catechin, an important component isolated from extract of *C. sinensis*. Despite the fact that EGCG is the major compound of the ethyl acetate fraction from green-tea (GEAc), this fraction was less effective than isolated EGCG for restoring the gastric mucus. This may be related to the presence of other compounds in this fraction, which could interfere in the pharmacokinetics of the active substance or antagonize its effects.

Alterations on GSH levels were also assayed with isolated EGCG and rutin. Both EGCG (at 0.204 mg/kg, p.o.) as rutin (at 0.0133 mg/kg, p.o) did not alter significantly the GSH levels after the induction of gastric lesions (156.5 ± 30.4 and 265.0 ± 41.4, respectively) when compared to the control group (158.9 ± 29.3) (Table 5). Similar results were found by Adhikary et al. (2011), which treated rats with EGCG at 200 mg/kg for 3 days and observed that the gastric mucus and GSH levels were restored in the indomethacin-induced lesions. Moreover, rutin (200 mg/kg) has restored only the GSH amounts in the gastric lesions induced by indomethacin but had not effect on lesions induced by ethanol (Abdel-Raheem, 2010; La Casa et al., 2000).

4. Conclusion

On the basis of chemical analysis, a wide range of compounds could be identified in hydro-alcoholic and fractions from green and black-teas (*C. sinensis*) as catechins and their gallate and oxidation derivatives, glycosylated flavonoids, gallic or hydroxycinnamic acids and also esters of quinic acid and, as well as lipids, saponins and alkaloids. The hydro-alcoholic extracts of green- and black-teas (GEt and BEt) presented gastroprotective action, being GEt 2.8 times more potent than BEt, and the ethyl acetate fraction from green-tea (GEAc) and aqueous fraction from black-tea (BAq) were, respectively, 6 and 10 times more potent than their initial extracts. Moreover, epigallocatechin gallate (EGCG), the main component of GEAc, seems to be responsible for the biological effect of green-tea, but the same did not occurs with rutin, one of the major compounds of BAq. These effects could be associated to maintenance of mucus and GSH levels of gastric mucosa. The difference in composition between both teas results from processing of the leaves during the manufacturing of teas and the different ability to prevent gastric lesion may be related to different compounds present in each analyzed fraction.

Table 5
Effect of oral administration of EGCG and rutin on mucus and GSH levels in acute gastric lesions induced by ethanol P.A. in rats.

Treatment	Mucus (μg of Alcian Blue/g of tissue)	GSH (μg/g of tissue)
NL	915.2 ± 141.2	365.0 ± 21.9
Control (1 ml/kg)	469.2 ± 85.0[#]	158.9 ± 29.3[#]
Omeprazole (40 mg/kg)	915.0 ± 98.2*	353.9 ± 48.7*
EGCG (0.204 mg/kg)	847.8 ± 119.0*	156.5 ± 30.4
Rutin (0.0133 mg/kg)	533.3 ± 29.9	265.0 ± 41.4

The results were expressed as mean ± S.E.M. and statistical comparison was performed using ANOVA followed by Bonferroni's test.
[#] $P < 0.05$ when compared to non-lesionated group (NL).
* $P < 0.05$ when compared with the control group.

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Chemical characterization of heteropolysaccharides from green and black teas (*Camellia sinensis*) and their anti-ulcer effect

Camila T. Scoparo [a], Lauro M. Souza [d], Nessana Dartora [b], Guilherme L. Sassaki [a], Arquimedes P. Santana-Filho [a], Maria Fernanda P. Werner [c], Débora G. Borato [c], Cristiane H. Baggio [c,*], Marcello Iacomini [a,*]

[a] Department of Biochemistry and Molecular Biology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil
[b] Department of Biology, Sector of Biological Sciences, State University of Centro-Oeste, Guarapuava, PR, Brazil
[c] Department of Pharmacology, Sector of Biological Sciences, Federal University of Paraná, Curitiba, PR, Brazil
[d] Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdade Pequeno Príncipe, Curitiba, PR, Brazil

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ABSTRACT

In order to obtain polysaccharides from green and black teas (*Camellia sinensis*), commercial leaves were submitted to infusion and then to alkaline extraction. The extracts were fractionated by freeze-thawing process, giving insoluble and soluble fractions. Complex arabinogalactan protein from the soluble fractions of both teas (GTPS and BTPS) were determined by methylation analysis and $^{1}H/^{13}C$-HSQC spectroscopy, showing a main chain of (1 → 3)-β-Gal*p*, substituted at *O*-6 by (1 → 6)-linked β-Gal*p* with side chains of α-Ara*f* and terminal units of α-Ara*f*, α-Fuc*p* and α-Rha*p*. A highly branched heteroxylan from the insoluble fractions (GTPI and BTPI) showed in methylation analysis and $^{1}H/^{13}C$-HSQC spectroscopy the main chain of (1 → 4)-β-Xyl*p*, substituted in *O*-3 by α-Ara*f*, β-Gal*p* and α-Glc*p* units. Evaluating their gastroprotective activity, the fractions containing the soluble heteropolysaccharides from green (GTPS) and black teas (BTPS) reduced the gastric lesions induced by ethanol. Furthermore, the fraction of insoluble heteropolysaccharides of green (GTPI) and black (BTPI) teas also protected the gastric mucosa. In addition, the maintenance of gastric mucus and reduced glutathione (GSH) levels was involved in the polysaccharides gastroprotection.

1. Introduction

Tea is commonly referred to as an infusion of processed leaf of *Camellia sinensis*, and among all different kinds, the green and black teas are the most consumed [1]. The differences between the teas arise from their processing and their growth and geographic conditions. Teas obtained from different harvest or post-harvest processing are black (oxidized), oolong (semi-oxidized), green and white (non-oxidized) [2,3]. Recently a technique of anaerobic microbial fermentation is being employed, originating the pu-erh tea (fermented) [4].

Currently, several studies showed different biological activities related to *C. sinensis*, such as antioxidant [5], anticarcinogenic [6,7], anti-inflammatory [8], antiobesity [9] and antidiabetic [10] activities, action on cardiovascular disease [11,12], ultra-violet protection [13], anti-ulcer effect [14] and protection against sepsis in murine model [15]. These properties are attributed to the chemical composition that is quite diversified in this plant, which includes polyphenols (catechins, flavonoids, proanthocyanidins), alkaloids (caffeine, theobromine, theophylline), terpenoids, amino acids, polysaccharides, among others [3,16,17]. Due to their bioactive potentials, the polysaccharides from leaves and flowers of the *C. sinensis* have attracted great interest [18].

Regarding to the gastroprotective ability, many polysaccharides from different sources have been reported to protect the gastric mucosa, such as arabinogalactans types I and II from *Cereus peruvianus* [19], soybean meal [20], *Cochlospermum tinctorium* [21] and *Maytenus ilicifolia* [22], pectic polysaccharides from *Bupleurum falcatum* [23], acidic heteroxylans from *M. ilicifolia* and *Phyllanthus niruri* [24], heteroxylan of gum exudate of the palm *Scheelea phalerata* (uricuri) [25] and arabinoxylan from sugarcane bagasse [26].

Only a handful of studies were dedicated to elucidate the chemical structures of polysaccharides from *C. sinensis*. Nevertheless, some polysaccharides were identified as the neutral (1 → 4)-β-galactan and pectin-type polysaccharide formed by

* Corresponding authors.
E-mail addresses: crisbaggio@gmail.com (C.H. Baggio), iacomini@ufpr.br, marcelloiacomini@yahoo.com.br (M. Iacomini).

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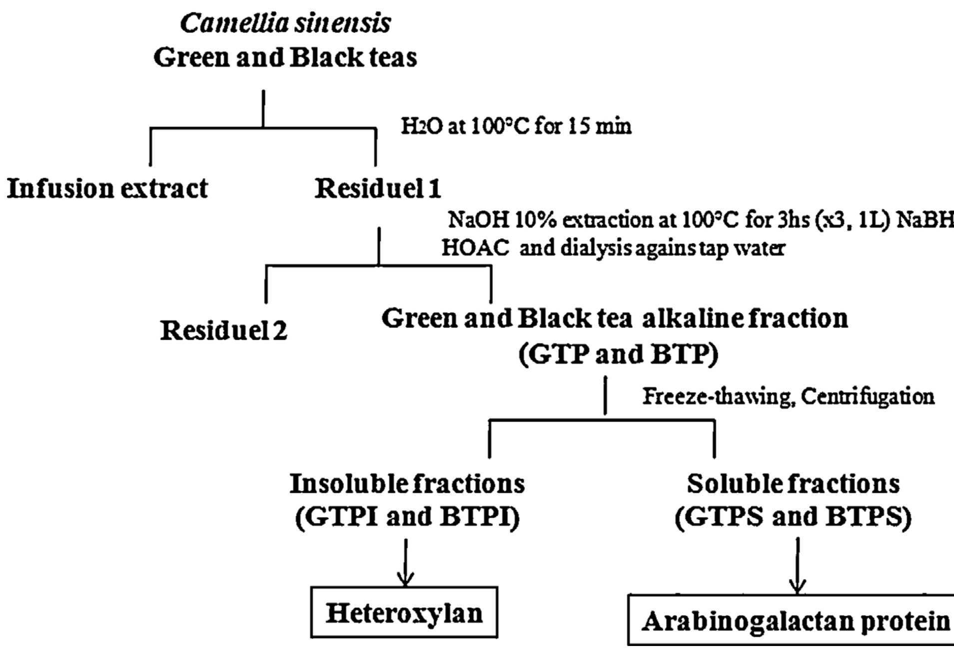

Fig. 1. Scheme of extraction and purification of arabinogalactan protein (GTPS and BTPS) and heteroxylan (GTPI and BTPI) isolated of green and black teas from *Camellia sinensis*.

blocks of (1 → 4)-α-D-galacturonic acids interconnected by rhamnose residues bonded by (1 → 2) glycosidic linkage [27,28]. Also, a sequence of (1 → 4)-6-*O*-Me-α-D-Gal*p*A interrupted by α-L-Rha*p* residues and substituted by type II arabinogalactan chains was identified [15]. Some polysaccharides associated with proteins have also been identified in *C. sinensis* [29,30]. Several properties are attributed to the tea polysaccharides from *C. sinensis* [18,31,32], nevertheless, the ability of polysaccharides from *C. sinensis* of reducing the gastric lesions has not yet been reported.

Thus, considering the lack of information about polysaccharides from *C. sinensis*, the goal of the present investigation was to compare the structure of polysaccharides obtained by alkaline extraction of leaves from green tea and black tea, as well as to investigate the gastric healing effects of isolated polysaccharides, using a model of chronic gastric ulcer induced by acid acetic in rats and the possible mechanism involved in the action.

2. Materials and methods

2.1. Plant material, extraction and fractionation

The green and black teas were purchased in a local market (Curitiba, State of Paraná, Brazil) as commercially processed leaves. A quantity of 100 g of each leave was submitted to infusion (100 °C, 500 mL, 15 min). The residues were submitted to alkaline extraction (10% aq. NaOH). The extract was then neutralized with HOAc, evaporated to small volume and dialyzed in membrane (12,000 Da cuttof). The crude fractions from the alkaline extraction, (GTP from green tea and BTP from black tea), were submitted to freezing, followed by gentle thawing at 4 °C [33], yielding the soluble fractions from green tea (GTPS) and black tea (BTPS) and the insoluble fractions from green (GTPI) and black teas (BTPI), which were separated by centrifugation (8,500 rpm, 20 min, 25 °C) (Fig. 1).

2.2. Monosaccharide analysis

GTPS, BTPS, GTPI and BTPI (2 mg) were hydrolyzed with 2 M TFA at 100 °C for 8 h, the solution was evaporated and the residue dissolved in water (1 mL). The resulting monosaccharides were submitted to thin layer chromatography (TLC) in silica gel-60 (Merck), with ethyl acetate:acetic acid:*n*-propanol:water (4:2:2:1 v/v) as eluent, followed by staining with orcinol–sulfuric acid. Then, the monosaccharides were reduced with 2 mg $NaBH_4$ yielding alditols which were acetylated in Ac_2O-pyridine (1:1 v/v, 0.5 mL) at room temperature for 12 h [34,35]. The resulting alditol acetates were extracted with $CHCl_3$, and analyzed by gas chromatography–mass spectrometry (GC–MS—Varian, Saturn 2000R), using a DB-225-MS column (30 m × 0.25 mm × 0.25 μm) programmed from 50 to 220 °C at 40 °C/min (20 min total run), with He as carrier gas. Monosaccharides were identified by their relative retention times and typical electron ionization spectra (EI–70 eV).

2.3. Methylation analysis

Tea polysaccharides (GTPS, BTPS, GTPI and BTPI) (5 mg) were per-*O*-methylated in DMSO (0.5 mL), powdered with NaOH (50 mg) and iodomethane (0.5 mL), adapting from Ciucanu and Kerek [36]. The suspension was neutralized with acetic acid and dialyzed (12,000 Da cutoff) to eliminated reagents. The water was lyophilized and the alkylated polysaccharides were then hydrolyzed with 45% aqueous formic acid (1 ml) for 12 h at 100 °C, filtered and evaporated to dryness [37]. The partially methylated monosaccharides were reduced with NaB^2H_4 and acetylated as described above to give a mixture of partially *O*-methylated alditol acetates (PMAA), which were analyzed by GC–MS similarly to the description above, with the exception of the temperature program (50–210 °C, 30 min). The identification of PMAA was based on their *m/z* spectra (EI–70 eV), and by comparing them with standards, beingthe results expressed as a relative percentage of each component [38].

2.4. Quantification of protein and amino acid analysis

The concentration of total protein in samples was measured using the Bradford Assay Kit [39]. Initially, the protein reagent was constituted of 40 μg Coomassie Briliant Blue G250, 20 μl of ethanol 95% v/v and 40 μl of *O*-phosphoric acid was diluted 3-fold in purified water. Posteriorly were added 40 μg (40 μL solution of 1 mg/ml) of the samples and then the resulting solution was analyzed by spectrophotometry at 595 nm. The standard curve was prepared varying weights, 0.4–40 μg of bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA).

In order to analyze the amino acid composition, the protein-positive samples were hydrolyzed with 6 N HCl at 100 °C for 12 h. The sample was dried and to the mixture of amino acids were added 50 μl of pyridine, 50 μL of MTBSTFA (*N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide), vortexed for 30 sec and the reaction was held at 60 °C for 30 min [40]. Then the derivatized amino acids were analyzed by GC–MS, equipped with capillary column 30 m × 0.25 mm i.d. (VF-1MS—Varian), with injector at 250 °C, oven initially at 50 °C, held for 2 min, heated to 90 °C (20 °C min^{-1}, held for 1 min) and then to 280 °C (5 °C min^{-1}, held for 2 min). Amino acids were identified by their relative retention times and typical EI profile (70 eV). Post-run analysis was performed with a Saturn Workstation 5.1

2.5. Nuclear Magnetic Resonance (NMR) analysis

Mono- (^{13}C, ^{1}H) and bi-dimensional $^{1}H/^{13}C$ (HSQC) NMR spectra were developed in a Bruker Avance *III* 400 MHz spectrometer, with 5 mm inverse probe. The samples were dissolved in D_2O and DMSO-*d*6 at 40 mg/mL and the analysis was carried out at 70 °C. The chemical shifts were expressed in ppm (δ) standardized with TMSP-d_4 (2,2,3,3-tetradeuterium-3-trimetilsililpropionate) $\delta = 0$ for ^{13}C and ^{1}H (15,20,24).

2.6. Animals

Female Wistar rats (180–200 g) were provided by the Federal University of Paraná and housed at standard laboratory conditions, with a cycle of 12 h light/dark, at temperature of 22 ± 2 °C and free access to food and water. The animals were deprived of food (~18 h) prior to the experiments. All experimental procedures were previously approved by the local Animal Ethics Committee of Federal University of Parana (CEUA/BIO-UFPR; approval number 718).

2.7. Induction of acute gastric lesion

The acute gastric lesion was induced by ethanol P.A. according to Robert et al. [41]. One hour before oral administration of ethanol P.A. (0.5 ml/200 g), the animals were orally pretreated with vehicle (Control: water, 1 ml/kg), omeprazole (Ome: 40 mg/kg) or polysaccharides (GTPS, BTPS, GTPI and BTPI, at doses of 3, 10 and 30 mg/kg). After 1 h of ethanol administration, the animals were sacrificed and their stomachs were removed to measure the area of lesions (mm^2) by the software Image Tool 3.0®.

2.8. Determination of gastric wall mucus

Samples of gastric mucosa were weighed and incubated at room temperature in 0.1% Alcian Blue solution for 2 h. The excess of Alcian Blue was removed by two successively rinses with 0.25 M sucrose, the first for 15 min and the second for 45 min. After that, the dye complexed with gastric wall mucus was extracted with a 0.5 M magnesium chloride solution and the segments of gastric mucosa were shaken for 1 min at 30 min intervals for 2 h. The resultant blue extract was mixed with an equal volume of diethyl ether and then centrifuged at 3,600 rpm by 10 min. Absorbance was determined by spectrophotometry at 598 nm and the quantification of mucus levels was performed using standard curve of Alcian Blue (6.25–100 μg). The results were expressed in μg of Alcian Blue/g of tissue [42].

Table 1
Monosaccharides and amino acids composition of GTPS, BTPS, GTPI and BTPI.

	GTPS[a] (%)	BTPS[b] (%)	GTPI (%)	BTPI (%)
Monosaccharides[c]				
Rhamnose	6	7	–	–
Fucose	7	8	–	–
Arabinose	47	53	12	11
Xylose	–	–	70	77
Galactose	40	32	6	5
Glucose	–	–	12	7
Amino acids[d]				
Alanine	7.9	8.1	–	–
Valine	7.5	7.2	–	–
Leucine	7.6	8.2	–	–
Isoleucine	6.9	6.1	–	–
Serine	10.9	12.4	–	–
Proline	6.3	5.4	–	–
Phenylalanine	2.1	7.2	–	–
Threonine	9.8	10.3	–	–
Cysteine	9.6	0.0	–	–
OH-Proline	15.2	17.8	–	–
Glutamic Acid	8.3	9.9	–	–
Tyrosine	7.9	7.4	–	–

[a] Protein content of GTPS was 12%.
[b] Protein content of BTPS was 19%.
[c] Relative percentage of alditol acetates obtained by successive hydrolysis, $NaBH_4$ reduction, and acetylation, followed by GC–MS analysis.
[d] Relative percentage of amino acids obtained by successive hydrolysis, pyridine and MTBSTFA treatment, followed by GC–MS analysis.

2.9. Determination of reduced glutathione content

The glandular segment of gastric mucosa was weighed and homogenized with potassium phosphate buffer (200 mM, pH 6.5) to determine the reduced glutathione (GSH) levels following Sedlak and Lindsay method [43] with modifications. Aliquots of homogenate were mixed with trichloroacetic acid (12.5%), vigorously shaken and centrifuged at 3,000 rpm for 15 min. A buffer Tris–HCl (0.4 M, pH 8.9) and 0.01 M DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] was added to the supernatant aliquots in 96-well plate. The procedures were performed at 4 °C. The absorbance was measured by spectrophotometry at 415 nm and the individual values interpolated into a standard curve of GSH (0.375–3 μg). The results were expressed as μg of GSH/g of tissue.

2.10. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) with 6–8 animals per group. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni's test. Differences in *P* values <0.05 were considered significant. The ED_{50} values (effective dose capable of inhibiting the gastric lesions formation by 50% relative to the control value) were determined by nonlinear regression analysis and reported as geometric mean. Calculations were performed with Graph-pad software (GraphPad software, San Diego, CA, USA).

3. Results and discussion

3.1. General analysis of polysaccharides from green and black teas

Polysaccharides from infusions of green and black teas were previously studied [15]. Now, after submitting the green and black teas to the infusion, the residues were subjected to alkaline extraction to obtain the heteropolysaccharides from *C. sinensis*. After dialysis against tap water, the crude polysaccharide extracts from green tea (GTP-17%) and black tea (BTP-19%) were obtained.

Fractionation and partial purification of crude polysaccharides were carried out by a freeze-thawing procedure, resulting in their respective cold-water soluble polysaccharide from green

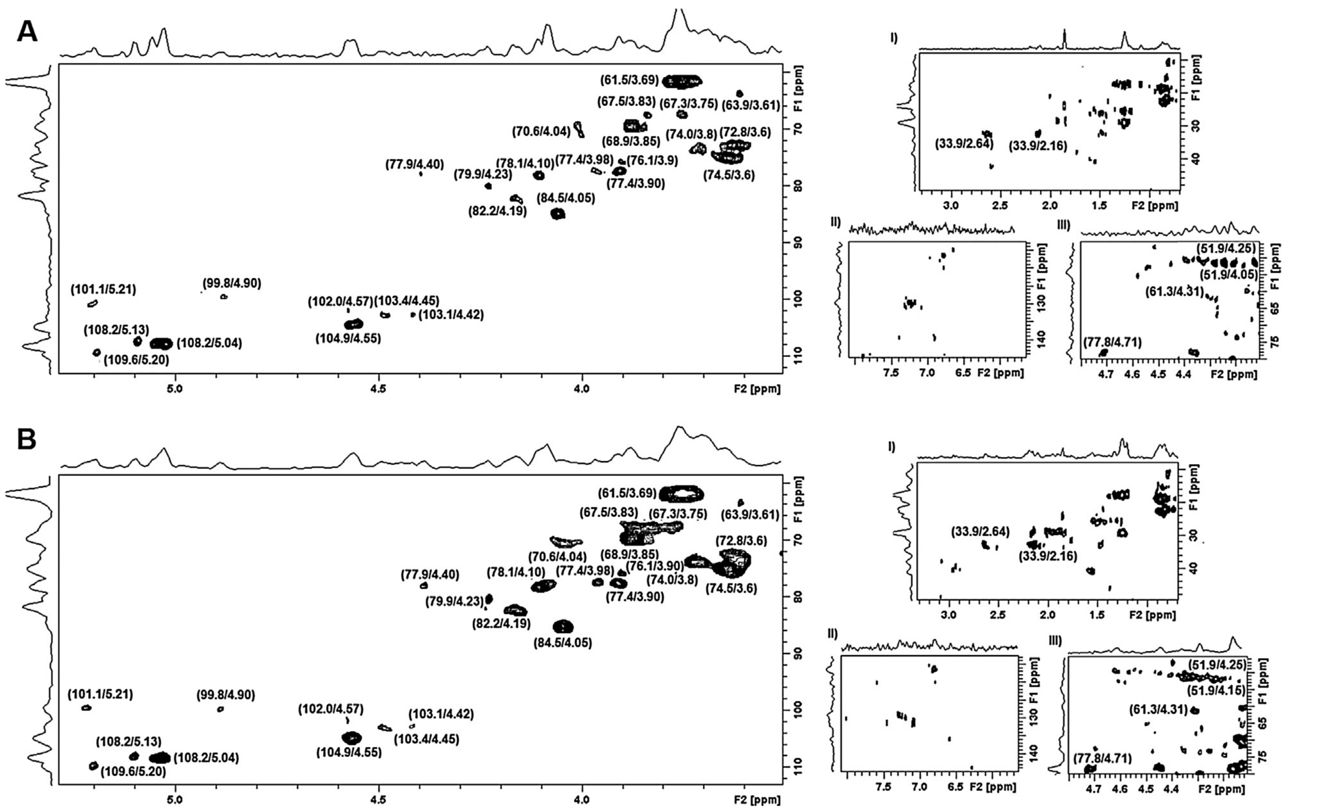

Fig. 2. $^1H/^{13}C$ HSQC NMR spectrum of GTPS (A) and BTPS (B), solvent D_2O at 70 °C. Inserts represents the amino acids signals at: (I) region with δ 0.7–3.3 (^1H) and at δ 10.0–50.0 (^{13}C) are from CH_3 and CH_2 from the amino acids, and the signals numbered are from hydroxyproline; (II) region with δ 8.0–5.7 (^1H) and at δ 115–145 (^{13}C) from double bonds of aromatic amino acids; and III) region with δ 4.1–4.8 (^1H) and at δ 45.0–80.0 (^{13}C) from α-carbon of amino acids, and the signals numbered are from hydroxyproline.

Table 2
Partially *O*-methylated alditol acetates (PMAA) of the GTPS and BTPS polysaccharides.

PMAA[a]	Rt[b]	GTPS (%)	BTPS (%)	Structure[c]
2,3,5-Me_3-Ara	8.4	12.0	9.9	Ara*f*-(1→
2,3,4-Me_3-Fuc	9	6.0	7.0	Fuc*p*-(1→
3,5-Me_2-Ara	9.8	7.5	5.8	→2)-Ara*f*-(1→
2,5-Me_2-Ara	10.1	7.8	4.2	→3)-Ara*f*-(1→
2,3-Me_2-Ara	10.7	12.0	18.7	→5)-Ara*f*-(1→
2,3,4,6-Me_4-Gal	11	8.6	12.7	Gal*p*-(1→
3,4-Me_2-Rha	12.6	5.0	6.7	→2)-Rha*p*-(1→
2-Me-Ara	13.1	8.0	13.1	→3,5)-Ara*f*-(1→
2,4,6-Me_3-Gal	13.7	11.5	10.5	→3)-Gal*p*-(1→
2,4-Me_2-Gal	21.3	21.6	11.4	→3,6)-Gal*p*-(1→

[a] Analyzed by GC–MS, after methylation, total acid hydrolysis, reduction with $NaBD_4$ and acetylation.
[b] Rt = retention time relative to 2,3,4,6-Me_4Glc acetate.
[c] Based on derived *O*-methylalditol acetates.

(GTPS-75%) and black teas (BTPS-78%) and insoluble polysaccharide from green (GTPI-25%) and black teas (BTPI-22%).

The soluble polysaccharide fractions (GTPS and BTPS) and insoluble polysaccharides fractions (GTPI and BTPI) were analyzed for their monosaccharide composition. The polysaccharides of GTPS and BTPS were mainly composed by arabinose, galactose, rhamnose and fucose, while the GTPI and BTPI resulted in xylose, arabinose, galactose and glucose (Table 1). The monosaccharide composition of polysaccharides from green and black teas were very similar, however, a slight difference was observed between the soluble polysaccharides, consistent in the fact that the black tea had a greater concentration of arabinose and lower concentration of galactose compared to the green tea, and the insoluble polysaccharides from the black tea also had higher amounts of xylose and lower amounts of glucose and galactose.

Proteins was found in the soluble fractions from green and black teas, GTPS (12%) and BTPS (19%). These proteins were analyzed for their amino acid composition, and in GTPS were found hydroxyproline (Hyp), serine (Ser), threonine (Thr) and cysteine (Cys) as major amino acids. In BTPS the most abundant amino acids were Ser, Thr, Hyp and glutamic acid (Glu). It is interesting that the cysteine content varied considerably from green tea (9.6%) to black tea, which showed no significant amounts of this amino acid. This is another slight difference between the two kinds of tea analyzed. The results are shown in Table 1. These differences can arise from their oxidative processing of black tea [3].

3.1.1. Structural analysis of soluble polysaccharides from green and black teas

The $^1H/^{13}C$-HSQC spectra of soluble polysaccharides GTPS and BTPS (Fig. 2A and B, respectively) indicated the presence of a similar structure, composed mainly of arabinose, galactose, as well as amino acid signs, suggesting that the polysaccharides chains could be composed of an arabinogalactan protein. Arabinogalactan proteins (AGP) generally are *O*-glycosylated at one or more hydroxyproline or serine residues by branches of arabinogalactans type II [44].

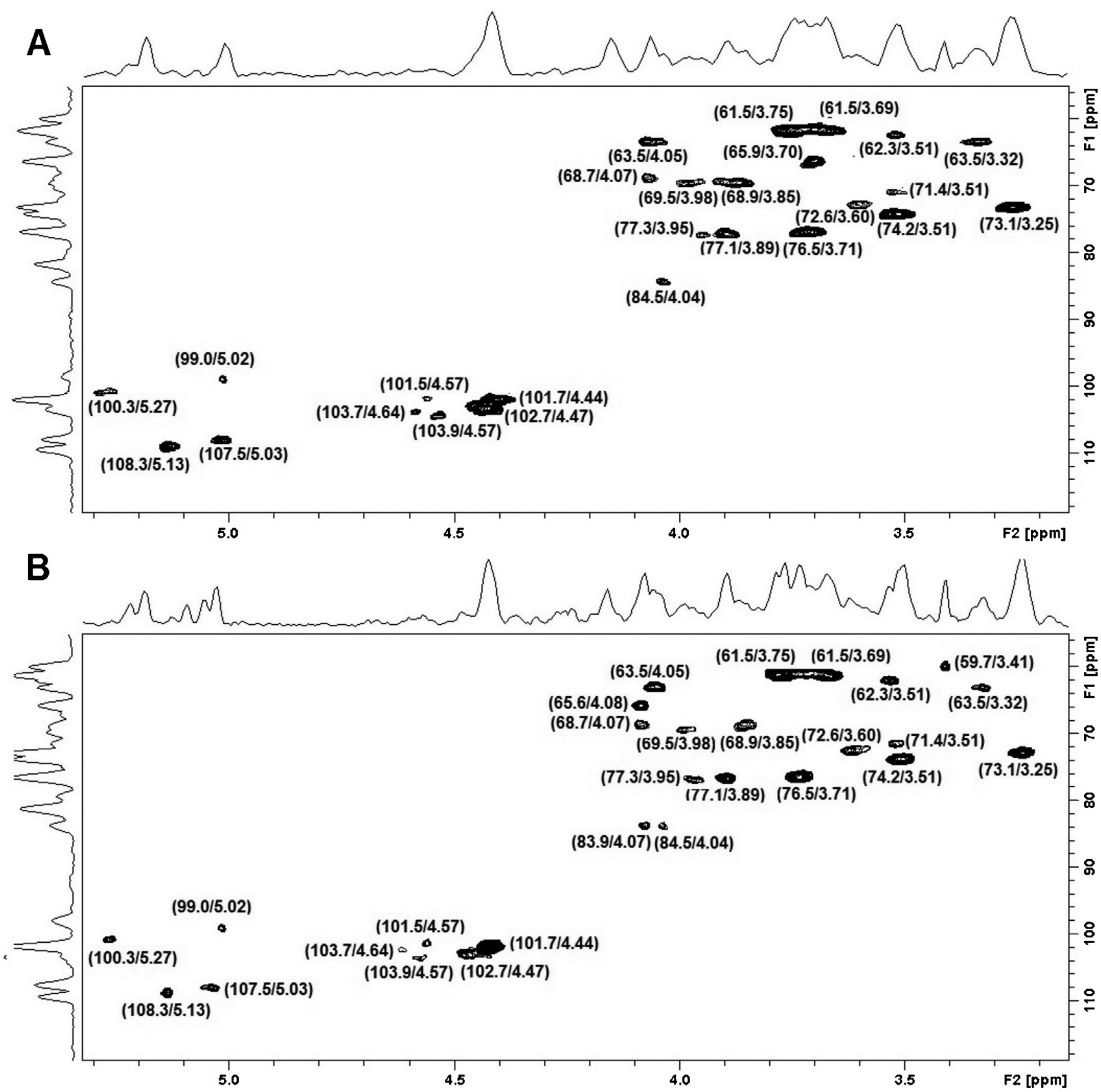

Fig. 3. ^{1}H/^{13}C HSQC NMR spectrum of GTPI (A) and BTPI (B), solvent DMSO-d6 at 70 °C.

The spectra of GTPS and BTPS (Fig. 2A and B) contained signals consistent with (1 → 3)- and branches (1 → 6)-linked β-Gal*p* units, appearing at δ 104.9/4.55, δ 103.4/4.45 and δ 103.1/4.42 (C1/H1), δ 82.2/4.19 (C3/H3) and δ 68.9/3.85 (C6/H6) [45]. Methylation analysis of soluble polysaccharide (GTPS and BTPS) confirms the NMR data, showing galactopyranosyl units mainly non reducing end-units, (1 → 3)-β-Gal*p* and (1 → 3, →6)-β-Gal*p* (Table 2).

The arabinose units appeared linked to *O*-3 or *O*-6 of β-Gal*p* units, yielding side chains of (1 → 3) and (1 → 5)-linked Ara*f* units. The HSQC spectra of GTPS and BTPS had typical signals of (1 → 3)- and (1 → 5)-linked α-Ara*f* units, appearing at δ 109.6/5.2 and δ 108.2/5.04 (C1/H1) respectively, in addition the signal at δ 108.2/5.13 were attributed to (1 → 2)-linked α-Ara*f*. Moreover, the signals at δ 84.5/4.04 (C2/H2), δ 74.0/3.8 (C3/H3) and δ 63.9/3.61 (C5/H5) confirmed the substituted α-Ara*f* [27,45]. Corroborating to these data, methylation analysis confirmed the arabinosyl units as (1 → 2)-, (1 → 3) and (1 → 3, →5)-linked (Table 2).

The signals at δ 101.1/5.21 (C1/H1) and δ 76.1/3.9 (C2/H2) confirmed the presence of rhamnose (1 → 2)-linked. Furthermore, the signal in the anomeric region at δ 99.8/4.9 was consistent with C1/H1 of α-Fuc*p* [46], and was also confirmed by methylation analysis that showed that Rha*p* units are substituted on *O*-2 by non-reducing end units of Fuc*p* (Table 2). These data indicate the presence of many substitutions in the main chain of arabinogalactan protein from *C. sinensis*, corroborating previous studies [29,30,47].

HSQC signals consistent with chemical shifts characteristic of amino acids were found in both samples (GTPS and BTPS), being further confirmed by GC–MS. The signals at region with δ 0.7–3.3 (^{1}H) and at δ 10.0–50.0 (^{13}C) are consistent with CH_3 and CH_2 from amino acids (Fig. 2 A and B, I), while the signals at region with δ 8.0–5.7 (^{1}H) and at δ 115–145 (^{13}C) were consistent with double bonds from aromatic amino acids (Fig. 2 A and B, II), and signals at δ 4.1–5.8 (^{1}H) and at δ 45.0–80.0 (^{13}C) from α-carbon of amino acids (Fig. 2 A and B, III) [48]. Furthermore, in the HSQC spectra were observed signals at δ 102.0/4.57 (C1/H1), which suggest the presence of units of β-Gal attached to hydroxyproline (Hyp), whereas the signals at δ 77.8/4.71, 61.3/4.31, 33.9/2.16, 2.64 and 51.9/4.15, 4.25 could be attributed to C4/H4, C2/H2, C3/H3 and C5/H5 of Hyp (Fig. 2 A and B, I and III). These data are in agreement with the previous study from Tan et al. [49] that characterized a structure of a hydroxyproline-linked arabinogalactan from *Nicotiana tabacum*,

Table 3
Partially *O*-methylated alditol acetates (PMAA) of the GTPI and BTPI polysaccharides from *C. sinensis.*

PMAA[a]	Rt[b]	GTPI (%)	BTPI (%)	Structure[c]
2,3,5-Me_3-Ara	8.4	9.0	9.3	Ara*f*-(1→
2,3,4-Me_3-Xyl	8.9	1.9	1.5	Xyl*p*-(1→
3,5-Ara	10.11	2.3	1.0	→2)-Ara*f*-(1→
2,3,4,6-Me_4-Glc	10.47	7.1	4.5	Glc*p*-(1→
2,3,4,6-Me_4-Gal	11.04	3.7	4.0	Gal*p*-(1→
2,3-Me_2-Xyl	11.15	49.5	57.7	→4)-Xyl*p*-(1→
2-Me-Xyl	14.28	21.5	18.9	→3,4)-Xyl*p*-(1→
2,3,4-Me_3-Gal	16.1	2.1	0.7	→6)-Gal*p*-(1→
2,3,4-Me_3-Glc	19.1	2.9	2.4	→4,6)-Glc*p*-(1→

[a] Analyzed by GC–MS, after methylation, total acid hydrolysis, reduction with $NaBD_4$ and acetylation.
[b] Rt = retention time relative to 2,3,4,6-Me_4Glc acetate.
[c] Based on derived *O*-methyl alditol acetates.

in which the HMBC spectrum showed that β-D-Gal*p* residue has appeared *O*-linked to C-4 of Hyp. As observed from GC–MS analysis, both galactose and hydroxyproline have appeared at high levels, supporting the suggested structure. Also, other reports cite the presence of polysaccharides associated with proteins from seeds, flowers and leaves from *C. sinensis* [29,30,49–52].

Thus, on the basis of methylation results and NMR analysis of GTPS and BTPS, arabinogalactan proteins have been described. The polysaccharide consist of a main chain (1 → 3)-β-Gal*p* with branching in the *O*-6 of –β-Gal*p*. In addition, branches of (1 → 2)-, (1 → 3)- and (1 → 5)-α-Ara*f* and (1 → 2)-α-Rha*p* were identified, as well as non-reducing terminal units α-Ara*f* and α-Fuc.

3.1.2. *Structural analysis of insoluble polysaccharide from green and black teas*

The analysis of GTPI and BTPI indicated the absence of protein, moreover the HSQC spectra (Fig. 3A and B, respectively) suggest the presence of a similar heteroxylan structure. The signals at δ 102.7/4.47 and δ 101.7/4.44 and δ 101.5/4.57 correspond to C1/H1 of β-Xyl*p* with different substitution pattern [53–55]. Signals at δ 76.5/3.71 (C4/H4) and δ 74.2/3.51 (C3/H3) were consistent with Xyl*p* units (1 → 4)-linked and *O*-3 substituted. The signals at δ 73.1/3.25 (C2/H2), δ 63.5/3.32 and δ 63.5/4.05 (C5/H5), were consistent with non-substituted Xyl*p* units [53]. These data are in accordance to methylation analysis, that presented non-reducing end units of Xyl*p* and greater amounts of (1 → 4)- and (1 → 3,→4)-Xyl*p* (Table 3).

In the HSQC spectra, the signals at δ 108.3/5.13 and δ 107.5/5.03 arose from C1/H1 of α-Ara*f* units with different linkages [56,57], and the signal at δ 84.5/4.04 (C2/H2) is from 2-*O* substituted α-Ara*f*. The signals at δ 72.6/3.60 (C3/H3), δ 71.4/3.51 (C4/H4) and δ 62.3/3.51 (C5/H5) were from non-substituted α-Ara*f* units [24,58]. The methylation analysis also confirms that arabinose was presented as non-reducing end units and substituted on *O*-2 (Table 3).

Moreover, the spectra of GTPI and BTPI (Fig. 3A and B) contained signals consistent with β-Gal*p* units, appearing at δ 103.9/4.57 and δ 103.7/4.64 (C1/H1) and δ 68.9/3.85 (C6\H6) [45], and, agreeing to these data, it was observed in the methylation analysis

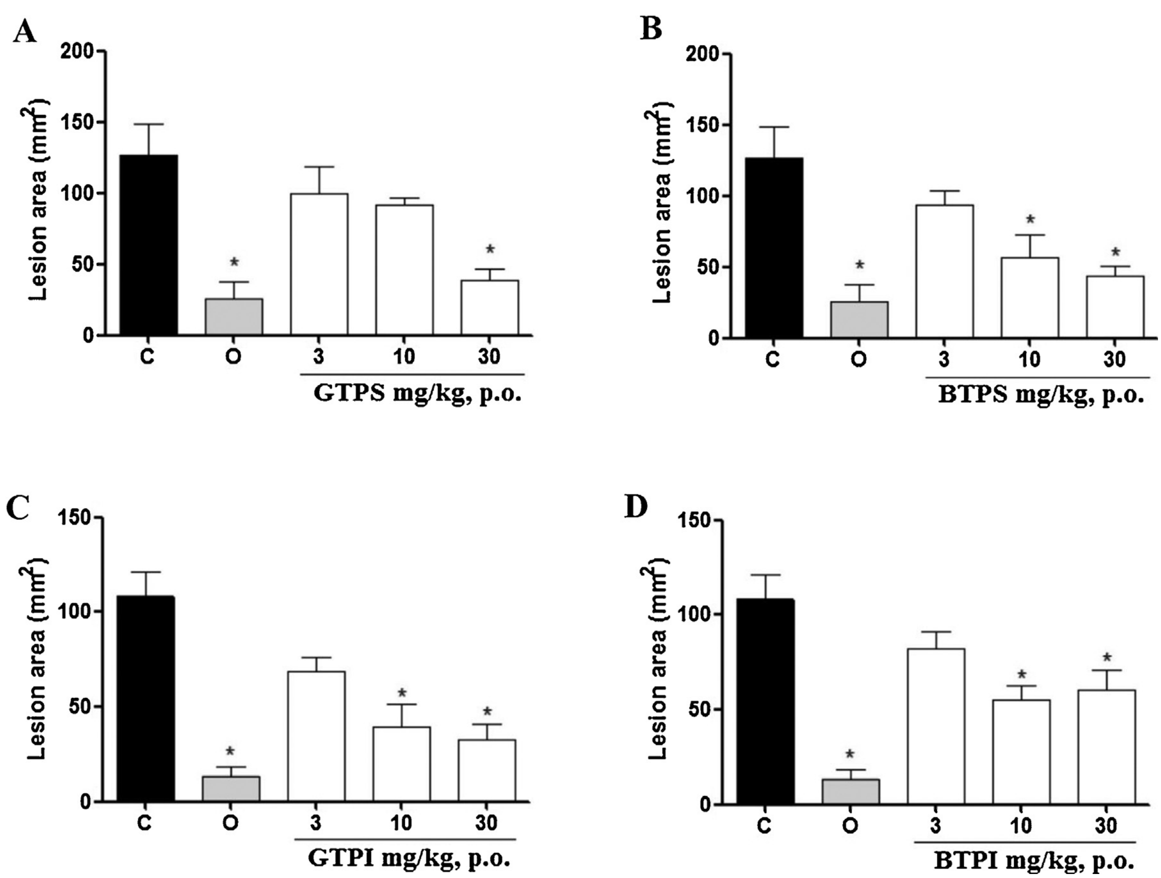

Fig. 4. Effect of green (GTPS) (A) and black (BTPS) (B) soluble polysaccharides, and green (GTPI) (C) and black (BTPI) (D) insoluble polysaccharides from alkaline extraction of green and black teas from *C. sinensis* on acute gastric lesions induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 ml/kg), omeprazole (Ome: 40 mg/kg), GTPS, BTPS, GTPI or BTPI (3, 10 and 30 mg/kg), 60 min before oral administration of ethanol P.A. (0.5 ml/200 g). Results are expressed as mean ± S.E.M. (n = 6–8) and statistical comparison was performed using one-way ANOVA followed by post hoc Bonferroni's test. *P < 0.05 compared to the control group.

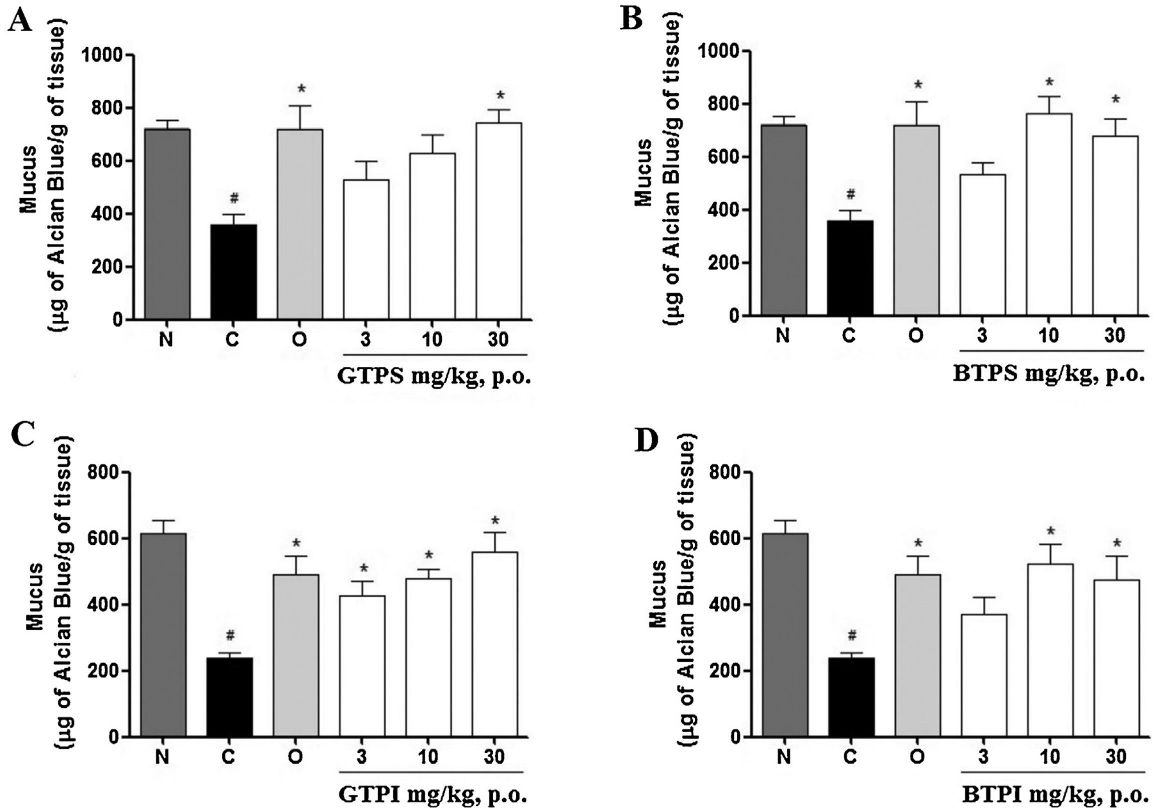

Fig. 5. Effect of green (GTPS) (A) and black (BTPS) (B) soluble polysaccharides and green (GTPI) (C) and black (BTPI) (D) insoluble polysaccharides from alkaline extraction of green and black teas from *C. sinensis* on gastric mucus levels in acute gastric lesions induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 ml/kg), omeprazole (Ome: 40 mg/kg), GTPS, BTPS, GTPI or BTPI (3, 10 and 30 mg/kg), 60 min before oral administration of ethanol P.A. (0.5 ml/200 g). Results are expressed as mean ± S.E.M. ($n = 6$–8) and statistical comparison was performed using one-way ANOVA followed by post hoc Bonferroni's test. $^{\#}P < 0.05$ compared to the non-lesionated group (NL), $^{*}P < 0.05$ compared to the control group (C).

galactopyranosyl units as non-reducing end units and substituted at *O*-6 (Table 3), confirming the presence of Gal*p* (1 → 6)-linked.

The C1/H1 signal at δ 99.0/5.02 was consistent with 4,6-di-*O*-substituted α-Glc*p* units whereas δ 100.3/4.27 was consistent with terminal units [24]. The methylation analysis confirmed the glucopyranosyl units as non-reducing ends and *O*-4, *O*-6 substituted (Table 3).

Therefore, from the methylation results and NMR analysis of GTPI and BTPI, these heteropolysaccharides from *C. sinensis* were first described, consisting of a similar heteroxylan containing a main chain of (1 → 4)-linked β-Xyl*p* units, branched in 3-*O*- by chains of (1 → 2)-α-Ara*f*, (1 → 6)-β-Gal*p* and (1 → 4,6)-α-Glc*p* units, and terminal units of Ara*f*, Gal*p* and Glc*p* were also observer.

3.2. Effect of green and black teas polysaccharides on acute gastric lesions induced by ethanol

Considering the gastroprotective properties of these polysaccharides, we investigated whether the arabinogalactans protein (AGP) and heteroxylans isolated from *C. sinensis* also present this biological activity. Since ethanol-induced acute gastric lesion in rats has been used to evaluate the protective effect of new agents on gastric mucosa, we have investigated whether green (GTPS and GTPI) and/or black (BTPS and BTPI) teas polysaccharides had potential in this experimental model. The capacity of ethanol to induce gastric injury is due to its deleterious effects on gastric mucus layer, reduced glutathione levels and secretion of bicarbonate [59].

Our results demonstrated that oral administration of soluble polysaccharides (AGP) from green (GTPS, 30 mg/kg) and black teas (BTPS, 10 and 30 mg/kg) inhibited the gastric lesion area in 71, 55 and 66%, respectively, when compared to control group [C: 136.1 ± 23.6 (GTPS) and 126.6 ± 22.1 mm^2 (BTPS)] (Fig. 4A and B). Corroborating this present study, early studies demonstrated that an arabinogalactan from *M. ilicifolia* and *C. tinctorium*, significantly reduced the ethanol-induced gastric lesions in rats when administered by oral or intraperitoneal routes [21,22,60].

Similarly, the treatment of animals with the insoluble polysaccharides (heteroxylans) from green (GTPI; 3, 10 and 30 mg/kg) and black teas (BTPI, 10 and 30 mg/kg) by oral route also reduced the gastric lesions induced by ethanol by 51, 72, 76, 61 and 62%, respectively, compared to control group [C: 139.4 ± 30.2 (GTPI) and 140.0 ± 35.6 mm^2 (BTPI)] (Fig. 4C and D). Omeprazole (40 mg/kg, p.o.), the positive control, protected the gastric mucosa reducing the lesion area up to 90% (Fig. 4). These data are in accordance with Mellinger-Silva et al. [26] that isolated an arabinoxylan from sugarcane bagasse, which inhibited the gastric lesion area on experimentally induced gastric ulcers, and Simas-Tosin et al. [61] that showed a glucuronoarabinoxylan from coconut palm gum exudates witch exhibited gastroprotective effect against gastric hemorrhagic lesions induced by ethanol administration.

Altogether, these results are in accordance with previous data described before, confirming the gastroprotective actions of arabinogalactans proteins and heteroxylans from green and black teas of *C. sinensis*.

3.2.1. Effect of green and black teas polysaccharides on gastric wall mucus

Ethanol depletes the gastric mucus barrier which constitutes the first line of defense against acid and luminal pepsin. This protection is provided by formation of a viscous, elastic, adherent and

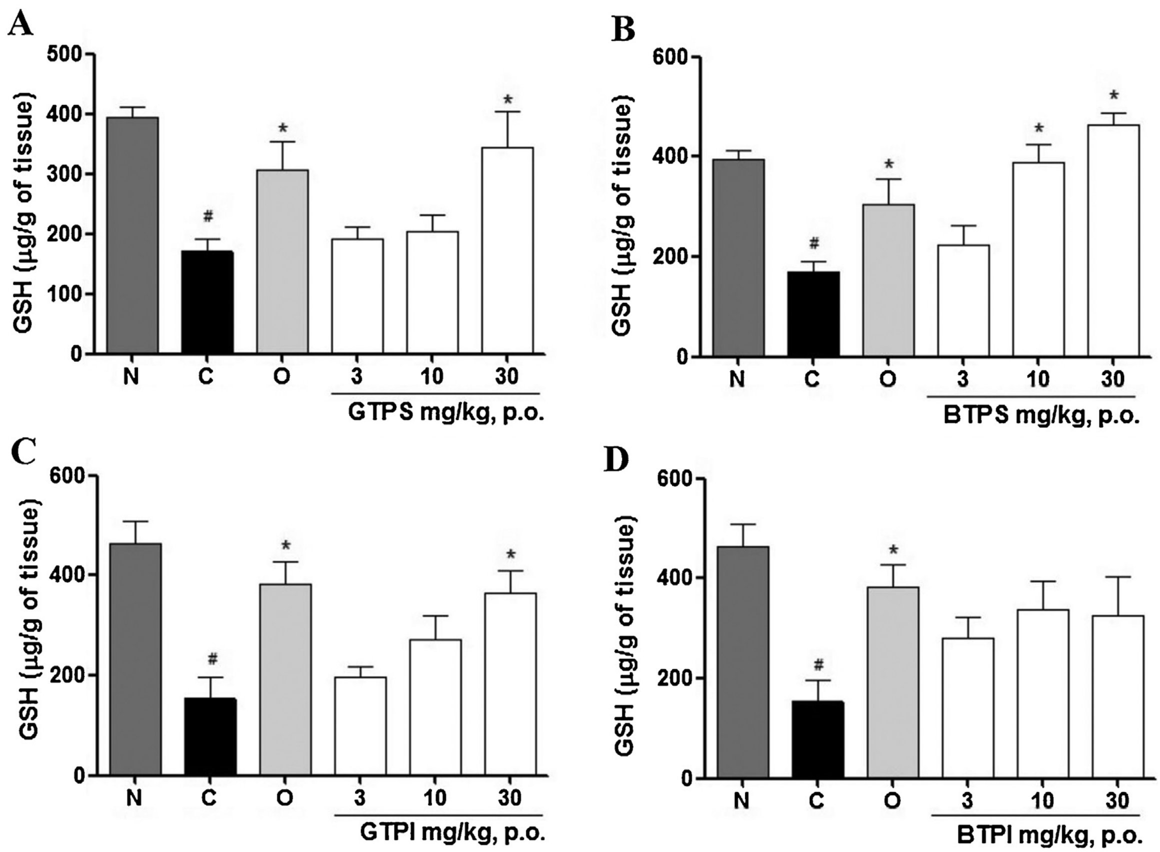

Fig. 6. Effect of green (GTPS) (A) and black (BTPS) (B) soluble polysaccharides and green (GTPS) (C) and black (BTPS) (D) insoluble polysaccharides from alkaline extraction of green and black teas from *C. sinensis* on gastric GSH levels in acute gastric lesions induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 ml/kg), omeprazole (Ome: 40 mg/kg), GTPS, BTPS, GTPI or BTPI (3, 10 and 30 mg/kg), 60 min before oral administration of ethanol P.A. (0.5 ml/200 g). Results are expressed as mean± S.E.M. (n = 6–8) and statistical comparison was performed using one-way ANOVA followed by post hoc Bonferroni's test. $^{\#}P < 0.05$ compared to the non-lesionated group (NL), $^{*}P < 0.05$ compared to the control group (C).

transparent gel over the gastric mucosa, creating a physical barrier [62]. As expected, the oral administration of ethanol P.A. decreased the levels of gastric mucus up to 62% when compared to non-lesionated group (NL) (Fig. 5). However, the pretreatment of animals with the soluble polysaccharide GTPS restored the gastric mucus levels to 629.0 ± 68.7 and 742.3 ± 49.6 µg of Alcian Blue/g of tissue, and BTPS, at doses of 10 and 30 mg/kg, p.o., to 761.8 ± 65.5 and 680.1 ± 61.4 µg of Alcian Blue/g of tissue, respectively, when compared to control group [C: 359.8 ± 39.5 (GTPS) and 370.2 ± 33.9 µg (BTPS) of Alcian Blue/g of tissue] and non-lesionated group [NL: 720.3 ± 34.38 (GTPS) and 714.18 ± 27.8 µg (BTPS) of Alcian Blue/g of tissue] (Fig. 5A and B).

Similar results were obtained with insoluble polysaccharides from green and black teas. The administration of GTPI (3, 10 and 30 mg/kg, p.o.) were able to maintain the mucus levels in 425.4 ± 45.1, 480.6 ± 26.0 and 557.4 ± 61.3 µg of Alcian Blue/g of tissue, and BTPI (10 and 30 mg/kg, p.o.) in 523.7 ± 60.0 and 475.2 ± 71.3 µg of Alcian Blue/g of tissue, respectively, compared to control group [C: 244.2 ± 19.5 (GTPI) and 239.4 ± 17.1 µg of Alcian Blue/g of tissue (BTPI)] and non-lesionated group [NL: 613.1 ± 39.74 (GTPI) and 619.2 ± 19.81 (BTPI) µg of Alcian Blue/g of tissue] (Fig. 5C and D). The positive control of the test, omeprazole (40 mg/kg, p.o.), also prevented the decrease of gastric mucus levels (Fig. 5).

Mellinger-Silva et al. [26] also observed the maintenance of gastric mucus promoted by the arabinoxylan from sugarcane bagasse in gastric ulcers. So, our results, in accordance to previous literature, indicate the participation of this important protective factor in the gastroprotection promoted by these polysaccharides.

3.2.2. Effect of green and black-teas polysaccharides on GSH levels

The evaluation of protective mechanisms involved in the gastroprotection can also be measured by quantification of the amounts of GSH restored in gastric mucosa by green and black teas polysaccharides after induction of lesions by ethanol. GSH acts as a non-enzymatic antioxidant and provides cellular protection against damage induced by reactive oxygen species (ROS) [63].

As observed in mucus levels, the administration of ethanol P.A. also decreased the amount of GSH up to 68% compared to non-lesionated group (Fig. 6). The oral administration of GTPS (30 mg/kg) and BTPS (10 and 30 mg/kg) maintained the GSH levels to 343.5 ± 61.5, 389.2 ± 35.4 and 464.8 ± 22.4 µg/g of tissue, respectively, compared to control group [C: 154.9 ± 18.6 (GTPS) and 170.1 ± 21.9 (BTPS) µg/g of tissue] and to non-lesionated group [NL: 393.9 ± 18.66 (GTPS) and 398.8 ± 19.9 (BTPS) µg/g of tissue] (Fig. 6A and B).

Regarding to insoluble fractions, only GTPI at dose of 30 mg/kg was able to restore the GSH levels in 364.6 ± 46.9 µg/g of tissue, when compared to control group (C: 190.7 ± 51.4 µg/g of tissue) and non-lesionated group [NL: 462.8 ± 46.96 µg/g of tissue] (Fig. 6C). BTPI in all tested doses did not alter the levels of GSH (Fig. 6D). Omeprazole (40 mg/kg, p.o.) also restored the GSH amounts to basal levels (Fig. 6). It is important to note that our results are in accordance with data obtained by Mellinger-Silva et al. [26], where the arabinoxylan from sugarcane bagasse also preserve the GSH levels, suggesting the involvement of this protective factor in the gastroprotective activity of xylans. Thus corroborating the literature, our experiments indicate this possible mechanism acting in gastroprotection of evaluated polysaccharides from *C. sinensis*.

4. Conclusion

Heteropolysaccharides from alkaline extraction of green and black teas (*C. sinensis*) were identified. The soluble polysaccharides (GTPS and BTPS) are consistent of an arabinogalactan protein with a main chain of (1 → 3) and (1 → 6)-linked β-Gal*p* units, and α-Ara*f* units. Also, several amino acids were identified, characterized for their association to the structure of the main chain. Regarding to the insoluble polysaccharides (GTPI and BTPI), they consist of an heteroxylan that contain a main chain of (1 → 4)-linked β-Xyl*p* units, which side chains of α-Ara*f*, β-Gal*p* and α-Glc*p* units.

GTPS, BTPS, GTPI and BTPI (30 mg/kg) were able to inhibit ethanol-induced gastric lesions indicating that they could be effective gastroprotective agents. This beneficial effect seems to be, at least in part, due to a restoration of the GSH and of the gastric mucus levels, two important factors on gastroprotection. These results demonstrate the usefulness of polysaccharides of vegetable origin in the prevention of pathologies, emerging the *C. sinensis* as an important subject for scientific research and future application of its therapeutic potential.

5. Declaration of interest

The authors report no conflicts of interest.

Acknowledgments

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The protective effect of green and black teas (*Camellia sinensis*) and their identified compounds against murine sepsis

Camila T. Scoparo [a], Lauro M. de Souza [a,b], Yanna D. Rattmann [c], Elaine C. Kiatkoski [a], Nessada Dartora [d,*], Marcello Iacomini [a,**]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, 81531-980 Curitiba, PR, Brazil
[b] Instituto de Pesquisa Pelé Pequeno Príncipe, Faculdade Pequeno Príncipe, 80250-200 Curitiba, PR, Brazil
[c] Departamento de Saúde Comunitária, Universidade Federal do Paraná, 80060-240 Curitiba, PR, Brazil
[d] Departamento de Biologia, Universidade Estadual do Centro-Oeste, 85040-080 Guarapuava, PR, Brazil

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ABSTRACT

Several compound classes could be identified in the infusion of green tea (GTI) and black tea (BTI) from *Camellia sinensis*, mainly catechins and their gallate derivatives in GTI and their oxidation products, theaflavins and theasinensins, in BTI. We also managed to identify other components, such as proanthocyanidins, flavonoid glycosides, alkaloids, hydroxybenzoic and hydroxycinnamic acids and saponins. The infusions were fractionated and the ethyl acetate fractions [green tea ethyl acetate fraction (GEAF) and black tea ethyl acetate fraction (BEAF)] have concentrated the catechins, theaflavins and caffeine. So, the infusions, fractions and major compounds identified from both teas were assayed against murine sepsis, in order to assess whether they are effectively protecting from this pathology. The infusions at 70 mg/kg resulted in a decrease of sepsis mortality in 43% (GTI) and 40% (BTI), respectively. The ethyl acetate fractions were also efficient, allowing a survival rate of 50% (GEAF) and 66.6% (BEAF) at concentrations of 30 and 70 mg/kg, respectively. The main components from GEAF and BEAF were also assayed; epigallocatechin gallate (10 mg/kg), theaflavins (9 mg/kg) and caffeine (18 mg/kg) showed a survival rate of 50%, 40% and 20%, respectively. This protective effect can be explained, at least in part, by some of these compounds' ability to modulate some important inflammatory parameters, such as neutrophil influx, avoiding its accumulation in lungs, depletion of the pro-inflammatory cytokines (TNF-α and IL-6), increase the anti-inflammatory cytokine (IL-10) and, also, a significant decrease in tissue expression of iNOS and COX-2.

1. Introduction

Teas from *Camellia sinensis* are widely consumed for more than fifty centuries in the world (Chantre & Lairon, 2002). Different teas from *C. sinensis* are obtained on the basis of the auto-oxidation process catalyzed by polyphenol oxidase and peroxidase enzymes. Thus, the teas are classified as non-oxidized (green and white tea), semi-oxidized (oolong tea), oxidized (black tea) and post-fermented (pu-erh tea). The most consumed teas are the green and black teas (Engelhardt, 2010).

Several classes of compounds have been identified in different types of *C. sinensis* teas, mainly the products of secondary metabolism, such as, terpenes, alkaloids and, particularly, phenolic compounds, as well as volatile compounds, carotenoids, tocopherols, vitamins (A, K, B, C), and minerals such as fluorine, potassium, magnesium, iron, manganese, and phosphorus, among others (Engelhardt, 2010; Scoparo et al., 2012, 2014). Also the primary metabolism products have been identified, such as polysaccharides, proteins and lipids (Nie & Xie, 2011; Scoparo et al., 2013; Yang et al., 2007).

Moreover, the compounds found in teas present different biological properties, as established by the number of in vivo and in vitro studies (Sharangi, 2009), the main ones being: antioxidants (Cooper, Morré, & Morré, 2005a), anti-inflammatory (Gonzalez, Ramirez-mares, & Puangpraphant, 2009), antisepticemic (Scoparo et al., 2013), anticarcinogenic (Cooper, Morré, & Morré, 2005b; Wu, Lu, & Chang, 2003; Wu, Yu, & Tseng, 2003; Yang et al., 2011), hypocholesterolemic (Sae-Tan, Grove, & Lambert, 2011), antiobesity (Wu, Lu, et al., 2003; Wu, Yu, et al., 2003), UV protection for the skin (Pandya, Magliocco, & Malaviya, 2004), reduced risk of cardiovascular disease (Mineharu et al., 2011; Wang, Gong, & Yan, 2010; Yang, Lu, & Wu, 2004), improved cognitive function (Kuriyama, Hozawa, Ohmori, Shimazu, & Matsui, 2006) and gastroprotection (Scoparo et al., 2014).

Recently, it was demonstrated that epigallocatechin gallate (EGCG), had anti-inflammatory effects against murine sepsis (Wheeler et al.,

* Correspondence to: N. Dartora, Departamento de Biologia, Setor de Ciências Biológicas, Universidade Estadual do Centro-Oeste, Rua Simeão Camargo Varela de Sá, 03, Guarapuava, PR 85040-080, Brazil.
** Correspondence to: M. Iacomini, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, PO Box 19031, Curitiba, PR 81531-980, Brazil.
E-mail addresses: nessa.dartora@gmail.com (N. Dartora), iacomini@ufpr.br (M. Iacomini).

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2007). Sepsis is a set of systemic severe manifestations, defined as a systemic inflammatory response syndrome (SIRS), caused by infection, mainly by bacteria. Nevertheless, it can also be the result of infection by fungi, helminths and viruses. The most common consequence of sepsis is the syndrome of multiple organ dysfunction (MODS) (Riedemann, Guo, & Ward, 2003).

Despite the therapeutic arsenal available in the clinic, sepsis remains the leading cause of mortality in intensive care units. One of the events that trigger sepsis is the access of bacteria to the bloodstream and the consequent release of their endotoxins. These endotoxins activate immune cells, especially macrophages, initiating the inflammatory response through the release of pro-inflammatory mediators such as nitric oxide (Fink & Payen, 1996; Vincent, Zhang, Szabo, & Preiser, 2000), interferon (IFN)-γ (Yin, Gribbin, & Wang, 2005), macrophage migration inhibitory factor (MIF) (Calandra et al., 2000) and cytokines, especially the cytokines TNF-α and IL-1β, IL-2, IL-6, IL-8 and IL-12 that are considered crucial for the development of sepsis (Cohen, 2002; Dejager, Pinheiro, Dejonckheere, & Libert, 2011). However, anti-inflammatory interleukins, IL-4, IL-5, IL-10, IL-11 and IL-13 are also produced (Cohen, 2002).

Thus, natural products containing various components that act in different inflammatory cascades may have an advantage on sepsis treatment over drugs that target a single mediator. So, one can infer that natural products have been the subject of intensive research regarding their effects on the pathology of sepsis. Thus, the *C. sinensis*, which presents numerous pharmacological properties since they have a wide range of chemical compounds, is an important subject of study for a thorough and extensive investigation as to their activity in sepsis. Thereby, in the present investigation, we have evaluated the effect of infusion extracts and the ethyl acetate fractions from green and black teas, which exhibit a large number of compounds identified, as well as the major components, EGCG, caffeine and theaflavins, on the decrease of lethality, cytokine activities, macrophage migration and levels of expression of pro-inflammatory enzymes in sepsis, using a murine model.

2. Materials and methods

2.1. Plant material and chemicals

Standards of (−)-epigallocatechin-3-*O*-gallate (EGCG), theaflavins and caffeine were purchased from Sigma-Aldrich. The solvents utilized were HPLC-grade methanol, formic acid, ethyl acetate, chloroform and *n*-butanol, which were acquired from Tedia. The ultra-pure water was obtained for Millipore. General solvents were from Merck.

The commercially processed leaves from *C. sinensis* samples, green and black teas, (Green tea Yamamotoyama, Midori Indústria de Chá, São Miguel Arcanjo, São Paulo, Brazil and Chá Preto Amaya, Chás Amaya, Registro, São Paulo, Brazil) were purchased in a local market (Curitiba, Brazil).

2.2. Extraction and liquid/liquid partition

Green and black tea leaves (100 g of each) from *C. sinensis* were submitted to extraction by infusion (water) for 15 min. The solvent was evaporated under reduced pressure and the resulting infusion extracts were named GTI (33%) for green tea and BTI (30%) for black tea infusion extracts, which were stored at −20 °C.

GTI and BTI (10 g) were subjected to liquid/liquid partition. The samples were dissolved in H_2O (200 ml) and the ethyl acetate (200 ml) was added and vigorously stirred. The organic layer was removed to yield green tea ethyl acetate fraction (GEAF, 45%) and black tea ethyl acetate fraction (BEAF, 48%). Subsequently, *n*-butanol (200 ml) was added to the aqueous layer, stirred and separated to give rise to green tea butanolic fraction (GBF, 37%), black tea butanolic fraction (BBF, 35%), green tea aqueous fraction (GAF, 18%) and black tea aqueous fraction (BAF, 17%). The fractions were dried under reduced pressure lyophilized and stored at −20 °C. A scheme of the liquid/liquid partition performed is shown in Fig. 1.

2.3. Phytochemical analysis

The analysis was developed with an ultra-high performance liquid chromatography system (Acquity-UPLC™, Waters, Milford, MA) equipped with a binary solvent pump, a column oven, an auto-sampler and a photodiode array detector (PDA). An Acquity BEH-C18 column, with 50 mm × 2.1 mm i.d. and 1.7 μm particle size (Waters), was employed. The separation has occurred at 60 °C (column temperature) using water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid, with a linear gradient of solvent B from 0 to 38% over 10 min, then to 60% at 13 min and 100% at 14 min, held to 15 min at flow rate of 400 μl/min. After returning to initial conditions (0% B, in 16 min) the system was re-equilibrated for 2 min.

The infusion extracts and fractions from green and black teas were prepared in MeOH–H_2O (1:1, v/v) at 1 mg/ml. The injection volume of the samples was 10 μl and detection was provided by PDA (200–400 nm) and high resolution mass spectrometry (HR-MS).

The positive (+) and negative (−) HR-MS analysis was carried out with electrospray ionization (ESI) at atmospheric pressure ionization (API) in a LTQ-OrbiTrap-XL (Thermo-Scientific), using N_2 for sample desolvation with sheath gas at a flow rate of 60 arbitrary units (abu) and auxiliary gas at 20 abu, with a source temperature of 350 °C. The ionization was performed following the operational parameters: for positive polarity, electrospray voltage at 4 kV, capillary voltage 40 V, tube lens offset 100 V and for negative polarity electrospray at 3.5 kV, capillary at −30 V and tube lens at −120 V. External calibration (m/z 100–2000) was performed prior to the sample analysis.

To quantify the major components, the EGCG, theaflavins and caffeine, calibration curves were constructed (R2 > 0.98), each prepared at 50, 100, 250, and 500 μg/ml. After that, the detection was carried out with PDA (210–400 nm), and then processing at 280 nm. The quantification was carried out in triplicate.

2.4. Animals

The vivarium of the Federal University of Paraná provided for the biological tests, with the approval of the Institutional Ethics Committee of the university (authorization number 718), albino Swiss mice (male), at 3 months and weighing 25 to 30 g. The animals were maintained under standard laboratory conditions (22 ± 2 °C and constant 12 h light/dark cycle). The availability of food (standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water was ad libitum.

2.5. Procedure to induce sepsis by cecal ligation and puncture (CLP)

The groups of mice were organized as follows: Sham-operation, CLP plus Vehicle (water p.o.); CLP plus GTI and BTI at 30 mg/kg, p.o. and 70 mg/kg, p.o.; CLP plus GEAF and BEAF at 30 mg/kg, p.o. and 70 mg/kg, p.o.; and CLP plus EGCG (10 mg/kg), theaflavins (9 mg/kg) and caffeine (18 mg/kg), p.o. The compound dosages were calculated based on their quantification in ethyl acetate fractions. Each group consisted of 8 mice. Each test-solution was administered according to their body weight.

In order to anesthetize the mice prior to the surgical procedures, ketamine (80 mg/kg) and xylazine (20 mg/kg) were injected intraperitoneally. The induction of polymicrobial sepsis by CLP was performed as previously described for Rittirsch, Huber-Lang, Flierl, and Ward (2009), consisting, in synthesis: the abdomen undergoes a midline incision of ~1.5 cm, exposing the cecum and performing a ligation of 50% of the distal moiety and then punctured (3 times with a 16G needle). The perforations were confirmed by a light manual pressure, which resulted in a small amount of leakage of intestinal contents. After these procedures, the perforated intestine was immediately placed back into the

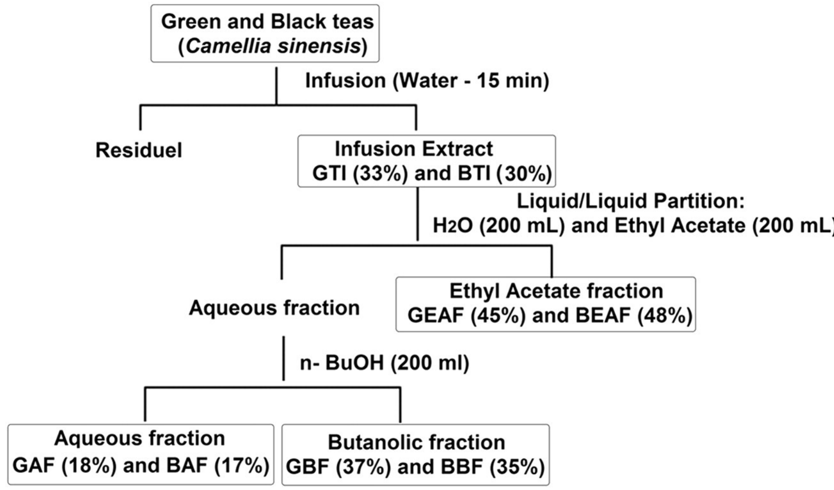

Fig. 1. Scheme of liquid/liquid partition of green and black-teas from *Camellia sinensis*. The respective yields of each fraction were demonstrated.

abdominal cavity and the incision was surgically sutured. The group Sham-operation was treated identically, but no cecal ligation or puncture was carried out, only the valve was exposed.

After surgery, all animals received a subcutaneous injection of isotonic saline (1 ml/100 g) for fluid resuscitation and were placed in heated boxes (35 °C) until full recovery from anesthesia. The treatment of each group was maintained for seven days following surgery. During this period, the mortality of the animals was measured every 12 h. Subsequently, the mortality of the different groups was compared. During this period, daily, food and water were provided at will and the water and treatments were administered orally.

In another experimental set (1 h before surgery), mice were orally treated with Vehicle, GEAF (30 mg/kg), BEAF (70 mg/kg), EGCG (10 mg/kg), theaflavins (9 mg/kg) and caffeine (18 mg/kg) p.o., since these fractions resulted in better responses in the lethality test. The mice were sacrificed after 6 h post-operation. Their lung, ileum tissues and their blood were collected and frozen for further use to determine the myeloperoxidase (MPO) activity, the tissue expression of iNOS and cyclooxygenase-2 (COX-2), and the concentration of serum cytokine, respectively.

2.6. Myeloperoxidase activity

The determination of myeloperoxidase activity is used as a marker for neutrophil infiltration into tissues (Bradley, Priebat, Christensen, & Rothstein, 1982). The lung tissues were homogenized in HTAB buffer (0.5 ml of 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide), sonicated on ice, and centrifuged at 14,000 rpm for 15 min at controlled temperature (4 °C). To the supernatant was added 9.6 mM TMB (3,3,5,5-tetramethylbenzidine) and 150 nmol/l hydrogen peroxide in 50 mM potassium phosphate buffer, at a 1:20 dilution in the reaction buffer. The activity was then measured spectrophotometrically at 620 nm, at 37 °C using a microplate reader. The results are expressed as change in optical density per milligram of protein (measured by Bradford assay).

2.7. Determination of serum cytokine concentration

1 ml of blood was collected from the groups of animals 6 h after CLP, added to sodium citrate, anticoagulant and the blood plasma was separated by centrifugation and stored at −80 °C (Takahashi et al., 2001). Determination of serum concentrations of IL-10, TNF, and IL-6 was performed using the Mouse Inflammation Kit (BD™ Cytometric Bead Array), according to the manufacturer's instructions and analyzed by a flow cytometer FACSCalibur flow-BD Pharmingen, FCAPArray software v. 3.

2.8. Western blot analysis

Ileum samples from the animal groups were also collected after 6 h of CLP, washed twice with PBS and then homogenized and lyzed in extraction buffer. The composition of the buffer consists in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogene). The total proteins (20 μg) were separated for 2 h on 8% SDS-PAGE (Sigma) at 80 V. Separated proteins were transferred for 120 min electrophoretically onto polyvinylidene difluoride membranes (Bio-Rad) at 100 V. Membranes were blocked with blocking buffer containing 3% low fat milk powder, Tris-buffered saline solution (Bio-Rad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h.

Then, membranes were incubated overnight at 4 °C with primary antibodies of both iNOS and COX-2 (dilution of 1:1000). After washing, membranes were incubated for 60 min with the secondary antibody (peroxidase-labeled anti-mouse IgG, dilution of 1:5000) at room temperature. The detection of β-actin protein was used for quantification and standardization of iNOS and COX2, respectively. A molecular weight marker (Invitrogen) was used and immunoreactive bands detected by chemiluminescence (Bio-Rad kit), and then evaluated qualitatively and quantitatively.

2.9. Statistical analysis

Data were expressed as means ± SE of five mice examined in each group. Statistical error was determined by one-way ANOVA; the post hoc test was Bonferroni's. Calculations were performed with Graphpad Prism 5.0. p values <0.05 were considered significant.

3. Results and discussion

3.1. Phytochemical analysis

The phytochemical composition of infusions from green and black teas was evaluated by liquid chromatography and the compound identification was performed on the basis of high resolution mass spectrometry and UV-spectra, by comparison with previous reports (Dou, Lee, Jason, & Lee, 2007; Lin, Chen, & Harnly, 2008; Scoparo et al., 2012, 2014).

Many compounds were identified in the infusions, including components from primary metabolism, such as oligosaccharides (peak 1), but

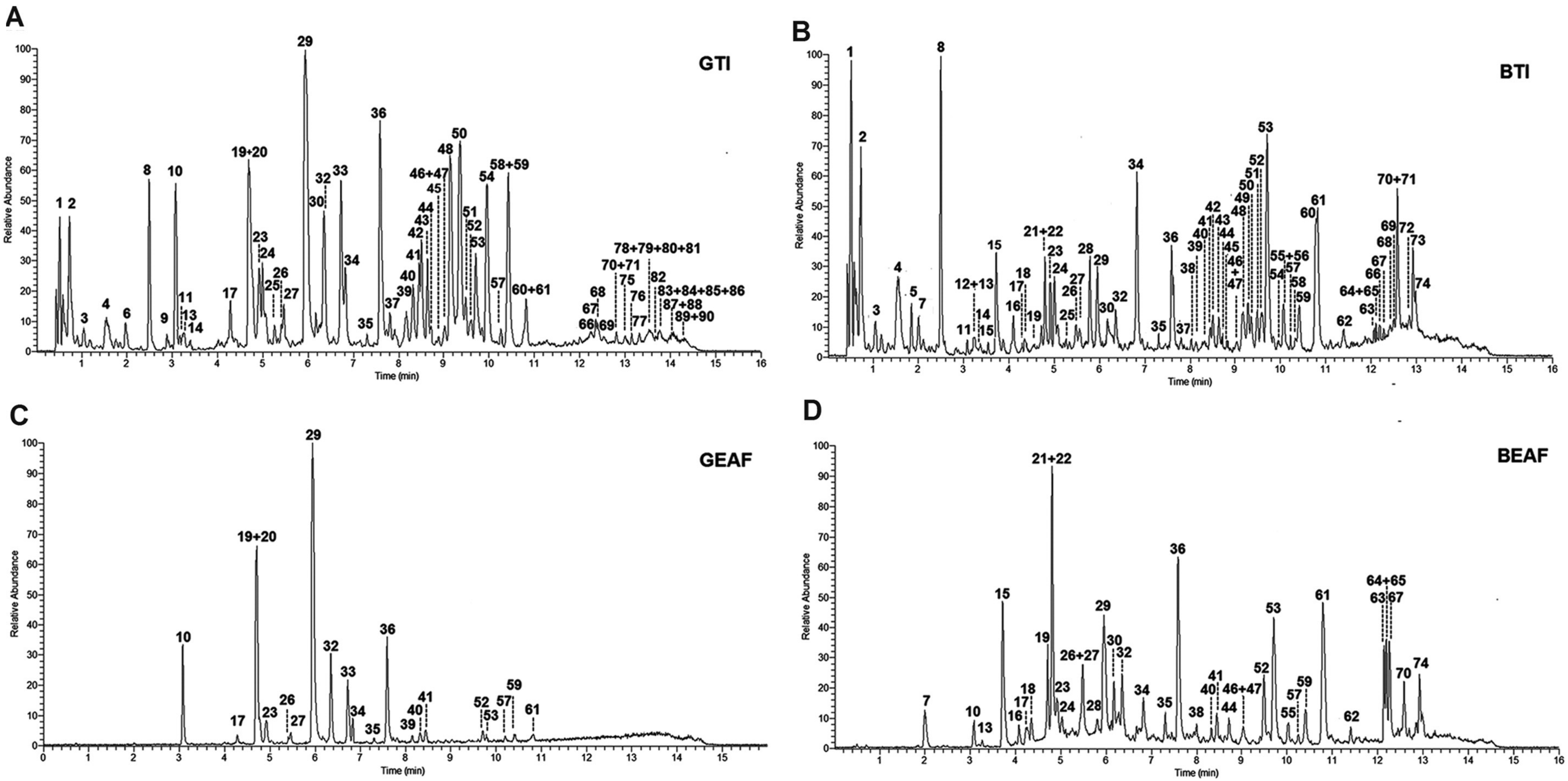

Fig. 2. UHPLC–MS chromatogram TIC (total ion current) from *C. sinensis* A) crude infusion (GTI) and ethyl acetate fraction (GEAF) of green tea; B) crude infusion (BTI) and ethyl acetate fraction (BEAF) of black tea.

mainly components from secondary metabolism were identified, such as saponins (peaks 75–90), caffeine (peak 31) and, particularly, several phenolic compounds, such as hydroxybenzoic acid (peaks 7, 8 and 9), hydroxycinnamic acids (peaks 11, 12 and 14), and flavonoids (peaks 19, 20, 29, 32, 36, 39, 40–62, 68–74) (Fig. 2A and B). The complete identification of compounds present in the infusions (GTI and BTI) can be found at Table S1 (supplementary data).

Considering the extreme complexity of the phytochemical composition from infusions, hindering the identification of possible active compounds, the infusions were submitted to a liquid/liquid fractionation, in order to separate the compounds according to their polarities. Moreover, the main compounds reported in green and black teas are flavonoids, mainly catechins, and these compounds can be concentrated in the ethyl acetate fraction (Scoparo et al., 2012, 2014). Since the catechins [e.g. catechin (C), epicatechin (EC), epigallocatechin (EGC), epigallocatechin-3-*O*-gallate (EGCG), and epicatechin-3-*O*-gallate (ECG)], have recognized biological activities, the ethyl acetate fractions from green and black tea infusions were assayed for the pharmacological properties.

These catechins were observed in the analysis of ethyl acetate fraction from green tea (GEAF), yielding negative ions $[M-H]^-$ on LC-MS, as gallocatechin at m/z 305 (peak 10), epigallocatechin at m/z 305 (peak 19), catechin at m/z 289 (peak 20), gallocatechin-3-*O*-gallate at m/z 457 (peak 29), catechingallate at m/z 441 (peak 36), epicatechingallate at m/z 441 (peak 39) and the epigallocatechin-3-*O*-gallate (EGCG) at m/z 457 (peak 29) (Fig. 2C, Table 1).

During the oxidation of black tea, it is well established that the monomeric flavanols undergo polyphenol oxidase-dependent polymerization, which leads to the conversion of catechins into theaflavins, theasinensins and thearubigins (Engelhardt, 2010; Obanda, Owuor, & Mang'oka, 2001, Robertson, 1992).

Thus, in the ethyl acetate fraction BEAF only traces of catechins were observed present, instead a series of condensation products was observed in the negative ionization $[M-H]^-$, as theaflavin at m/z 563 (peak 63), theaflavin-3-gallate at m/z 715 (peak 64), theaflavin-3′-gallate at m/z 715 (peak 65) and theaflavin-3,3′-digallate at m/z 863 (peak 67). Also during the tea oxidation, the accumulation of epigallocatechin quinone dimmers results in the formation of theasinensins, identified as theasinensin B with m/z 761 and theasinensin A and D with m/z 913 (peaks 16, 21 and 23) (Fig. 2D).

Flavonol glycosides were found in both, GEAF and BEAF. These are composed by myricetin, quercetin and kaempferol as mono-, di- and tri-glycosides (peaks 41, 41, 53, 59, 61, and 62) as well as the tetraglycosides of quercetin and kaempferol appearing acylated with *p*-coumaroyl (peaks 70 and 74). The amounts of flavonol glycosides in black tea seem to be higher than catechins, this occurs because the

Table 1
UHPLC–MS of the compounds in the ethyl acetate fraction (GEAF and BEAF) of green tea and black tea of *C. sinensis*.

Peak	R_t[a]	$[M-H]^-$ (m/z)	Structure	GEAF	BEAF
7	2.00	169	Gallic acid		x
10	3.07	305	(+)-Gallocatechin	x	x
13	3.26	609	Gallocatechin dimer		x
15	3.71	761	Prodelphinidin B-2-3′-*O*-gallate		x
16	4.09	761	Theasinensin B		x
17	4.28	761	Epigallocatechin-(4α ➔ 8)epigallocatechin-3-*O*-gallate	x	x
18	4.35	745	Gallocatechin-catechingallate		x
19	4.71	305	(−)-Epigallocatechin	x	x
20	4.73	289	(+)-Catechin	x	
21	4.80	913	Theasinensin A		x
22	4.83	577	Proanthocyanidin dimer	x	x
23	4.92	913	Theasinensin D		x
24	5.00	865	Proanthocyanidin trimer		x
26	5.46	577	Proanthocyanidin dimer	x	x
27	5.48	885	Proanthocyanidin trimer	x	x
28	5.79	729	Procyanidin B-2 (or 4)-3′-*O*-gallate		x
29	5.94	457	(−)-Epigallocatechin-3-*O*-gallate	x	x
30	6.16	729	Procyanidin B-2 (or 4)-3′-*O*-gallate		x
31	6.32	195[a]	Caffeine[a]	x	x
32	6.34	289	(−)-Epicatechin	x	x
33	6.72	457	(+)-Gallocatechin-3-*O*-gallate	x	
34	6.82	609	Gallocatechin dimer	x	x
35	7.35	635	Trigalloylglucose	x	x
36	7.59	441	(+)-Catechingallate	x	x
38	8.00	609	(−)-Gallocatechin-3–3′-di-*O*-gallate		x
39	8.15	441	(−)-Epicatechingallate	x	
40	8.32	479	Myricetin-3-*O*-galactoside	x	x
41	8.45	479	Myricetin-3-*O*-galactoside	x	x
44	8.72	425	(−)-Epiafzelechin gallate		x
46	9.00	615	Quercetin-galloyl-hexoside		x
47	9.04	431	Apigenin-C-glucoside		x
52	9.61	609	(−)-Epigallocatechin-3-3′-di-*O*-gallate	x	x
53	9.71	463	Quercetin-3-*O*-glucoside	x	x
55	10.04	851	Theaflavate A		x
57	10.23	599	Kaempferol-galloyl-hexoside	x	x
59	10.44	755	Kaempferol-3-*O*-glucosylrutinoside	x	x
61	10.83	593	Kaempferol-3-*O*-*p*-coumaroylglucoside	x	x
62	11.41	785	Kaempferol-3-*O*-acetyldirhamnosylhexoside		x
63	12.11	563	Theaflavin		x
64	12.14	715	Theaflavin-3-gallate		x
65	12.19	715	Theaflavin-3′-gallate		x
67	12.27	863	Theaflavin-3–3′-digallate		x
70	12.58	901	Quercetin 3-*O*-*p*-coumaroyldirhamnosylgalactoside	x	x
74	12.98	901	Quercetin 3-*O*-*p*-coumaroyldirhamnosylglucoside		x

[a] Identified in positive ESI/MS.

Table 2
Concentration of major compounds in green and black tea extracts and fractions.

Extract	Epigallocatechin-3-O-gallate[a]	Theaflavins[a]	Caffeine[a]
GTI	142.63 ± 0.45	0.00 ± 0.00	71.79 ± 0.641
BTI	28.04 ± 0.78	73.94 ± 0.876	168.12 ± 0.57
GEAF	329.92 ± 0.88	0.00 ± 0.00	120.45 ± 0.47
BEAF	66.39 ± 0.76	131.84 ± 0.96	262.33 ± 0.87

GTI, green tea infusion extract; GEAF, green tea ethyl acetate fraction; BTI, black tea infusion extract; BEAF, black tea ethyl acetate fraction.
[a] The yields are presented as μg/mg.

flavonol glycosides are not substantially decomposed during the manufacturing process, remaining in high proportions, unlike that of catechins that undergo oxidation observing a decrease in their proportion. Proanthocyanidins were also found in GEAF and BEAF, as dimers (peaks 13, 18, 22 and 26) and trimers (peaks 15, 17, 24, 27, 28 and 30). Theaflavate A (peak 55) was also identified, and as in our previous investigation (Scoparo et al., 2012), glycosides of myricetin, quercetin and kaempferol containing esters of gallic acid were observed, yielding quercetin-galloyl-hexoside at m/z 615 $[M–H]^-$ (peak 46) and kaempferol-galloyl-hexoside at m/z 599 $[M-H]^-$ (peak 57) (Fig. 2A and B, Table 1).

It is worth noting that EGCG is the major bioactive compound from green tea, as well as theaflavins were found in significant amounts in black tea, and caffeine was found at considerable abundance on both. So, in order to evaluate their influence in the pharmacological effects, these compounds were subjected to quantification in the samples and, subsequently, assayed in the pharmacological model. EGCG was present at 142.63 ± 0.45 μg/mg in the crude infusion (GTI) and appearing at high levels in GEAF 329.92 ± 0.88 μg/mg. In black tea, as discussed above, the processing leads to changes in the chemical composition, resulting in a decrease of EGCG concentrations, as 28.04 ± 0.78 μg/mg in BTI and 66.39 ± 0.76 μg/mg in BEAF. Caffeine was found at 71.79 ± 0.64 μg/mg in GTI and 120.45 ± 00.47 μg/mg in GEAF, whereas higher amounts were found in black tea, as 168.12 ± 00.57 μg/mg in BTI and 262.33 ± 0.87 μg/mg in BEAF. Theaflavins, present only in black tea, appeared at 73.94 ± 0.876 μg/mg in BTI, with a considerable increase in BEAF, 131.84 ± 0.96 μg/mg (Table 2).

Taking into consideration that the literature refers to the biological activities of the compounds found in green and black teas, and in this approach the flavonoids (i.e. catechins) are the main drivers of these studies due to numerous proven pharmacological properties of these compounds (Balentine, Wiseman, & Bouwens, 1997; Cabrera, Gimenez, & Lopez, 2003), the green and black tea extracts, as well as the ethyl acetate fraction from both teas and compounds found in significant amounts in this samples (EGCG, caffeine and theaflavins), were selected for pharmacological investigation on murine sepsis and its mechanism of action.

3.2. Effects of GTI, BTI, GEAF and BEAF on the survival rate of septic mice

GTI and BTI were tested at doses of 30 and 70 mg/kg, and their effect on the survival rate of infected mice was determined. As observed in Fig. 3A, the mortality rates have different profiles among groups. No death was observed in the group of false-operated animals (Sham), indicating that the incision and the abdominal suture were not enough to cause death. The area under the curve for this group was 16.800 arbitrary units. However, the group treated only with water (Vehicle group) exhibited symptoms of severe sepsis, such as reduced mobility, apathy, piloerection and nasal discharge. The mortality in this group reached 100% in 36 h after CLP, showing an area under the curve of 1.800 arbitrary units. Thus, Sham and Vehicle groups resulted in the expected reactions, proving that it is the CLP procedure that causes the development of sepsis and the consequent decrease of the survival rate.

The treatments with the green and black extracts resulted in a decrease of the mortality. The survival rate of the animals treated with

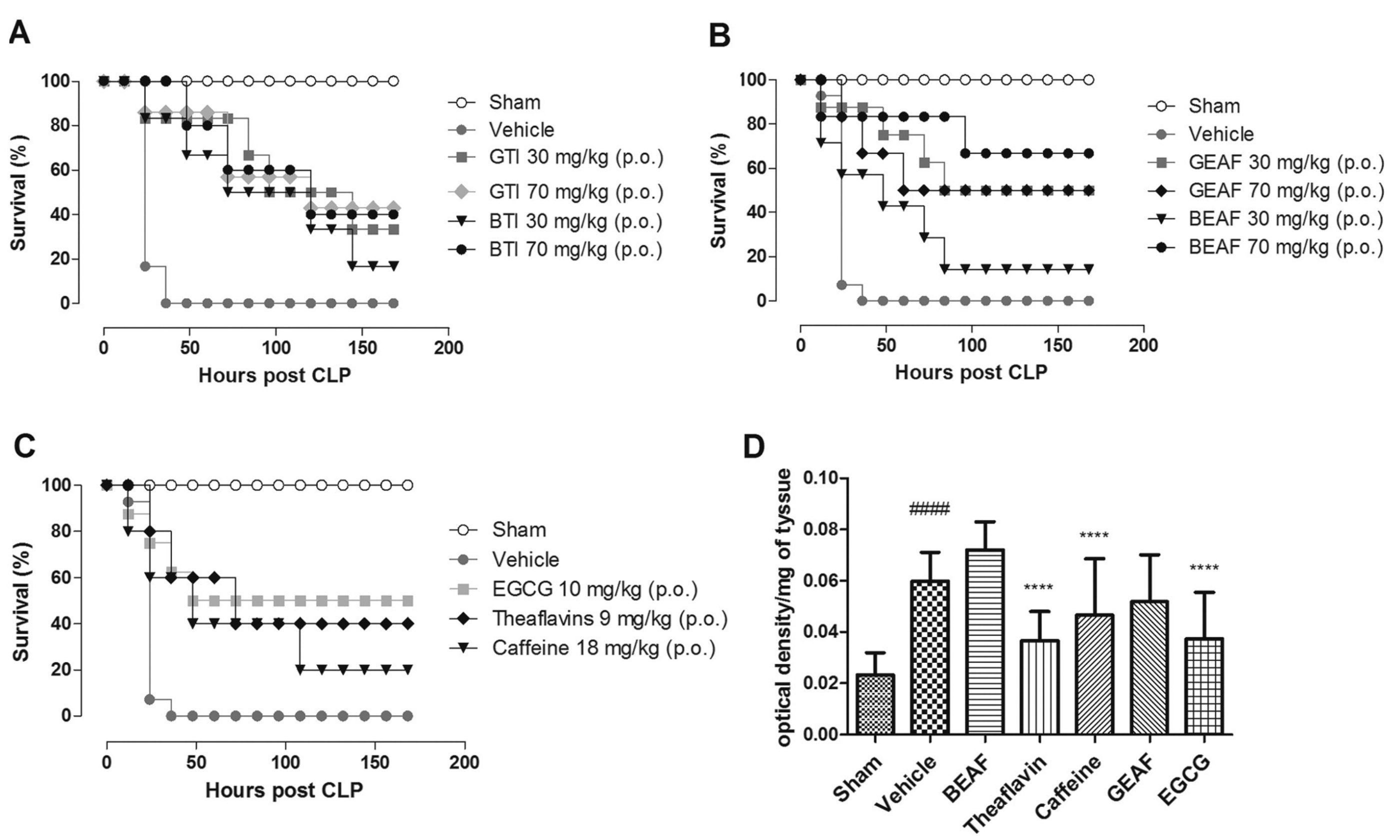

Fig. 3. Effect of GTI and BTI (A), GEAF and BEAF (B), EGCG, theaflavins and caffeine on survival rate (C) and on MPO activity (D) in sepsis. Values represent means ± SEM. ****$p < 0.0001$, indicated value versus CLP plus vehicle group; ####$p < 0.0001$, CLP plus vehicle versus sham. ANOVA followed by Bonferroni's test.

GTI at doses of 30 and 70 mg/kg was 33 and 43%, with areas under the curve of 11.000 and 10.986, respectively, whereas the survival rate of mice treated with BTI was 16.7% and 40% at doses 30 and 70 mg/kg, with areas under the curve of 9.100 and 11.160 (Fig. 3A).

Because of the complexity of phytochemical composition of infusions, liquid/liquid partitions were carried out and the ethyl acetate fractions, containing the main bioactive components were evaluate for their pharmacological potential. Both GEAF and BEAF had a significant effect on survival of mice (Fig. 3B). The lethality was markedly reduced in mice treated with GEAF and their areas under the curve were increased to 10.800 and 10.100 (arbitrary units) after administrating 30 and 70 mg/kg, respectively, when compared to the group treated with vehicle. At the end of the period, the overall survival in these GEAF groups was 50%. In the group treated with BEAF with a dose of 70 mg/kg, survival was greater, achieving 66.7%, with an area under the curve of 12.800. As shown in Fig. 3B, no death was observed in the Sham-operated mice and its corresponding area under the lethality curve was 16.800 (arbitrary units).

As observed by UHPLC analysis, caffeine was present at high contents in both, GEAF and BEAF, whereas higher amounts of EGCG appeared in GEAF and the theaflavins were exclusive from BEAF. Therefore, we have evaluated the effects of these major compounds in the CLP assays, using standards of (−)-epigallocatechin-3-*O*-gallate, theaflavins and caffeine. The doses of each standard were calculated on the basis of their content in the ethyl acetate fractions. The survival rate at the end of the treatment period was 50% for the group treated with EGCG (10 mg/kg), 40% for the group treated with the theaflavins (9 mg/kg) and 20% for the group treated with caffeine (18 mg/kg), with areas under curve of 9.600, 9.000 and 6.720, respectively (Fig. 3C).

These results demonstrate that EGCG may be the most responsible for protection induced by GEAF against sepsis. As shown by the results of mice lethality (Fig. 3B and C), both GEAF and EGCG reduced lethality by 50%. Similarly, theaflavins and caffeine seem to possess a synergistic effect on BEAF effects. Obviously, it can be considered that multiple bioactive compounds present in the extracts and fractions can contribute to the final effects.

Since the ethyl acetate fractions were more efficient in the treatment of murine sepsis, GEAF, BEAF and its major components (i.e. EGCG, caffeine and theaflavins) were evaluated on important inflammatory parameters, in order to elucidate the possible mechanisms of action.

3.2.1. Myeloperoxidase activity

MPO activity has been used as a marker for neutrophil infiltration into tissues, during septicemia and the lungs are directly affected by the migration and activation of neutrophils, with intense oxidative activity, involved in the formation of reactive oxygen species thus resulting in tissue damage and impaired organ function (Bradley et al., 1982; Landry & Oliver, 2001; Zemans, Colgan, & Downey, 2009).

Many secondary metabolites, especially phenolic compounds, have their efficacy against murine sepsis proven by decreasing the production level of myeloperoxidase and consequently the level of reactive species of oxygen. Among these we can cite an isolated fraction of *Eugenia uniflora*, containing a lot of glycosides of quercetin and myricetin (Rattmann et al., 2012). Myricytrin, a 3-O-rhamnoside of myricetin, also demonstrated a strong inhibiting power of myeloperoxidase (Meotti et al., 2008). Anthocyanins from blackberry also reduced the death of animals with sepsis by decreasing the activity of myeloperoxidase in the lungs and ileum (Shapiro, Lev, Cohen, & Singer, 2009).

CLP surgery markedly increased the MPO levels in the lung tissues in comparison with the Sham group (61.1%) (Fig. 3D). This rise in tissue MPO was significantly prevented by caffeine, theaflavins and EGCG (at doses cited above), with an inhibition of 50.4, 42.6 and 41.8%, respectively, in comparison to the Vehicle group (Fig. 3D). In order to evaluate the established inhibition rate, the results were also normalized relative to the Sham group, using a ratio factor based on basal response (Sham):CLP response with a numerical value of 0.389. This normalization gave the follow inhibitions: 129.5%, 109.5% and 105.4%, respectively.

Treatment of animals with ethyl acetate fractions of green (GEAF) and black teas (BEAF) did not result in significant modification of MPO activity, with results similar to the Vehicle group. It can be inferred that the fractions showed no effect on the infiltration of neutrophils in the lungs of mice with sepsis, at doses (30 mg/kg and 70 mg/kg, respectively) and time of 6 h after CLP. However, the EGCG, caffeine and theaflavins were more efficient in expression of MPO. This may be due to an inhibitory effect which may be caused by the presence of

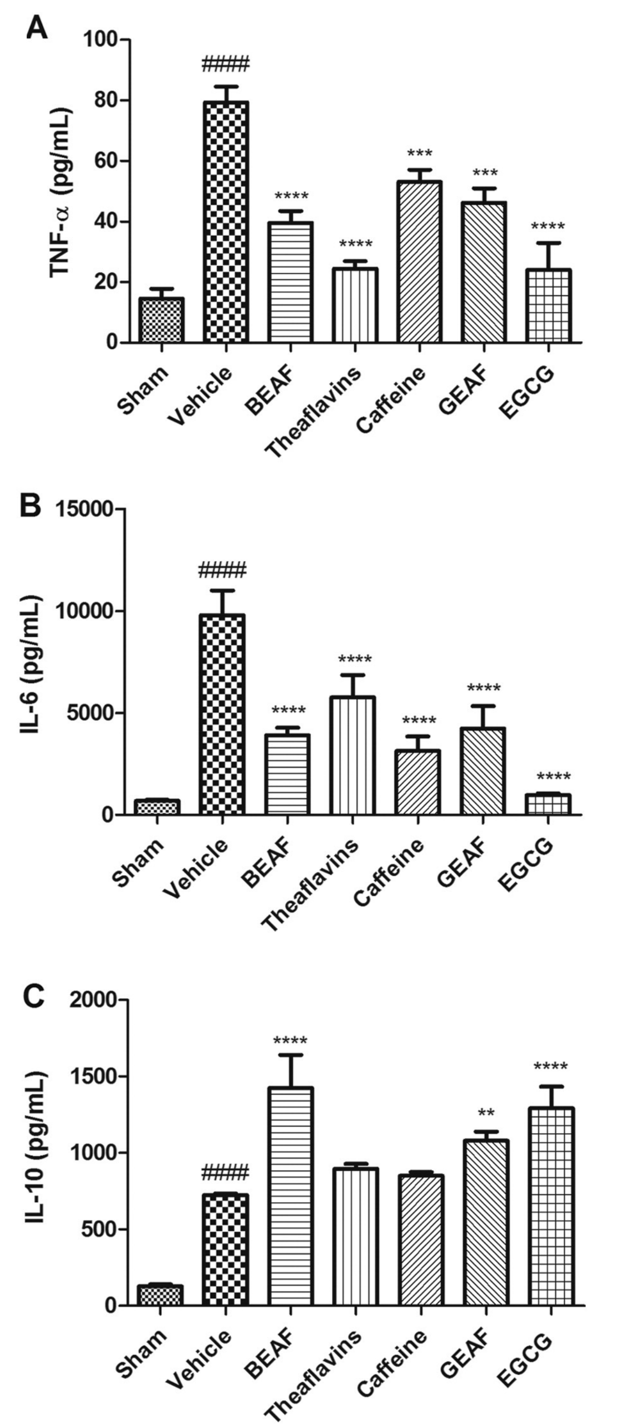

Fig. 4. Effect of BEAF, theaflavins, caffeine, GEAF and EGCG on pro-inflammatory cytokine levels: TNF-α (A), IL-6 (B) and IL-10 (C). Values represent means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$, indicated value versus CLP plus vehicle group; $^{\#\#\#\#}p < 0.0001$, CLP plus vehicle versus sham. ANOVA followed by Bonferroni's test.

numerous compounds identified in the fractions, which together may be reducing the effect noted for standard compounds. Furthermore, the compounds present in the fractions can act preventing sepsis by some other protection mechanism, as assessed at the following experiments. Corroborating this, some studies have shown effective compounds against sepsis, but which nevertheless have no effect on the activity of myeloperoxidase. A good example is the Rofecoxib, a molecule with a protective effect in sepsis that did not alter the activity of myeloperoxidase, acting as a selective inhibitor of COX-2 (pro-inflammatory enzyme) (Kara et al., 2004).

3.2.2. Evaluation of proinflammatory cytokines (IL-6, TNF-α) and anti-inflammatory cytokines (IL-10)

Sepsis is caused by over-activation of the innate immune system, leading to the release of large amounts of inflammatory mediators including cytokines and chemokines. Several studies have demonstrated that some cytokines, especially IL-6 and TNF-α are strongly associated with sepsis syndrome, therefore inhibiting the pro-inflammatory cytokine overproduction during early sepsis may reduce its risks (Song & Kellum, 2005). In the present study, the GEAF and BEAF fractions and their major components were able to decrease significantly the serum levels of TNF-α and IL-6 (Fig. 4A and B), and could be related to the increased survival rate.

The levels of TNF-α and IL-6 were lower in Sham control. In contrast, 6 h after CLP surgery, both cytokines showed an increase of 81.7 and 93.3%, respectively (Fig. 4A and B). Treatment with BEAF, theaflavins and caffeine reduced levels of TNF by 50.1%, 69.2% and 33.0% respectively (or 250.5%, 346.0% and 165.0% normalized by Sham group) and IL-6 levels were also inhibited by 60.2%, 41.3% and 68.1% respectively (or 872.4%, 598.5% and 986.9% when

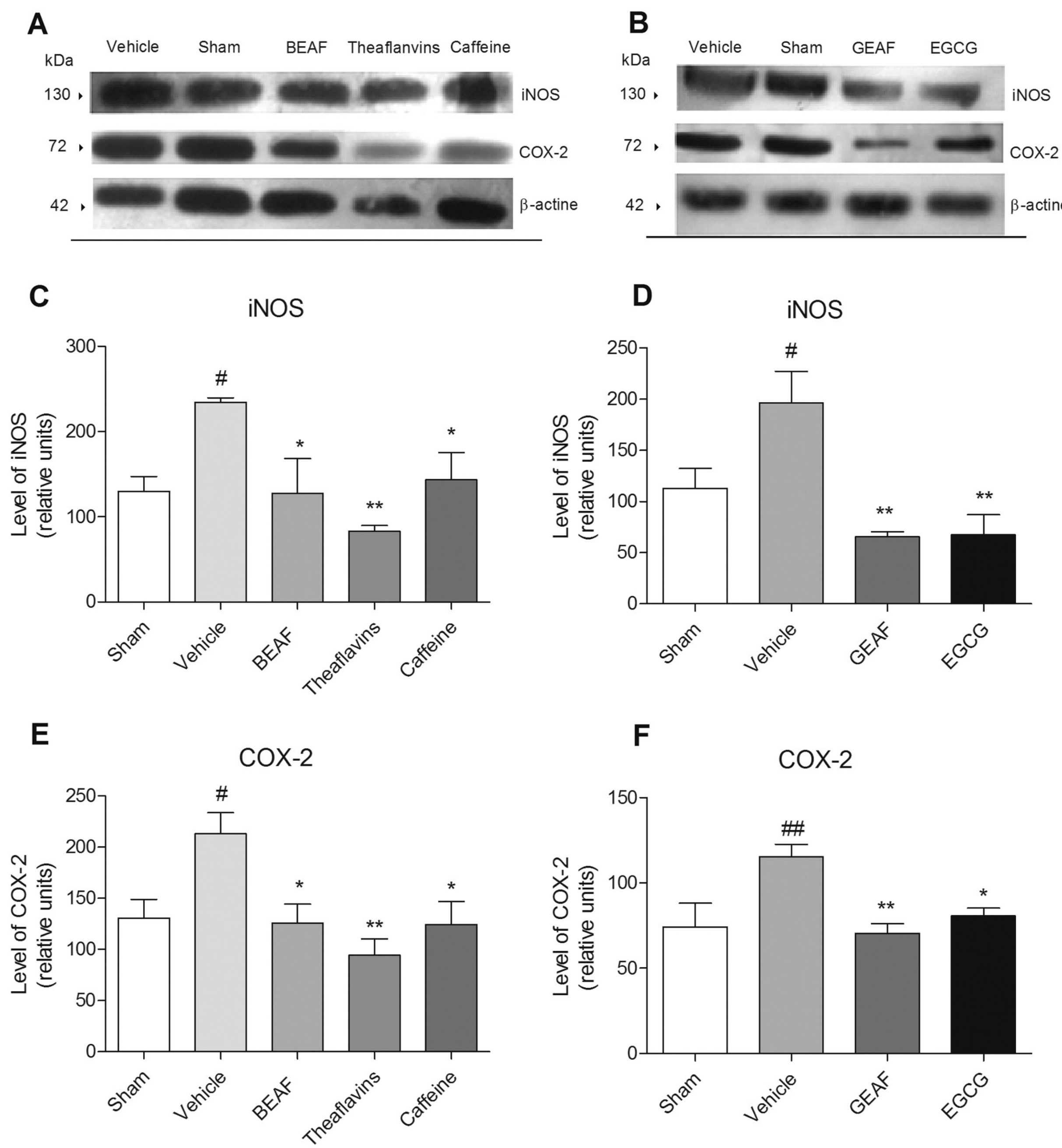

Fig. 5. Effect of BEAF, theaflavins and caffeine on iNOS (B) and COX-2 (C) and effect of GEAF and EGCG on iNOS (D) and COX-2 (E) expression in the ileum tissue of infected mice. The levels of iNOS and COX-2 were determined by Western blot analysis. (A and B) Representative immunoblots. Results are shown as the means ± SEM of 3 to 4 different experiments. #p < 0.05 CLP plus vehicle versus sham. *p < 0.05 and **p < 0.01, treatments versus vehicle.

normalized by Sham group) (Fig. 4A and B). The treatment with GEAF and EGCG, reduced levels of TNF expression in 41.8% and 69.7% (or 209.0% and 348.5% when normalized by Sham group) and IL-6 expression levels in 56.8% and 90.2% (or 823.2% and 1307.2% normalized by Sham group) (Fig. 4A and B).

Included in the initial hyper-inflammatory response is a nearly simultaneous production of anti-inflammatory proteins, such as IL-10, which dampen and terminate the inflammatory response. It was observed an increase in IL-10 expression of 89.2%, 13.2% and 17.2% after treatment with BEAF, caffeine and theaflavins, compared to the Vehicle group (or 527.8%, 78.1% and 101.7% normalized by Sham group). On the other hand, the treatment with GEAF and EGCG caused an increase of 43.6% and 71.7% in levels of expression of IL-10 (or 258.0% and 424.3% normalized by Sham group).

It has been observed that the phenolics and catechins present a decrease in pro-inflammatory cytokines (TNF-α and IL-6) which can be accompanied by an increase in IL-10, this cytokine with anti-inflammatory activity (Chen, Kuo, Chai, Ou, & Yang, 2007; Di Paola et al., 2006; Giamarellos-Bourboulis et al., 2006; Kawaguchi et al., 2004; Kanno et al., 2006; Li et al., 2007). This data is in accordance with present results that the protective effect of fractions and its components can be caused in part by the reduction of pro-inflammatory cytokines (IL-6 and TNF-α) and increased anti-inflammatory cytokine (IL-10).

3.2.3. Inhibition of iNOS and COX-2 expression

Besides the cytokines, the nitric oxide (NO) and eicosanoids are also mediators involved in the excessive pro-inflammatory response during sepsis. Overproduction of NO by induced nitric oxide synthase (iNOS) is associated with septic shock, considered the main cause of mortality among the septic patients (Landry & Oliver, 2001). Thus, compounds that inhibit iNOS expression or its activity will have anti-inflammatory properties (Min-Sun et al., 2012). Another important maker is cyclooxygenase-2 (COX-2), which is absent or present at low levels in most tissues, but highly expressed in response to endotoxins, viruses, inflammatory stimuli, pro-inflammatory cytokines and reactive oxygen species of mitochondria in various organs, tissues and cells (Aronoff, 2012; Ejima et al., 2003).

In order to investigate the expression of pro-inflammatory enzymes in the ileum, the levels of iNOS and COX-2 were determined by immunoblotting after different treatments (Fig. 5A and B). In a first treatment, CLP increased the levels of iNOS in comparison with the Sham group in 44.4%, and BEAF, theaflavins and caffeine decreased the levels of iNOS by 45.6%, 64.5% and 38.8% respectively (or 82.0%, 116.0% and 69.8% normalized by Sham group) (Fig. 5C).

In the second experiment, the treatment of animals with the vehicle (water) increased iNOS expression by 42.5% whereas GEAF and EGCG reduced this in 66.7% and 65.7% when compared to the Vehicle group (or 116.0% and 114.3% normalized by Sham group) (Fig 5D).

The COX-2 expression in the ileum was reduced by 40.9%, 55.8% and 41.5% (or 66.6%, 90.0% and 67.6% — normalized by 0.614 ratio factor) after treating with BEAF (30 mg/kg), theaflavins (9 mg/kg) and caffeine (18 mg/kg) respectively (Fig. 5E). GEAF and EGCG also affected COX-2 expression, reducing by 39.1% and 30.2% (60.9% and 47.0% normalized by Sham group), respectively (Fig. 5F). In contrast, for all the experiments, the level of β-actin (constitutive isoform) remained the same (Fig. 5A and B).

Once the tested fractions, as its major components, reduced levels of expression of proinflammatory cytokines (TNF-α and IL-6) and since these are associated with the expression of proinflammatory enzymes, the decrease of expression levels of both enzymes (iNOS and COX-2) was observed. Indeed, the effect of phenolic compounds was associated with the attenuation of local and systemic production of NFkB-dependent inflammatory mediators, including TNF-α, iNOS and COX-2 (Shapiro et al., 2009).

4. Conclusion

Green and black tea extracts and ethyl acetate fractions have several compounds, including carbohydrates, flavonoids, alkaloids and saponins with recognized therapeutic potential. The use of natural extracts confers an advantage, as they are cheaper than synthetic drugs and easier to obtain than isolated compounds. Considering that the overwhelming inflammatory and immune responses during the early stage of sepsis involve a vast array of mediators, it is critical to control this complex inflammatory cascade and, consequently to manage the sepsis effects (Cohen, 2002). Green and black tea infusions, ethyl acetate fractions as well as the standard compounds resulted in a decrease in mortality caused by sepsis. We realize that this protective effect could well be promoted, in part, by the decrease in MPO activity, reduction of proinflammatory cytokines (IL-6 and TNF-α) and increased anti-inflammatory cytokine (IL-10) and also a decrease in expression levels both enzymes (iNOS and COX-2), demonstrating the possible control of the complex inflammatory cascade and thus the management of sepsis effects.

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