Rui Pinho Moreira dos Santos

Avaliação da actividade antioxidante da folha e fruto da espécie *Dracaena draco* L.

Universidade Fernando Pessoa

Faculdade de Ciências da Saúde

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Trabalho original realizado por:

Trabalho apresentado à Universidade Fernando Pessoa como parte dos requisitos para obtenção do grau de Mestre em Ciências Farmacêuticas.

Orientador:

Professora Doutora Márcia Carvalho

Co-orientador:

Professora Doutora Branca Silva

RESUMO

O dragoeiro (*Dracaena draco* L.) é uma espécie arbórea, da família das Dracaenaceas, pertencente à flora da Macaronésia. Trata-se de uma das mais raras espécies arbóreas de Portugal, já mal representada tanto nos Açores como na Madeira. Tradicionalmente, a sua casca preparada por decocção era usada contra a atonia do tubo digestivo, diarreia, males estomacais, impurezas do sangue, catarros pulmonares, hemorragias e também como vermífugo e tónico. Em banhos e fomentos, este decocto era utilizado pela medicina popular no combate a tumores sifílicos. A seiva do tronco e dos ramos, conhecida por *sangue-de-drago* ou *sangue-de-dragão,* foi muito utilizada na medicina popular devido às suas propriedades antioxidantes e adstringentes. No entanto, até à data pouco se sabe no que concerne as propriedades biológicas da folha e fruto desta espécie. Por este motivo, o presente trabalho apresenta como objectivo a avaliação da actividade antioxidante de extractos aquosos de folha e fruto de dragoeiro através da sua acção protectora relativamente aos danos oxidativos induzidos por radicais livres em eritrócitos humanos. O 2,2´-azo-bis(2-amidinopropano) (AAPH) foi usado como sistema gerador de radicais livres que atacam a membrana eritrocitária causando várias alterações oxidativas, as quais foram avaliadas neste estudo pela indução da hemólise.

Os ensaios realizados mostram uma actividade antioxidante para o extracto do fruto superior ao da folha. Ambos os extractos protegem a membrana do eritrócito da hemólise induzida pelo AAPH de uma forma dependente da concentração de extracto e do tempo de incubação, obtendo-se valores de IC_{50} de 2,56 ± 0,97 µg/mL para o fruto e de 39,05 ± 11,54 µg/mL para a folha. Dada a reconhecida actividade antioxidante do morango, a actividade anti-hemolítica do fruto de dragoeiro foi comparada com a do morango. O valor de IC_{50} para o extracto de fruto de dragoeiro foi significativamente superior ao calculado para o extracto de morango (273,84 ± 49,38 µg/mL), o que enfatiza a forte actividade antioxidante do fruto.

Em conclusão, os resultados obtidos neste trabalho indicam que a espécie *D. draco* L., particularmente o fruto, apresenta um considerável potencial antioxidante e sequestrador de radicais livres, o que sugere a sua eventual aplicação na prevenção e/ou tratamento de diversas patologias nas quais os radicais livres estão implicados.

ABSTRACT

The dragon tree (*Dracaena draco* L.) is a tree that belongs to the Dracaenaceae family, which is natural in the flora of Macaronesia. This is one of the rarest tree species in Portugal, already poorly represented both in the Azores and Madeira. Traditionally its bark was prepared by decoction used against atony of the digestive tract, stomach ailments, impurities of blood, lung phlegm, hemorrhagic diseases and also as a vermifuge and tonic. The decoction was used by popular medicine to fight syphillc tumors in baths and encouragements. The sap from the trunk and branches, also known as the dragon´s blood, was widely used in folk medicine for its astringent and antioxidant properties. However, little is known thus far regarding the biological properties of its leaves and fruits. For this reason, this work is mainly focused in the evaluation of the protective effects of aqueous extracts of the fruit and leaf of the Dragon tree in the oxidative damage induced by free radicals in human erythrocytes. The 2,2'-azo-bis(2-amidinopropane) (AAPH) generates peroxyl free radicals that attack the erythrocyte membrane and cause various oxidative changes, which were evaluated in this study by induction of hemolysis.

Our results show that fruit extract presents an antioxidant effect more potent than the leaf. Both extracts protect the erythrocyte membrane from hemolysis induced by AAPH in a time- and concentration-dependent manner, with IC_{50} values of 2.56 ± 0.97 µg/mL and 39.05 ± 11.54 µg/mL for fruit and leaf extracts, respectively. Strawberry extract was used as a control for comparison purposes, since it is a well documented antioxidant red fruit with recognized biologically significant effects. The IC_{50} value calculated for *D. draco* fruit extract was significantly higher than that of strawberry extract (273.84 ± 49.38 µg/mL), which emphasize the strong antioxidant activity of the fruit.

In conclusion, these results suggest *D. draco* L. species, mainly its fruit, as a promising source of natural antioxidants with potential use in the prevention and/or treatment of diseases mediated by free radicals.

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ÍNDICE GERAL

ÍNDICE DE FIGURAS …… x
ÍNDICE DE GRÁFICOS …….. xi
ÍNDICE DE TABELAS ……... xii
ABREVIATURAS …….. xiii

I. INTRODUÇÃO 1

1.1 ENQUADRAMENTO E OBJECTIVOS 1
1.2 PLANO GERAL 3
II. A ESPÉCIE DRACAENA DRACO L. 4

2.1 CARACTERÍSTICAS 4
2.2 DISTRIBUIÇÃO GEOGRÁFICA 6
2.3 COMPOSIÇÃO QUÍMICA E BIOACTIVIDADE 7
III. PARTE EXPERIMENTAL: Avaliação da actividade antioxidante da folha e fruto da espécie Dracaena draco L. usando o eritrócito humano como modelo *in vitro* 12

3.1 O ERITRÓCITO COMO MODELO IN VITRO PARA A AVALIAÇÃO DA ACTIVIDADE ANTIOXIDANTE 12
3.2 MATERIAS E MÉTODOS 12
3.2.1 Preparação dos extractos 12
3.2.2 Avaliação da actividade antioxidante 13
3.2.2.1 Preparação da suspensão de eritrócitos 13
3.2.2.2 Incubação com AAPH 13
3.2.2.3 Avaliação da percentagem de hemólise 16
3.3 RESULTADOS 17
3.3.1 Efeito protector da folha de D. draco na hemólise induzida pelo AAPH. 17
3.3.2 Efeito protector do fruto de D. draco na hemólise induzida pelo AAPH. 19
3.3.3 Efeito protector do extracto de morango na hemólise induzida pelo AAPH……...22
3.4 DISCUSSÂO DOS RESULTADOS ..... **Erro! Marcador não definido.**
IV. CONCLUSÕES GERAIS 28

V. REFERÊNCIAS BIBLIOGRÁFICAS 29

VI. ANEXOS 33

ÍNDICE DE FIGURAS

Fig.1 – Árvore do Dragoeiro
Imagem disponível em: *UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)* 4
Fig.2 – Folhas de dragoeiro
Imagem disponível em: *UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)* 5
Fig.3 – Frutos de dragoeiro
Imagem disponível em: *UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)* 5
Fig.4 – Resina de dragoeiro
Imagem disponível em: *UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)* 6
Fig. 5 – Representação esquemática da Região Geográfica da Macaronésia.
Imagem disponível em: http://noticias.sapo.cv/info/artigo/1111764.html (acedido a 11/12/10) 7
Fig. 6 – Estrutura química do (25*R*)-espirost-5-en-3β-ol 3-*O*-{*O*-α-L-ramnopiranosil-(1→2)-β-D-glucopiranósido} (Mimaki et al., 1999). 8
Fig. 7 – Esturura química do (23S,24S)-espirosta-5,25(27)-diene-1β,3β,23,24-tetrol 1-O-{O-(2,3,4-tri-O-acetil-α-L-ramnopiranosil-(1→2)-β-L-arabinopiranosil} 24-O-β-D-fucopiranósido (Mimaki et al., 1999)..... 8
Fig. 8 – Estrutura química da draconina A (González et al., 2003). 8
Fig. 9 – Estrutura química da draconina B (González et al., 2003)..... 9
Fig. 10 – Estrutura química da icogenina (Hernández et al., 2004). 9
Fig. 11 – Estrutura química da dioscina (Wang et al., 2007). 9
Fig. 12 –Estrutura química do icodesido (Hernández et al., 2006). 10
Fig. 13 – Compostos fenólicos presentes na resina do dragoeiro: 1 - 2,4,4'-trihidroxidihidrochalcona; 2 - 3-(4-hidroxibenzil)-5,7-dimetoxicromano; 3 - 7-hidroxi-3-(4-hidroxibenzil)cromona (González *et al.*, 2000)..... 11
Fig. 14 – Estrutura química do dracoflavilio (Melo et al., 2007). 11
Fig. 15 – Estrutura química do dracol (Hernández et al., 2006)..... 11
Fig. 16. Reacção de oxidação do AAPH (adaptado de Dunlap *et al.*, 2003)..... 14

ÍNDICE DE GRÁFICOS

Gráfico 1. Gráfico do efeito protector do extracto da folha de dragoeiro na hemólise induzida pelo AAPH..... 16

Gráfico 2. Gráfico do efeito protector do extracto do fruto de dragoeiro na hemólise induzida pelo AAPH..... 21

Gráfico 3. Gráfico do efeito protector do extracto de morango na hemólise induzida pelo AAPH 24

ÍNDICE DE TABELAS

Tabela 1. Tabela usada na preparação dos tubos de hemólise para o ensaio da actividade anti-hemolítica da folha de dragoeiro……15

Tabela 2. Tabela usada na preparação dos tubos de hemólise para o ensaio da actividade anti-hemolítica do fruto de dragoeiro…….15

Tabela 3. Tabela usada na preparação dos tubos de hemólise para o ensaio da actividade anti-hemolítica do extracto de morango……16

Tabela 4. Resultados da percentagem de hemólise obtidos para o extracto da folha de dragoeiro……17

Tabela 5. Percentagem de inibição de hemólise obtida para o extracto da folha de dragoeiro…….19

Tabela 6. Resultados da percentagem de hemólise obtidos para o extracto do fruto de dragoeiro……..20

Tabela 7. Percentagem de inibição de hemólise obtida para o extracto do fruto de dragoeiro……22

Tabela 8. Resultados da percentagem de hemólise obtidos para o extracto de morango……23

Tabela 9. Percentagem de inibição de hemólise obtida para o extracto de morango……..25

LISTA DE ABREVIATURAS

D. *draco* – Nome da espécie em estudo Dracaena *Draco sp*

AAPH - 2,2´-azo-bis(2-amidinopropano)

UNEP – United Nations Environment Programme

HL-60 – Células de leucemia promielocíticas humanas

A-431 – Células de carcinoma epidermóide humano de cabeça e pescoço

Memmert UL6D – Modelo da estufa utilizada no ensaio laboratorial

ºC – Graus centigrados

g – Unidade de pesagem gramas

mL – Unidade de volume, mililitros

min – Unidade de tempo, minutos

% - Percentagem

rpm – Rotações por minuto

PBS – Solução tampão fosfato

pH – Indicador ácido-base

mM – Concentração em milimoles

μg/mL – Concentração em microgramas por mililitro

μL – Unidade de volume, microlitro

Vf – Volume final

nm – Unidade de comprimento para medição de comprimentos de onda, nanómetro

IC_{50} – Índice de concentração mínima letal

SD – Desvio padrão

P – Probabilidade de significância

DNA – Ácido desoxirribonucleico

I. INTRODUÇÃO

1.1 ENQUADRAMENTO E OBJECTIVOS

Diversos estudos demonstraram que a maioria das acções terapêuticas exibidas pelas plantas medicinais é devida à sua actividade antioxidante (Gupta *et al.*, 2008). Variadas classes de compostos de origem vegetal conferem protecção contra doenças degenerativas cerebrais, tais como Alzheimer, doenças cardiovasculares, vários tipos de cancro, infecções e sistema imunitário debilitado, doenças ósseas, envelhecimento precoce, entre outras, uma vez que são poderosos antioxidantes, prevenindo os danos provocados pelos radicais livres (Sparg *et al*., 2004). Acresce o facto de muitas destas biomoléculas serem utilizadas pelas próprias plantas que as produzem como elementos de defesa contra microrganismos e pragas, dado que são amargos, capazes de inibir certos sistemas enzimáticos e de quelatar metais, exibindo assim propriedades antibacterianas, antivirais, antifúngicas e insecticidas (Gupta *et al.*, 2008).

O dragoeiro (*Dracaena draco* L.) é uma espécie arbórea, da família das Dracaenaceas, pertencente à flora da Macaronésia. Trata-se de uma das mais raras espécies arbóreas de Portugal, já mal representada tanto no Arquipélago dos Açores como no da Madeira.

As suas aplicações são diversas na medicina popular. Por exemplo, a sua casca preparada por decocção era usada em banhos e fomentos no combate a tumores sifílicos. O decocto era também utilizado contra a atonia do tubo digestivo, males estomacais, diarreias, impurezas do sangue, catarros pulmonares, hemorragias e também como vermífugo e tónico (Gupta *et al.*, 2008; Mimaki *et al.*, 1999; González *et al.*, 2000; Ballabio, 2004).

A seiva do tronco e dos ramos, denominada de *sangue-de-drago* ou *sangue-de-dragão*, devido à sua cor vermelha, era usada para curar feridas e úlceras. Estudos recentes comprovaram que esta resina possui propriedades antioxidantes e adstringentes, tendo utilização em fitoterapia pela sua acção antidiarreica, antisséptica, antitumoral, anti-inflamatória, analgésica, hemostática, cicatrizante, antibacteriana,

antifúngica e antiviral, entre outras (Mimaki *et al.*, 1999; González *et al.*, 2000; Ballabio, 2004).

Até à data praticamente não existem referências bibliográficas disponíveis relativas a esta espécie, principalmente ao seu fruto. Contudo, foram realizados estudos fitoquímicos preliminares que demonstram que se trata de uma boa fonte de compostos bioactivos, nomeadamente de compostos fenólicos e ácidos orgânicos. Devido à sua composição química é de prever que esta espécie vegetal tenha um considerável potencial anti-radicalar, podendo intervir na prevenção de doenças nas quais os radicais livres estão envolvidos. Desta forma, o presente trabalho apresenta como objectivo a avaliação da actividade antioxidante da espécie *D. draco* através da sua acção protectora relativamente aos danos oxidativos induzidos por radicais livres em eritrócitos humanos.

O eritrócito é considerado um modelo adequado ao estudo dos efeitos de radicais livres, devido à elevada concentração em ácidos gordos polinsaturados na sua membrana e ao seu papel específico como transportador de oxigénio (Risco et al., 2003; Gupta et al., 2008). Assim, o eritrócito humano será usado neste estudo como modelo *in vitro* para avaliar o efeito antioxidante do extracto de folha e fruto de *D. draco* sobre os danos nas membranas biológicas provocados pelos radicais livres. Para este efeito, o 2,2´-azo-bis(2-amidinopropano) (AAPH) será utilizado como um sistema gerador de radicais livres do tipo peroxilo que atacam a membrana eritrocitária causando várias alterações oxidativas, tais como formação de peróxidos lipídicos, redução da deformabilidade, mudanças na morfologia, ligação cruzada e fragmentação de proteínas, hemólise e alterações no metabolismo intracelular (Risco et al., 2003; Gupta et al., 2008). Neste estudo, o efeito protector dos extractos da folha e fruto da espécie *D. draco* será avaliado pela inibição da hemólise mediada pelos radicais livres nos eritrócitos humanos.

1.2 PLANO GERAL

A presente dissertação encontra-se estruturada em cinco capítulos:

No presente capítulo ("Introdução") procedeu-se ao enquadramento do trabalho e são apresentados os objectivos a que o trabalho se propõe.

No Capítulo 2 ("A espécie *Dracaena draco* L.") é elaborada uma revisão da literatura no que diz respeito às características, interesse, composição química e bioactividade.da espécie *D. draco*.

No Capítulo 3 ("Parte Experimental") são explanadas as razões que determinam a escolha do eritrócito humano como um modelo celular adequado para a avaliação do efeito antioxidante de alguns compostos. São ainda apresentados todos os materiais e métodos seguidos no procedimento experimental, bem como os resultados obtidos e a discussão dos mesmos.

No Capítulo 4 ("Conclusões Gerais") são sumariadas as conclusões retiradas dos estudos incluídos neste trabalho.

Por último, no Capítulo 5, é apresentada uma listagem de todas as referências bibliográficas citadas ao longo do texto.

De salientar que, em anexo, são ainda apresentados dois artigos já publicados em revistas científicas internacionais com factor de impacto. Estas publicações incluem o trabalho experimental desta dissertação.

II. A ESPÉCIE DRACAENA DRACO L.

2.1 CARACTERÍSTICAS

Taxonomicamente a espécie botânica *Dracaena draco* L., de nome comum dragoeiro, pertence à divisão Magnoliophyta, classe Liliopsida, ordem Asparagales e família Dracaenaceae (Marrero e outros, 1998). Trata-se de uma árvore que pode atingir mais de 15 m de altura, de tronco cilíndrico muito robusto e ramificado após a produção de inflorescência. A sua ramificação é dicotómica após surgimento da inflorescência terminal, formando uma copa ampla (Figura 1). As folhas são verde-acinzentadas, dispostas em roseta terminal, coriáceas e de ápice agudo (Figura 2). As flores nascem em Agosto e Setembro e formam cachos de pétalas branco-esverdeadas bastante aromáticas. As suas bagas são globulosas e de cor vermelha-alaranjada (Figura 3). A seiva é uma resina de cor vermelha e, por isso, denominada de *sangue-de-draco* ou *sangue-de-dragão* (Figura 4) (Veloso, 2005).

Fig.1 – Árvore do Dragoeiro

Imagem disponível em: ***UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)***

Fig.2 – Folhas de dragoeiro

Imagem disponível em: ***UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)***

Fig.3 – Frutos de dragoeiro

Imagem disponível em: ***UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)***

Fig.4 – Resina de dragoeiro

Imagem disponível em: ***UNEP, Mohamed Moslih Sanabani, Topham Picturepoint (acedido a 04/02/2010)***

A sua utilização remete-nos para os séculos XV e XIX onde era usada com interesse comercial como espécie tintureira. A resina tem sido utilizada como substância corante e na produção de tintas, lacres e vernizes. Encontra-se igualmente referido o uso desta resina na medicina popular (Mimaki et al., 1999; González et al., 2003; Ballabio, 2004; Gupta et al., 2008).

2.2 DISTRIBUIÇÃO GEOGRÁFICA

O dragoeiro é uma espécie arbórea pertencente à flora da Macaronésia. Trata-se de uma das mais raras espécies arbóreas de Portugal, já mal representada tanto no Arquipélago dos Açores como no da Madeira. Esta espécie tem sido considerada endémica dos Arquipélagos das Canárias, da Madeira e de Cabo Verde e da região noroeste de África, numa zona restrita do sul de Marrocos, e ainda dos Açores, onde permanece a dúvida se é nativa deste arquipélago ou se foi posteriormente introduzida na sua flora (González et al., 2003; Gupta et al., 2008).

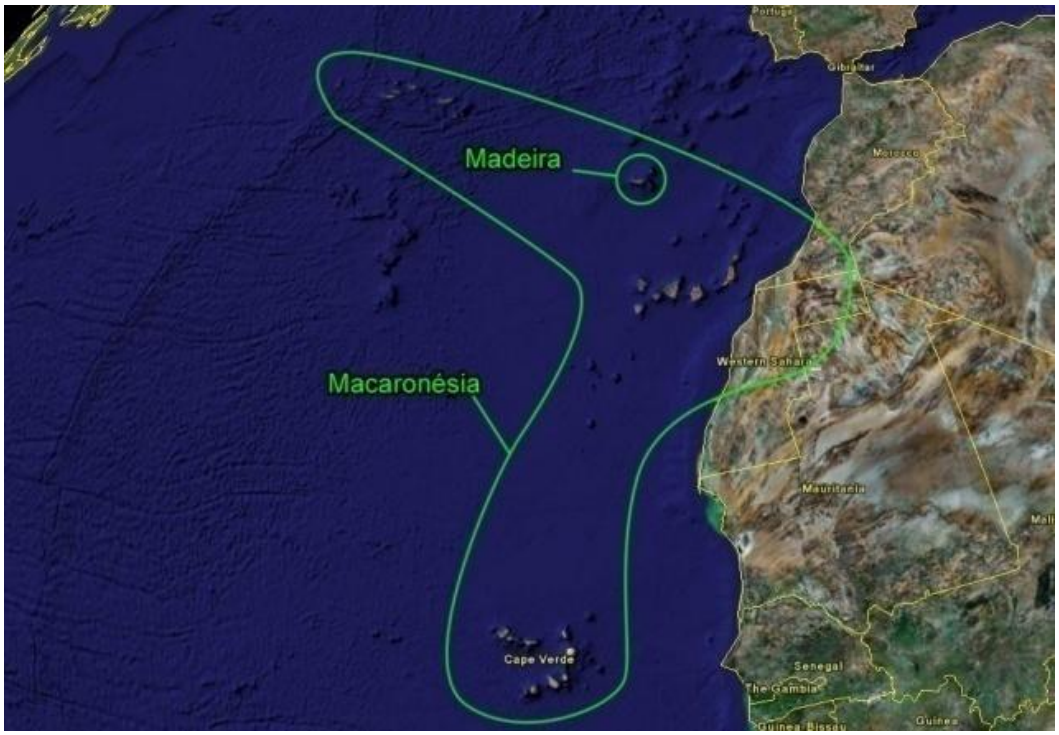


Fig. 5 – Representação esquemática da Região Geográfica da Macaronésia.
Imagem disponível em: http://noticias.sapo.cv/info/artigo/1111764.html (acedido a 11/12/10)

2.3 COMPOSIÇÃO QUÍMICA E BIOACTIVIDADE

A investigação sobre esta planta é bastante limitada e maioritariamente direccionada para o estudo dos seus saponósidos (Mimaki et al., 1999; González et al., 2003; Hernández et al., 2004, 2006; Sparg et al., 2004; Gupta et al., 2008). De facto, as diferentes partes morfológicas desta espécie botânica são, actualmente, consideradas excelentes fontes de saponinas de origem esteróide com propriedades citostáticas e/ou citotóxicas (Gupta et al., 2008).

No final dos anos 80, Darias et al. (1989) reportaram, pela primeira vez, a actividade anticancerígena da resina de *D. draco*. Posteriormente, Mimaki et al. (1999) demonstraram a potente actividade citostática das saponinas (25R)-espirost-5-en-3β-ol 3-O-{O-α-L-ramnopiranosil-(1→2)-β-D-glucopiranósido} (Figura 5) e (23S,24S)-espirosta-5,25(27)-diene-1β,3β,23,24-tetrol 1-O-{O-(2,3,4-tri-O-acetil-α-L-ramnopiranosil-(1→2)-β-L-arabinopiranosil} 24-O-β-D-fucopiranósido (Figura 6) extraídas das partes aéreas da planta relativamente a células humanas de leucemia promielocítica aguda (HL-60). Mais tarde, González et al. (2003) relataram ainda a forte

actividade citotóxica das draconinas A e B (Figuras 7 e 8, respectivamente) obtidas a partir da casca do dragoeiro em relação ao mesmo tipo de células. O mecanismo de toxicidade foi estudado e parece ocorrer via activação de processos apoptóticos (González et al., 2003).

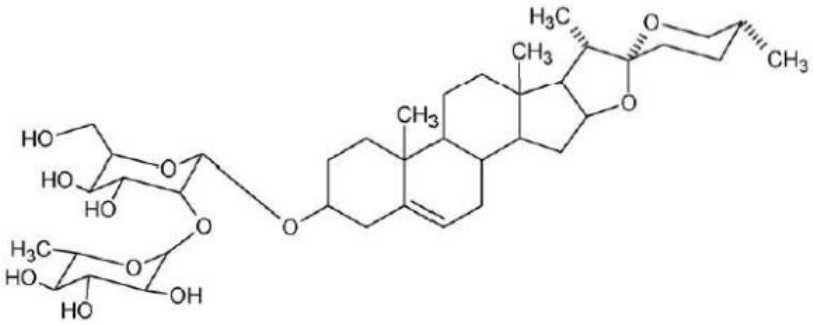


Fig. 6 – **Estrutura química do (25*R*)-espirost-5-en-3β-ol 3-*O*-{*O*-α-L-ramnopiranosil-(1→2)-β-D-glucopiranósido} (Mimaki et al., 1999).**

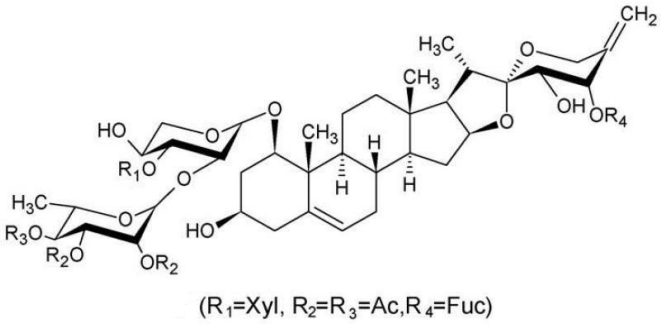


Fig. 7 – Esturura química do (23S,24S)-espirosta-5,25(27)-diene-1β,3β,23,24-tetrol 1-O-{O-(2,3,4-tri-O-acetil-α-L-ramnopiranosil-(1→2)-β-L-arabinopiranosil} 24-O-β-D-fucopiranósido (Mimaki et al., 1999).

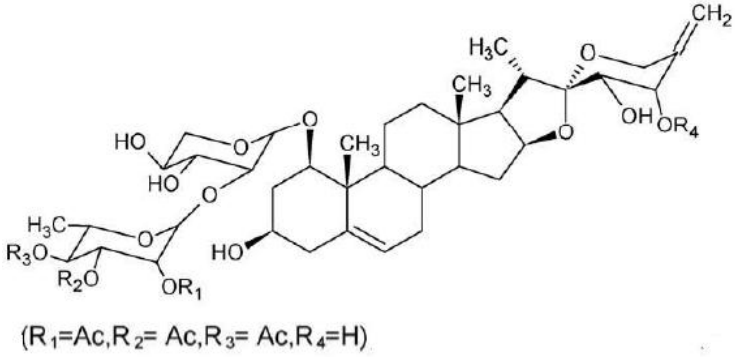


Fig. 8 – Estrutura química da draconina A (González et al., 2003).

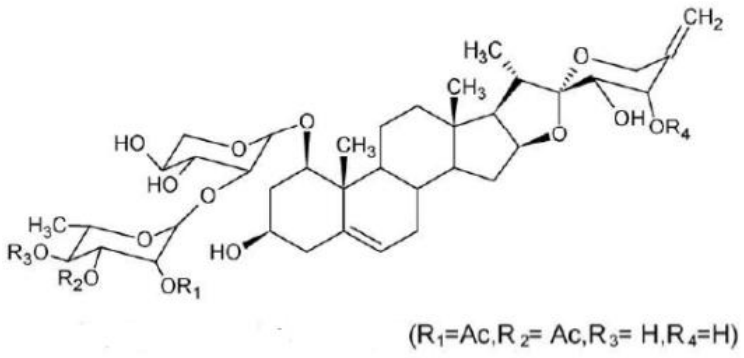


Fig. 9 – Estrutura química da draconina B (González et al., 2003).

A icogenina (Figura 9) e a dioscina (Figura 10) foram isoladas por Hernández et al. (2004) a partir da raíz de *D. draco*. Estes investigadores descobriram que estas duas saponinas também possuem actividade antiproliferativa em células HL-60 e igualmente via indução da apoptose (Hernández et al., 2004). Mais recentemente, este grupo identificou ainda o icodesido (Figura 11) das folhas, tendo sido demonstrada a sua moderada actividade citotóxica em células HL-60 e de carcinoma epidermóide humano de cabeça e pescoço (A-431) (Hernández et al., 2006).

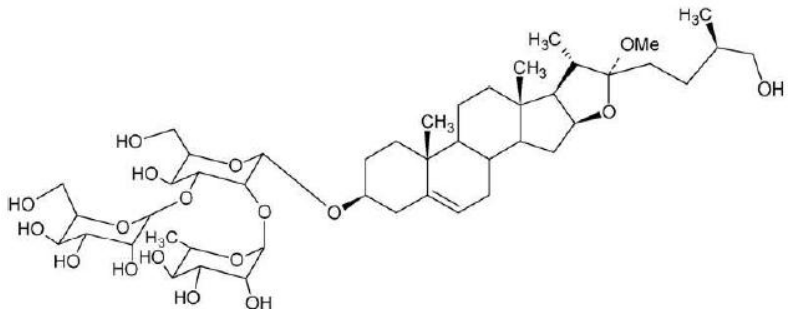


Fig. 10 – Estrutura química da icogenina (Hernández et al., 2004).

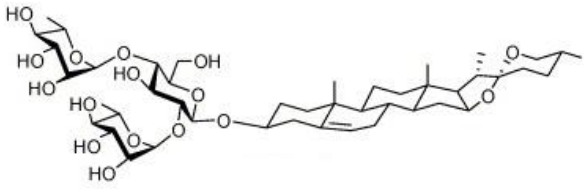


Fig. 11 – Estrutura química da dioscina (Wang et al., 2007).

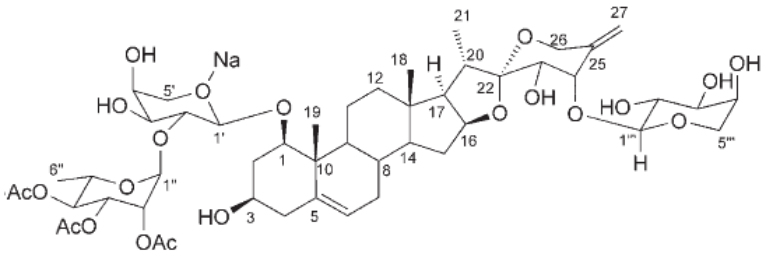
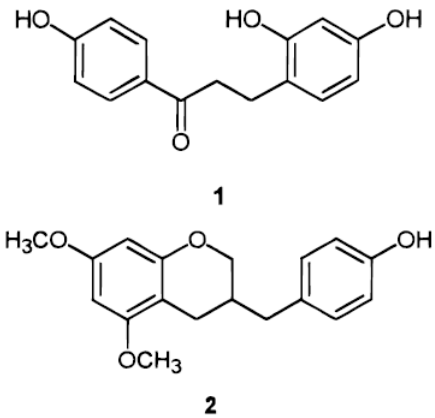


Fig. 12 –Estrutura química do icodesido (Hernández et al., 2006).

Existem outras propriedades do dragoeiro provavelmente devidas à presença de saponinas, nomeadamente as antifúngicas, antibacterianas, antivirais e antiparasitárias (Mimaki et al., 1999; González et al., 2000; Ballabio, 2004). A resina de *D. draco* apresenta ainda efeito adstringente e tem sido usada pela medicina tradicional desde a antiguidade devido às suas actividades antidiarreica e hemostática, entre outras (Mimaki et al., 1999; González et al., 2003; Ballabio, 2004; Gupta et al., 2008).

Para além dos estudos relativos aos saponósidos do dragoeiro, existem alguns acerca dos compostos fenólicos do *sangue-de-dragão* (Camarda et al., 1983; González et al., 2000, 2004; Melo et al., 2007), nomeadamente a identificação da 2,4,4'-trihidroxidihidrochalcona, do 3-(4-hidroxibenzil)-5,7-dimethoxicromano e da 7-hidroxi-3-(4-hidroxibenzil)cromona (Figura 12) (González et al., 2000). Recentemente, Melo et al. (2007) isolou e identificou o principal corante vermelho da resina, o dracoflavílio (Figura 13). Em 2006, Hernández et al. fizeram o isolamento e a identificação estrutural do dracol das folhas de *D. draco* (Figura 14).



1

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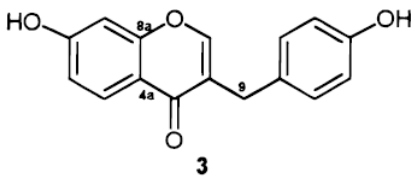

Fig. 13 – Compostos fenólicos presentes na resina do dragoeiro: 1 - 2,4,4'-trihidroxidihidrochalcona; 2 - 3-(4-hidroxibenzil)-5,7-dimetoxicromano; 3 - 7-hidroxi-3-(4-hidroxibenzil)cromona (González *et al.*, 2000).

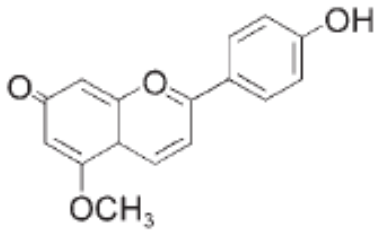

Fig. 14 – Estrutura química do dracoflavilio (Melo e tal., 2007).

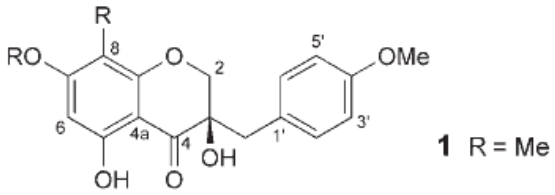

Fig. 15 – Estrutura química do dracol (Hernández et al., 2006).

III. PARTE EXPERIMENTAL: Avaliação da actividade antioxidante da folha e fruto da espécie Dracaena draco L. usando o eritrócito humano como modelo *in vitro*

3.1 O ERITRÓCITO COMO MODELO IN VITRO PARA A AVALIAÇÃO DA ACTIVIDADE ANTIOXIDANTE

O eritrócito constitui um sistema celular adequado para o estudo *in vitro* quer dos efeitos de radicais livres e espécies oxidantes nas membranas biológicas, quer para a investigação do efeito protector de diversos compostos, em virtude da sua simplicidade estrutural, acessibilidade e vulnerabilidade dos seus constituintes à oxidação. As principais estruturas eritrocitárias afectadas por estas espécies são os constituintes membranares e a hemoglobina. A membrana dos eritrócitos é rica em ácidos gordos poliinsaturados, os quais são muito susceptíveis à peroxidação lipídica mediada por radicais livres (Shiva et al., 2007). Outras alterações decorrentes da acção dos radicais livres nas membranas eritrocitárias incluem redução da deformabilidade, alteração na morfologia celular, fenómenos de cross-linking proteico, hemólise e alterações no metabolismo intracelular (Shiva et al., 2007; Begum e Terao, 2002; Sato et al., 1995; Sandhu et al., 1992). Estas alterações têm como principal consequência a diminuição do tempo médio de vida do eritrócito.

3.2 MATERIAS E MÉTODOS

3.2.1 Preparação dos extractos

As folhas de dragoeiro foram colhidas na ilha do Pico (Açores, Portugal), em Fevereiro de 2009 e secas numa estufa (Memmert UL6D) a 30 ± 2ºC durante 5 dias (ao abrigo da luz). As folhas secas (~5 g) foram extraídas com 250 mL de água a ferver durante 45 min. O extracto resultante foi posteriormente liofilizado e mantido num excicador ao abrigo da luz, até ao momento da análise. O rendimento para o processo extractivo foi de 28,8 ± 0,6%.

Os frutos de dragoeiro foram colhidos na ilha de Tenerife (Canárias, Espanha), em Agosto de 2008. As amostras foram imediatamente congeladas e liofilizadas (Ly-8-FM-ULE, Snijders) antes de se proceder à extracção. Três sub-amostras em pó (~5 g) foram extraídas com 250 mL de água fervente durante 45 minutos. O extracto resultante foi posteriormente liofilizado e mantido num excicador (ao abrigo da luz), até ao momento da utilização. O rendimento para o processo de extracção foi de 28,77 ± 1,19%.

Os morangos (*Fragaria* x *ananassa* Duch. cv. Camarosa) foram adquiridos no mercado Português, em Junho de 2009, e o extracto foi preparado como descrito para o fruto de dragoeiro. O rendimento da extracção foi de 45,53 ± 4,90%.

3.2.2 Avaliação da actividade antioxidante

3.2.2.1 Preparação da suspensão de eritrócitos

O sangue venoso humano foi colhido em citrato (anticoagulante) de dadores saudáveis, não fumadores, após obtenção do consentimento informado (Anexo I). Para obter os eritrócitos empacotados centrifugou-se o sangue total (5 a 10 mL) a 1.500 rpm durante 10 minutos a 4ºC. O plasma e a camada leucocitária (“buffy coat”) foram removidos por aspiração e os eritrócitos foram lavados 3 vezes com solução tampão de fosfato (PBS; pH 7,4), repetindo-se em cada lavagem a centrifugação nas condições referidas anteriormente. Após a última lavagem perfez-se o volume com PBS de modo a obter uma suspensão eritrocitária com hematócrito 5,2% (0,52 mL de eritrócitos compactados para um volume final de 10 mL em PBS).

3.2.2.2 Incubação com AAPH

Diversas substâncias oxidantes foram descritas em estudos anteriores como meio de desencadeamento do stress oxidativo em eritrócitos, sendo algumas delas: o peróxido de hidrogénio (Lii e Hung, 1997), o terc-butilhidroperóxido (Rice-Evans et al., 1996 e Zou et al., 2001), a primaquina (Grinberg e Samuni, 1994) e as hidrazinas (Biswas et al., 2005). No entanto, a substância mais amplamente utilizada é o 2,2´-azo-bis(2-amidinopropano) (AAPH) (Dai et al., 2006; Ma et al., 2000 e Shiva et al., 2007).

O AAPH é um composto hidrossolúvel que origina radicais livres do tipo peroxilo por decomposição térmica (a 37ºC) unimolecular em taxa constante (Figura 15). O AAPH origina radicais livres de modo dependente do tempo e da concentração (Zou et al., 2001).

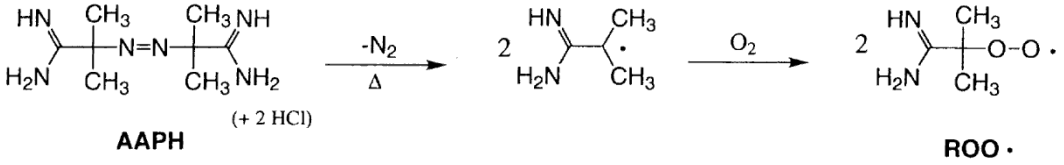

Fig. 16. Reacção de oxidação do AAPH (adaptado de Dunlap *et al*., 2003)

Neste estudo, a suspensão de eritrócitos foi incubada com AAPH na concentração final de 50 mM. Para avaliar o efeito protector da espécie *D. draco* foram estudadas várias concentrações do extracto da folha (20, 40 e 80 µg/mL) e do fruto (2,5, 5, 10 e 20 µg/mL).

Aos tubos de hemólise adicionou-se 500 µL da suspensão eritrocitária a 5,2% de modo a obter um hematócrito final de 2,0%, tendo em conta que o volume final nos tubos será de 1300 µL, sendo estes colocados a incubar a 37ºC durante 5 minutos. Após a incubação, adicionou-se os antioxidantes (extractos de folha e fruto de dragoeiro e de morango) ou não, de acordo com as tabelas apresentadas abaixo, sendo os tubos pré-incubados a 37ºC durante 30 minutos.

As concentrações testadas dos extractos da folha de dragoeiro foram obtidas a partir de uma solução stock de 1000 µg/mL em PBS, da qual se retiraram 2 mL aos quais foram adicionados 8 mL de PBS obtendo-se assim uma solução de 200 µg/mL. A partir desta solução e, de acordo com a Tabela 1, obtiveram-se as concentrações finais nos tubos de 20, 40 e 80 µg/mL. A actividade hemolítica do extracto foi avaliada expondo as células à concentração mais alta estudada (80 µg/mL) na ausência de AAPH (controlo do extracto).

Tabela 1. Tabela usada na preparação dos tubos de hemólise para o ensaio da actividade anti-hemolítica da folha de dragoeiro

	Eritrócito	**Extracto**	**PBS**	**AAPH**	**Vf**
Controlo	500 µL	----------	800 µL	----------	1300 µL
AAPH	500 µL	----------	670 µL	130 µL	1300 µL
C $_{20\ \mu g/mL}$	500 µL	130 µL	540 µL	130 µL	1300 µL
C $_{40\ \mu g/mL}$	500 µL	260 µL	410 µL	130 µL	1300 µL
C $_{80\ \mu g/mL}$	500 µL	520 µL	150 µL	130 µL	1300 µL
Controlo extracto	500 µL	520 µL	280 µL	----------	1300 µL

As concentrações testadas dos extractos do fruto de dragoeiro foram obtidas a partir de uma solução stock de 2500 µg/mL em PBS. A solução stock foi convenientemente diluída em PBS de modo a se obter uma solução de 50 µg/mL, a partir da qual e, de acordo com a Tabela 2, obtiveram-se as concentrações finais nos tubos de 2,5, 5, 10 e 20 µg/mL. Do mesmo modo, a actividade hemolítica do extracto de fruto foi avaliada expondo as células à concentração mais alta estudada (20 µg/mL) na ausência de AAPH (controlo do extracto).

Tabela 2. Tabela usada na preparação dos tubos de hemólise para o ensaio da actividade anti-hemolítica do fruto de dragoeiro

	Eritrócito	**Extracto**	**PBS**	**AAPH**	**Vf**
Controlo	500 µL	----------	800 µL	----------	1300 µL
AAPH	500 µL	----------	670 µL	130 µL	1300 µL
C $_{2,5\ \mu g/mL}$	500 µL	65 µL	605 µL	130 µL	1300 µL
C $_{5\ \mu g/mL}$	500 µL	130 µL	540 µL	130 µL	1300 µL
C $_{10\ \mu g/mL}$	500 µL	260 µL	410 µL	130 µL	1300 µL
C $_{20\ \mu g/Ml}$	500 µL	520 µL	150 µL	130 µL	1300 µL
Controlo extracto	500 µL	520 µL	280 µL	----------	1300 µL

Findo o tempo de pré-incubação, adicionou-se (amostras) ou não (controlos) o oxidante AAPH, de acordo com as tabelas acima apresentadas, de modo a obter a concentração final de AAPH de 50 mM. Os tubos de hemólise foram depois incubados durante 4 horas, a 37ºC, com agitação suave e constante e ao abrigo da luz.

Neste estudo o extracto de morango foi utilizado como fruto vermelho de referência, com o qual será comparado o efeito protector do fruto de dragoeiro. Para tal, preparou-se uma solução stock de 1000 µg/mL em PBS. A partir desta solução e, de acordo com a Tabela 3, foram realizadas diluições nos tubos para obter soluções com as concentrações finais de 100, 50 e 25 µg/mL.

Tabela 3. Tabela usada na preparação dos tubos de hemólise para o ensaio da actividade anti-hemolítica do extracto de morango

	Eritrócito	**Extracto**	**PBS**	**AAPH**	**Vf**
Controlo	500 µL	----------	800 µL	----------	1300 µL
AAPH	500 µL	----------	670 µL	130 µL	1300 µL
C 100 µg/mL	500 µL	130 µL	540 µL	130 µL	1300 µL
C 200 µg/Ml	500 µL	260 µL	410 µL	130 µL	1300 µL
C 400 µg/Ml	500 µL	520 µL	150 µL	130 µL	1300 µL

3.2.2.3 Avaliação da percentagem de hemólise

Durante as 4 horas de incubação, com intervalos de 1 hora, retiraram-se duas alíquotas de 50 µL de cada tubo. Uma das alíquotas é adicionada a 950 µL de água (B) e a outra a 950 µL de soro fisiológico (A), em eppendorfs previamente colocados no gelo (4ºC) de modo a parar a hemólise. De seguida, centrifugou-se os eppendorfs a 4.000 rpm durante 10 minutos, removendo-se depois cerca de 300 µL do sobrenadante para uma placa de 96 poços, de modo a proceder-se à leitura da absorvância a 545 nm no leitor de placas. A % de hemólise é calculada a partir da razão entre as duas leituras, ou seja, % hemólise = (A/B) x 100. A concentração de extracto que inibe 50% da hemólise (IC_{50}) ao fim de três horas foi calculada através do traçado do gráfico da percentagem de inibição de hemólise em função da concentração de extracto. Para estes cálculos foram realizados 4 ensaios independentes.

3.3 RESULTADOS

3.3.1 Efeito protector da folha de D. draco na hemólise induzida pelo AAPH

Na tabela seguinte são apresentados os valores da percentagem de hemólise nas células incubadas com as diferentes concentrações do extracto de folha de dragoeiro obtidos em quatro ensaios independentes.

Tabela 4. Resultados da percentagem de hemólise obtidos para o extracto da folha de dragoeiro

	Tempo (horas)			
	1	**2**	**3**	**4**
Controlo	4,5	5,0	5,5	3,6
	7,3	3,7	2,8	4,6
	3,3	1,1	1,2	1,2
	1,6	2,0	3,5	3,7
Média	**4,2**	**2,9**	**3,2**	**3,3**
SD	**2,4**	**1,7**	**1,8**	**1,5**
AAPH	3,9	51,3	88,3	96,5
	2,9	69,1	88,1	97,8
	5,3	88,5	79,4	88,9
	3,6	74,3	104,9	94,6
Média	**4,0**	**70,8**	**90,2**	**94,4**
SD	**1,0**	**15,4**	**10,7**	**3,9**
Folha dragoeiro 20 µg/mL	1,1	16,8	85,5	90,0
	3,3	6,9	63,1	90,0
	3,1	26,5	79,4	89,6
	3,7	18,4	74,1	92,1
Média	**2,8**	**17,1**	**75,5**	**90,4**
SD	**1,2**	**8,0**	**9,5**	**1,1**
Folha dragoeiro 40 µg/mL	2,2	5,5	57,7	89,2
	2,8	3,4	20,5	96,0
	3,0	5,4	55,0	89,6
	3,6	3,4	42,0	85,8
Média	**2,9**	**4,4**	**43,8**	**90,1**
SD	**0,6**	**1,2**	**17,0**	**4,2**
Folha dragoeiro 80 µg/mL	3,8	2,5	14,7	64,1
	3,4	3,4	4,4	19,7
	8,5	1,7	10,6	85,2
	1,4	2,2	4,8	59,8
Média	**4,3**	**2,5**	**8,6**	**57,2**
SD	**3,0**	**0,7**	**5,0**	**27,4**

Estes resultados são seguidamente apresentados na forma de gráfico (Gráfico 1). Cada valor representa a média ± SD de quatro ensaios independentes. *Representa resultados significativos (P <0,05) quando o grupo tratado foi comparado com o grupo AAPH, nos respectivos tempos. #Representa resultados significativos (P <0,05) quando o grupo tratado foi comparado com o grupo controlo, nos respectivos tempos.

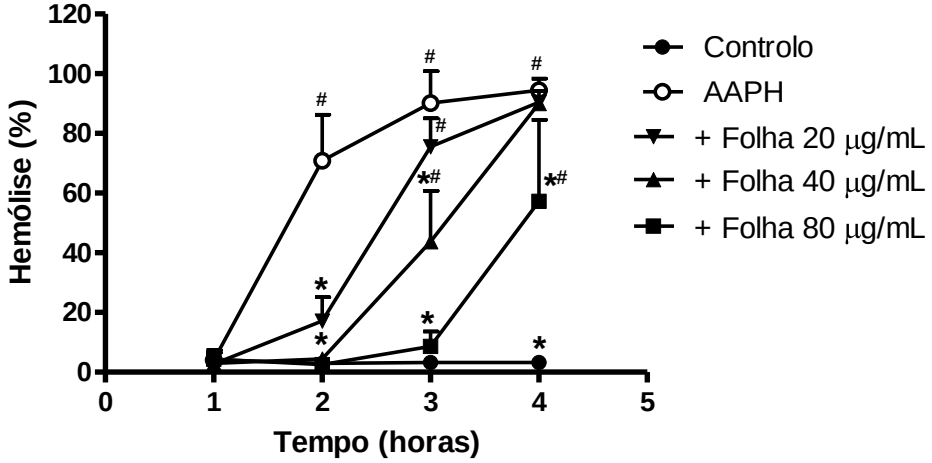

Gráfico 1. Gráfico do efeito protector do extracto da folha de dragoeiro na hemólise induzida pelo AAPH

Para o cálculo da concentração inibitória 50 (IC_{50}), ou seja, a concentração de extracto que inibe 50% da hemólise induzida pelo AAPH, determinou-se a percentagem de inibição de hemólise para cada concentração de extracto ao tempo 3 horas, tal como apresentado na tabela seguinte.

Tabela 5. Percentagem de inibição de hemólise obtida para o extracto da folha de dragoeiro

	% hemólise	-Controlo	% Inibição
Controlo	5,5		
	2,8		
	1,2		
	3,5		
AAPH	88,3	82,8	
	88,1	85,3	
	79,4	78,2	
	104,9	101,4	
Folha dragoeiro 20 µg/mL	85,5	80,0	3,4
	63,1	60,3	29,3
	79,4	78,2	0,0
	74,1	70,6	30,3
Folha dragoeiro 40 µg/mL	57,7	52,2	37,0
	20,5	17,7	79,2
	55,0	53,8	31,2
	42,0	38,5	62,0
Folha dragoeiro 80 µg/mL	14,7	9,2	88,9
	4,4	1,6	98,1
	10,6	9,4	88,0
	4,8	1,3	98,7

Em seguida, traçou-se o gráfico da % inibição de hemólise *versus* a concentração de extracto para cada ensaio independente. O valor de IC_{50} calculado para o extracto da folha de dragoeiro foi de 39,05 ± 11,54 µg/mL.

3.3.2 Efeito protector do fruto de D. draco na hemólise induzida pelo AAPH

Na tabela seguinte são apresentados os valores da percentagem de hemólise nas células incubadas com as diferentes concentrações do extracto do fruto de dragoeiro obtidos em quatro ensaios independentes.

Tabela 6. Resultados da percentagem de hemólise obtidos para o extracto do fruto de dragoeiro

	Tempo (horas)			
	1	**2**	**3**	**4**
Controlo	1,4	5,6	6,8	4,3
	4,7	10,1	11,7	5,1
	3,1	3,1	3,1	3,1
	6,0	7,2	3,1	4,8
Média	**3,8**	**6,5**	**6,2**	**4,3**
SD	**2,0**	**2,9**	**4,1**	**0,9**
AAPH	4,0	70,3	74,1	86,0
	4,7	64,0	76,7	78,9
	1,8	50,0	84,8	100,0
	2,3	56,1	91,7	93,6
Média	**3,2**	**60,1**	**81,8**	**89,6**
SD	**1,4**	**8,9**	**8,0**	**9,2**
Fruto dragoeiro 2,5 µg/mL	1,9	12,5	47,5	86,6
	3,0	7,2	44,7	73,7
	6,5	8,6	51,6	90,5
	2,9	3,3	33,2	80,6
Média	**3,6**	**7,9**	**44,3**	**82,8**
SD	**2,0**	**3,8**	**7,9**	**7,3**
Fruto dragoeiro 5 µg/mL	3,3	2,9	21,3	83,3
	1,9	3,3	29,0	75,6
	6,5	6,9	31,8	90,0
	2,9	3,6	13,5	66,1
Média	**3,6**	**4,2**	**23,9**	**78,8**
SD	**2,0**	**1,8**	**8,2**	**10,3**
Fruto dragoeiro 10 µg/mL	2,1	2,1	6,3	51,3
	1,8	4,6	8,8	64,3
	2,0	2,5	2,9	21,8
	2,4	3,1	3,8	16,7
Média	**2,1**	**3,1**	**5,5**	**38,5**
SD	**0,2**	**1,1**	**2,7**	**22,9**
Fruto dragoeiro 20 µg/mL	2,5	2,7	2,2	6,3
	2,1	3,4	1,8	9,4
	2,0	2,9	2,4	3,5
	5,6	2,8	2,9	4,6
Média	**3,0**	**2,9**	**2,3**	**6,0**
SD	**1,7**	**0,3**	**0,4**	**2,6**

Estes resultados são seguidamente apresentados na forma de gráfico. Cada valor representa a média ± SD de quatro ensaios independentes. *Representa resultados significativos ($P < 0,05$) quando o grupo tratado foi comparado com o grupo AAPH, nos

respectivos tempos. #Representa resultados significativos (P <0,05) quando o grupo tratado foi comparado com o grupo controlo, nos respectivos tempos.

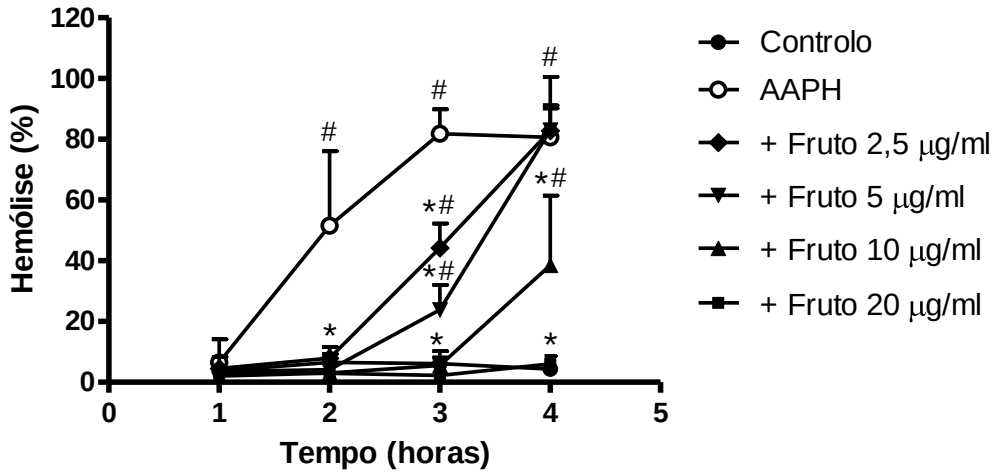

Gráfico 2. Gráfico do efeito protector do extracto do fruto de dragoeiro na hemólise induzida pelo AAPH

Para o cálculo do IC_{50} determinou-se a percentagem de inibição de hemólise para cada concentração de extracto ao tempo 3 horas, tal como apresentado na tabela seguinte.

Tabela 7. Percentagem de inibição de hemólise obtida para o extracto do fruto de dragoeiro

	% hemólise	-Controlo	% Inibição
Controlo	6,8		
	11,7		
	3,1		
	3,1		
AAPH	74,1	67,3	
	76,7	65,0	
	84,8	81,7	
	91,7	88,6	
Fruto dragoeiro 2,5 µg/mL	47,5	40,7	39,5
	44,7	32,9	49,3
	51,6	48,5	40,6
	33,2	30,1	66,1
Fruto dragoeiro 5 µg/mL	21,28	14,5	78,5
	29,0	17,3	73,4
	31,8	28,7	64,9
	13,5	10,4	88,3
Fruto dragoeiro 10 µg/mL	6,3	-0,5	100,7
	8,8	-2,9	104,5
	2,9	-0,2	100,2
	3,8	0,6	99,3
Fruto dragoeiro 20 µg/mL	2,23	0,0	100,0
	1,8	0,0	100,0
	2,4	0,0	100,0
	2,9	0,0	100,0

Em seguida, traçou-se o gráfico da % inibição de hemólise *versus* a concentração de extracto para cada ensaio independente. O valor de IC_{50} calculado para o extracto do fruto de dragoeiro foi de 2,56 ± 0,97 µg/mL.

3.3.3 Efeito protector do extracto de morango na hemólise induzida pelo AAPH

O extracto de morango foi utilizado neste estudo com a finalidade comparar a sua actividade antioxidante com a obtida para o extracto do fruto de dragoeiro, uma vez que se trata de um fruto vermelho com actividade antioxidante muito bem documentada e com efeitos biológicos significativos reconhecidos (García-Alonso et al., 2004; Seeram, 2008; Zang et al., 2008). Na tabela seguinte apresentam-se os valores da percentagem

de hemólise nas células incubadas com as diferentes concentrações do extracto de morango obtidos em 4 ensaios independentes.

Tabela 8. Resultados da percentagem de hemólise obtidos para o extracto de morango

	Tempo (horas)			
	1	**2**	**3**	**4**
Controlo	0,3	4,7	3,3	3,6
	8,7	6,5	3,3	3,9
	6,6	6,1	6,0	5,6
	8,3	4,3	5,9	4,6
Média	**6,0**	**5,4**	**4,6**	**4,4**
SD	**3,9**	**1,1**	**1,5**	**0,9**
AAPH	2,6	22,8	92,7	90,0
	10,5	64,4	77,2	80,0
	5,1	71,7	88,6	91,8
	2,6	82,5	90,4	90,0
Média	**5,2**	**60,4**	**87,2**	**88,0**
SD	**3,7**	**26,1**	**6,9**	**5,4**
Morango 100 µg/mL	3,1	4,4	78,8	81,5
	4,1	19,8	63,3	75,3
	7,0	13,1	73,1	82,4
	7,7	12,8	85,1	89,6
Média	**5,5**	**12,5**	**75,1**	**82,2**
SD	**2,2**	**6,3**	**9,3**	**5,9**
Morango 200 µg/mL	3,5	1,2	69,3	89,0
	2,3	4,8	55,9	73,6
	3,2	5,2	45,4	86,2
	6,4	13,0	63,2	94,9
Média	**3,9**	**6,1**	**58,5**	**85,9**
SD	**1,8**	**5,0**	**10,3**	**9,0**
Morango 400 µg/mL	3,3	1,5	12,6	84,6
	4,3	2,6	23,7	68,7
	5,8	3,0	13,2	66,1
	7,3	3,5	39,7	85,2
Média	**5,2**	**2,7**	**22,3**	**76,2**
SD	**1,8**	**0,9**	**12,7**	**10,2**

Estes resultados são seguidamente apresentados na forma de gráfico. Cada valor representa a média ± SD de quatro ensaios independentes. *Representa resultados significativos (P <0,05) quando o grupo tratado foi comparado com o grupo AAPH, nos respectivos tempos. #Representa resultados significativos (P <0,05) quando o grupo tratado foi comparado com o grupo controlo, nos respectivos tempos.

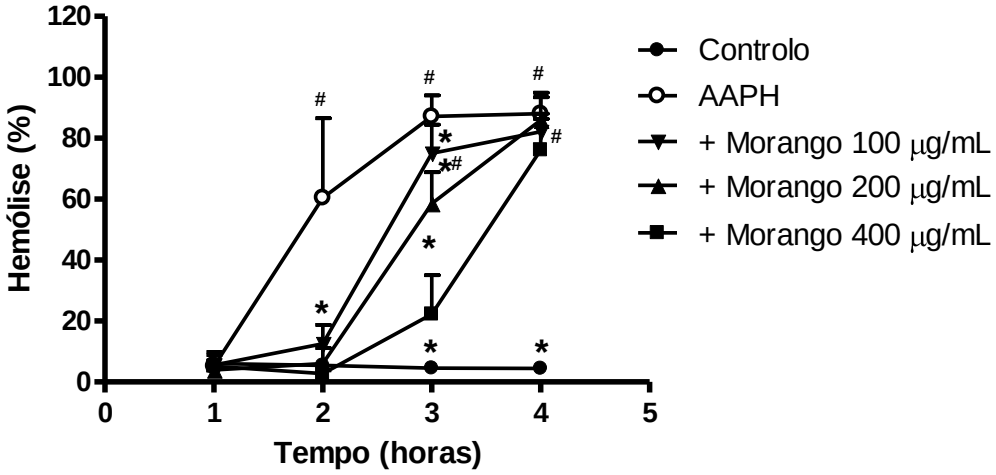

Gráfico 3. Gráfico do efeito protector do extracto de morango na hemólise induzida pelo AAPH

Para o cálculo do IC_{50} determinou-se a percentagem de inibição de hemólise para cada concentração de extracto ao tempo 3 horas, tal como apresentado na tabela seguinte.

Tabela 9. Percentagem de inibição de hemólise obtida para o extracto de morango

	% hemólise	-Controlo	% Inibição
Controlo	3,3		
	3,3		
	6,0		
	5,9		
AAPH	92,7	89,4	
	77,2	73,9	
	88,6	82,6	
	90,4	84,5	
Morango 100 µg/mL	78,8	75,5	15,5
	63,3	60,0	18,8
	73,1	67,1	18,8
	85,1	79,2	6,3
Morango 200 µg/mL	69,3	66,0	26,2
	55,9	52,6	28,8
	45,4	39,4	52,3
	63,2	57,3	32,2
Morango 400 µg/mL	12,6	9,3	89,6
	23,7	20,4	72,4
	13,2	7,2	91,3
	39,7	33,8	60,0

Em seguida, traçou-se o gráfico da % inibição de hemólise *versus* a concentração de extracto para cada ensaio independente. O valor de IC_{50} calculado para o extracto do morango foi de 273,84 ± 49,38 µg/mL.

3.4 DISCUSSÃO DOS RESULTADOS

Os gráficos 1 e 2 mostram o efeito anti-hemolítico dos extractos de folha (20-80 µg/mL) e fruto (2,5-20 µg/mL) de dragoeiro, respectivamente. Verificou-se que o grupo controlo (suspensão eritrocitária em tampão fosfato sem adição de AAPH) manteve-se estável, com uma percentagem de hemólise reduzida ao longo das quatro horas de incubação. No entanto, quando se adicionou o AAPH à suspensão eritrocitária, a indução de hemólise passou a ser proporcional ao tempo de ensaio decorrido. O início da hemólise induzida pelo AAPH foi retardado, indicando que os antioxidantes endógenos do eritrócito, principalmente a glutationa, vitamina E, ácido L-ascórbico e

enzimas como a catalase e a superóxido dismutase, são capazes de sequestrar os radicais livres, conferindo protecção contra estas espécies que induzem a hemólise (Zou et al., 2001). Quanto ao grupo controlo do extracto (eritrócitos incubados com a maior concentração testada de cada extracto na ausência de AAPH) verificou-se que a percentagem de hemólise obtida foi semelhante à do grupo controlo.

Os resultados obtidos neste estudo mostram que ambos os extractos protegem significativamente a membrana dos eritrócitos da hemólise induzida pelo AAPH, de um modo dependente da concentração de extracto e do tempo de incubação. No entanto, o extracto do fruto apresentou um efeito anti-hemolítico superior ao da folha, sendo o valor de IC_{50} determinado para o extracto do fruto após três horas de incubação de 2,56 ± 0,97 µg/mL e de 39,05 ± 11,54 µg/mL para a folha. Costa et al. (2009) estabeleceram para o extracto de chá verde um valor de IC_{50} de 24,3 ± 9,6 µg/mL utilizando as mesmas condições do presente ensaio, valor este que enfatiza a actividade antioxidante exibida pelo extracto da folha de dragoeiro.

Dada a reconhecida actividade antioxidante do morango, a actividade anti-hemolítica do extracto do fruto de dragoeiro foi comparada com a do extracto deste fruto vermelho. Os resultados obtidos mostram que o extracto do fruto de dragoeiro apresentou um efeito anti-hemolítico bastante superior ao do extracto de morango, como se pode observar no gráfico 3. O valor de IC_{50}, calculado a um tempo de incubação de 3 horas, para o extracto do fruto de dragoeiro foi significativamente inferior ao extracto de morango (2,56 ± 0,97 µg/mL e 273,84 ± 49,38 µg/mL, respectivamente; $P < 0.05$) nas mesmas condições de teste antioxidante, o que enfatiza a forte actividade antiradicalar do fruto. É ainda de realçar que o valor de IC_{50} obtido para a folha é significativamente superior ao do fruto, o que significa que esta última matriz é ainda mais interessante como agente antioxidante. De realçar que este é o primeiro estudo que avalia o potencial antioxidante da espécie *D. draco* neste modelo celular.

Várias investigações têm vindo a demonstrar que compostos polifenólicos aumentam a resistência dos eritrócitos ao stress oxidativo (Costa et al., 2009; Youdim, Shukitt-Hale, MacKinnon, Kalt, e Joseph, 2000; Magalhães, Silva, Pereira, Andrade, valentão, & Carvalho, 2009). Os polifenóis são sobejamente reconhecidos como

eficientes sequestradores de radicais livres (Bors et al., 1990 e Nanjo et al., 1996). Estes fitoquímicos actuam como antioxidantes na inactivação dos radicais livres nos compartimentos celulares lipofílicos e hidrofílicos, dada a capacidade destes compostos de doar átomos de hidrogénio e, desta forma, inibir as reacções em cadeia provocadas pelos radicais livres (Hartman et al., 1990; Arora et al., 1998). Nos artigos apresentados em anexo podemos ver que os extractos aquosos da folha e fruto de dragoeiro usados neste estudo apresentam quantidades significativas de compostos fenólicos. O extracto do fruto apresenta um perfil fenólico constituído por cinco compostos: os ácidos 5-*O*-cafeoilquínico, 3,5-*O*-dicafeoilquínico, ferúlico e sinápico e a quercetina-3-*O*-rutinósido (Silva et al., 2011). O extracto da folha foi caracterizado pela presença de nove compostos fenólicos, os mesmos encontrados no fruto acrescidos do ácido cafeico, ácido *p*-cumárico, campferol-3-*O*-glucósido e o campferol-3-*O*-rutinósido (Santos et al., 2011). O composto fenólico mais abundante em ambos os extractos foi a quercetina-3-*O*-rutinósido.

No modelo celular utilizado neste estudo, os compostos fenólicos do dragoeiro presentes no meio de incubação podem proteger contra a peroxidação lipídica sequestrando os radicais peroxilo formados durante a incubação, interrompendo desta forma a propagação em cadeia dos radicais peroxilo e evitando o ataque destes às membranas dos eritrócitos, ricas em ácidos gordos polinsaturados, e com isto inibindo a peroxidação lipídica e a consequente hemólise. Além dos polifenóis, outros compostos antioxidantes presentes nas folhas e frutos podem igualmente contribuir para a sua actividade antihemolítica, tais como os ácidos orgânicos identificados nas folhas – ácidos oxálico e cítrico – e nos frutos – ácidos málico, quinico e cítrico – que são igualmente antioxidantes eficazes. Esta eficácia é atribuída à forte capacidade destes compostos de quelatar os iões metálicos envolvidos na produção de radicais livres (Seabra et al., 2006). A actividade antioxidante foi igualmente descrita para alguns compostos voláteis, incluindo o maltol e limoneno (Wei, Mura, e Shibamoto, 2001; Wei, e Shibamoto, 2007; Grassmann, Hippeli, Vollmann & Elstner, 2003). Além disso, os efeitos sinérgicos de compostos fenólicos com outros antioxidantes têm sido descritos (Croft, 1998; Liao, & Yin, 2000) e, portanto, o efeito protector da folha e fruto da espécie *D. draco* contra os danos oxidativos em eritrócitos induzidos por radicais livres pode reflectir a sua acção combinada.

IV. CONCLUSÕES GERAIS

O stress oxidativo parece estar envolvido na maioria das doenças crónicas, tais como o cancro, doenças degenerativas e doenças cardiovasculares (García-Alonso et al., 2004; Magalhães et al., 2009; Seeram, 2008; Zhang et al., 2008). Os antioxidantes provenientes da alimentação são particularmente importantes na luta contra essas doenças uma vez que conferem protecção contra os danos induzidos pelos radicais livres no DNA celular, proteínas e lípidos (Cao et al., 1996; Costa et al., 2009; du Toit et al., 2001; García-Alonso et al., 2004; Magalhães et al., 2009; Marques et al., 2009; Seeram, 2008; Zhang et al., 2008).

Para uma melhor compreensão das propriedades antioxidantes dos extractos de folha e fruto da espécie D. *draco* em células humanas, o eritrócito humano foi utilizado como modelo celular *in vitro*. Os resultados obtidos neste estudo demonstraram, pela primeira vez, que os extractos da folha e fruto de dragoeiro possuem actividade antioxidante e de sequestro de radicais livres notáveis, conferindo deste modo protecção contra o dano oxidativo induzido pelos radicais livres em membranas biológicas. Estas bioactividades parecem ser o resultado da acção combinada de compostos voláteis, semi-voláteis, fenólicos e ácidos orgânicos presentes nestas amostras. A folha e o fruto constituem então agentes antioxidantes naturais promissores e ainda pouco explorados, com elevado potencial para prevenir ou retardar o progresso de doenças humanas mediadas pelo stress oxidativo.

Em conclusão, os nossos resultados indicam que a espécie *D. draco*, especialmente o fruto, apresenta um considerável potencial antioxidante e sequestrador de radicais livres, o que sugere a sua eventual aplicação na prevenção e/ou tratamento de diversas situações patológicas em que os radicais livres estão envolvidos.

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VI. **ANEXOS**

Anexo 1. Modelo da Declaração de Consentimento

DECLARAÇÃO DE CONSENTIMENTO

Considerando a "Declaração de Helsinquia" da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Designação do Estudo (em português):

Eu, abaixo-assinado, (nome completo do doente ou voluntário são) ----------
----------, compreendi a explicação que me foi fornecida acerca da minha participação na investigação que se tenciona realizar, bem como do estudo em que serei incluído. Foi-me dada oportunidade de fazer as perguntas que julguei necessárias e de todas obtive resposta satisfatória.

Tomei conhecimento de que, de acordo com as recomendações da Declaração de Helsínquia, a informação ou explicação que me foi prestada versou os objectivos e os métodos e, se ocorrer uma situação de prática clínica, os benefícios previstos, os riscos potenciais e o eventual desconforto. Além disso, foi-me afirmado que tenho o direito de recusar a todo o tempo a minha participação no estudo, sem que isso possa ter como efeito qualquer prejuízo pessoal.

Por isso, consinto que me seja aplicado o método ou o tratamento, se for caso disso, propostos pelo investigador.

Data: _____/_____/ 200__

Assinatura do doente ou voluntário são:_____

O Investigador responsável:

Nome:

Assinatura:

Comissão de Ética da Universidade Fernando Pessoa

Anexo 2. Artigo *Phytochemical profiles and inhibitory effect on free radical-induced human erythrocyte damage of Dracaena draco leaf: A potential novel antioxidant agent* publicado na revista *Food Chemistry*.

Anexo 3. Artigo *Dracaena draco L. fruit: Phytochemical and antioxidant activity assessment* publicado na revista *Food Research International.*

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Phytochemical profiles and inhibitory effect on free radical-induced human erythrocyte damage of *Dracaena draco* leaf: A potential novel antioxidant agent

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Antioxidant activity

ABSTRACT

The present study reports for the first time the metabolite profile and antioxidant activity of aqueous extract obtained from *Dracaena draco* L. leaf. Volatiles profile was determined by HS-SPME/GC-IT-MS, with 34 compounds being identified, distributed by distinct chemical classes: 2 alcohols, 5 aldehydes, 16 carotenoid derivatives and 8 terpenic compounds. Carotenoid derivative compounds constituted the most abundant class in leaf (representing 45% of total identified compounds). Phenolics profile was determined by HPLC/DAD and 9 constituents were identified: 2 hydroxycinnamic acid derivatives – 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids; 4 hydroxycinnamic acids – caffeic, *p*-coumaric, ferulic and sinapic acids and 3 flavonol glycosides – quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside. The most abundant phenolic compound is quercetin-3-*O*-rutinoside (representing 50.2% of total polyphenols). Organic acids composition was also characterised, by HPLC–UV and oxalic, citric, malic and fumaric acids were determined. Oxalic and citric acids were present in higher amounts (representing 47%, each). The antioxidant potential of this material was assessed by the ability to protect against free radical-induced biomembrane damage, using human erythrocyte as *in vitro* model. Leaf extract strongly protected the erythrocyte membrane from haemolysis (IC_{50} of 39 ± 11 μg/ml), in a time- and concentration-dependent manner. This is the first report showing that *D. draco* leaf is a promising antioxidant agent.

1. Introduction

The dragon tree (*Dracaena draco* L.; Dracaenaceae family) is a subtropical plant species endemic to Macaronesian region. Macaronesia is a biogeographic area, which combines the geological characteristics with fauna and, especially, flora specificities. This region consists of the set formed by the Archipelagos of Madeira and Azores (both belonging to Portugal), Canaries (which are part of Spain) and Cape Verde and by a small enclave of the Moroccan coast (opposite the Canaries Islands).

D. draco is an arboreal species characterised by a single or multiple trunk growing up to 12 m tall (rarely more), with a dense umbrella-shaped canopy of thick leaves. It grows very slowly, requiring about 10 years reaching 1 m tall. The leaves are green, tinged with red at the base, arranged in dense rosettes at the ends of the branches. The flowers, very fragrant, form large clusters of greenish-white petals. The fruits are orange red berries, with a single seed, which easily germinates. When *D. draco* trunk or branches are wounded it secretes a dark red resin so-called "Dragon's blood" (Mimaki et al., 1999). Of note is that this sap may also be obtained from other botanical sources (Gupta, Bleakley, & Gupta, 2008). The *D. draco* resin has been used since ancient times for artistic purposes and by traditional medicine for its antidiarrhetic and haemostatic activities, amongst others (Ballabio, 2004; González et al., 2003; Gupta et al., 2008; Mimaki et al., 1999). Because of overexploitation, this species is currently cited as vulnerable in the IUCN Red List of Threatened Species (http://www.iucnredlist.org/).

Research on this plant species is limited and mainly directed towards the study of its saponins (González et al., 2003; Gupta et al., 2008; Hernández, Léon, Estévez, Quintana, & Bermejo, 2006; Hernández, Léon, Quintana, Estévez, & Bermejo, 2004; Mimaki et al., 1999; Sparg, Light, & van Standen, 2004). In fact, *D. draco* morphological parts are now considered as rich sources of cytostatic and/or cytotoxic steroidal saponins (Gupta et al., 2008). Darias et al. (1989) reported, for the first time, the use of the sap of *D. draco*

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as an anticarcinogen. Later, Mimaki et al. (1999) have proved the intense cytostatic activity against human acute myeloid leukaemia cells (HL-60) of the two steroidal saponins (25*R*)-spirost-5-en-3β-ol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside} and (23*S*,24*S*)-spirosta-5,25(27)-diene-1β,3β,23,24-tetrol 1-*O*-{*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→2)-β-L-arabinopyranosyl} 24-*O*-β-D-fucopyranoside extracted from the aerial parts of *D. draco*. In addition, González et al. (2003) reported the strong cytotoxic effect on the same cell line of the two new steroidal saponins draconins A and B isolated from *D. draco* bark. The mechanism of these compounds cytotoxicity was established to be via activation of apoptotic process (González et al., 2003). Icogenin and dioscin were isolated from the root of *D. draco* and their structural elucidation was performed by Hernández et al. (2004). These researchers have found that these compounds also inhibit HL-60 cells growth by induction of apoptosis (Hernández et al., 2004). Recently, the same group has isolated icodeside from the *D. draco* leaves and showed that it presents moderate cytotoxicity against both HL-60 and human epidermoid carcinoma (A-431) cells (Hernández et al., 2006).

Beyond the phytochemical studies concerning the *D. draco* saponins, little research has been done on this plant species to know about its thorough chemical constituents and biological activities. Some phenolic compounds have been identified in *D. draco* resin (Camarda, Merlini, & Nasini, 1983; González, Léon, Sánchez-Pinto, Padrón, & Bermejo, 2000; González et al., 2004; Melo et al., 2007), including the 2,4,4′-trihydroxydihydrochalcone, 3-(4-hydroxybenzyl)-5,7-dimethoxychroman and 7-hydroxy-3-(4-hydroxybenzyl)-chromone, which were described for the first time in nature by González et al. (2000). Recently, dracoflavylium was isolated and identified as the major red colourant in this resin (Melo et al., 2007). In addition, Hernández et al. (2006) reported the isolation from the *D. draco* leaves and the structural determination of the new homo-isoflavonoid dracol.

The use of antioxidants as preventive and/or therapeutic agents in oxidative stress related diseases has been generally proposed. From this perspective, plants rich in antioxidant phytochemicals, such as phenolic compounds, organic acids, certain volatiles, carotenoids, tocopherols and tocotrienols, amongst others, may play an essential role in the prevention of many diseases. As part of our research on natural antioxidants, we have carried out a phytochemical screening of the aqueous extract obtained from the leaves of *D. draco*, on whose constituents nothing thus far has been reported. Volatiles, polyphenols and organic acids profiles were determined by GC/MS, HPLC/DAD and HPLC/UV, respectively. In addition, the antioxidant capacity of the extract was also studied for its ability to inhibit the 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative haemolysis of human erythrocytes. As far as we know, this is the first report of volatile and organic acid composition, along with antioxidant activity, of *D. draco* leaf.

2. Materials and methods

2.1. Standards and reagents

All chemicals used were of analytical grade. The standards compounds were purchased from various suppliers: 2-decenol, maltol, octanal, 6-methyl-5-hepten-2-one, limonene, valencene, β-caryophyllene, β-cyclocitral, nonanal, geranylcetona, 2,2,6-trimethylcyclohexanone, β-ionone trans-2-nonenal were obtained from Sigma–Aldrich (St. Louis, MO); hexanal and (*E*, *E*)-2,4-nonadienal were from SAFC (Steinheim, Germany); *β*-ionone and safranal were from Extrasynthése (Genay, France); β-damascenone was kindly fournished by Firmenish (Buchs, Switzerland). The 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) synthesis was attempted according to the method of Schneider, Razungles, Augier, and Baumes (2001). Methanol and formic acid were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Plant material and extraction

D. draco leaves were collected in Pico Island (Azores, Portugal), in February 2009. Leaves were dried in a stove (Memmert UL6D – Germany) at 30 ± 2 °C for 5 days (in the dark). Dried leaves (~5 g; 20 mesh) were extracted with 250 ml of boiling water for 45 min. The resulting extract was then lyophilised and kept in a dessicator (in the dark), until analysis. The yield for extraction process was 28.8 ± 0.6%.

2.3. Headspace solid-phase microextraction (HS-SPME) for volatile compounds analysis

2.3.1. SPME fibres

Several commercial fibres can be used to extract volatiles. According to bibliography, recommendations of supplier (Supelco, Bellefonte, PA, USA) and to our own knowledge (Guedes de Pinho et al., 2009a) the fibre used was coated with divinylbenzene/polydimethylsiloxane (DVB/PDMS), 65 μm.

2.3.2. Volatiles extraction

Approximately 0.1 g of freeze-dried powered sample was dissolved in 5 ml of water in a 15 ml vial and 0.5 g of anhydrous sodium sulphate was added to favour the release of analytes from the matrix. It was then sealed with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA). The mixture was then magnetically stirred at 760 rpm, at 45 °C, for 5 min. The fibre was then exposed to the headspace for 20 min, with agitation (800 rpm). Afterwards, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 2 min the fibre was removed and conditioned in another GC injection port for 15 min at 250 °C.

2.3.3. Gas chromatography-ion trap-mass spectrometry analysis

GC-IT-MS analysis was performed with a Varian CP-3800 gas chromatograph (USA) coupled to a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. A VF-5 ms 30 m × 0.25 mm × 0.25 μm (FactorFour) column from VARIAN was used in the analysis. The injector port was heated to 220 °C and injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 ml/min. Oven temperature was set at 40 °C (for 1 min), then increasing 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in electron impact (EI) positive mode. Ionisation was maintained off during the first minute. Transfer line, manifold and trap temperatures of the ion trap detector were set at 280, 50 and 180 °C, respectively. Covered mass ranged from 40 to 350 *m/z*, with a scan rate of 6 scans per second. The emission current was 50 μA and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionisation time was 25,000 μs, with an ionisation storage level of 35 *m/z*. The analysis was performed in FullScan mode. Compounds were identified by comparing their retention times with those of authentic compounds analysed under the same conditions and by comparison of the retention indices (as Kovats indices) with literature data (Guedes de Pinho et al., 2009a). The comparison of MS fragmentation pattern with those of pure compounds and mass spectrum

database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Confirmation was also accomplished using laboratory built MS spectral database, obtained from chromatographic runs of pure compounds performed with the same equipment and conditions. Peak areas were determined by reconstructed Full Scan chromatogram using for each compound some specific ions, quantification ions. By this way some peaks which were co-eluted in Full Scan mode (resolution value less than 1) could be integrated with a value of resolution higher than 1.

2.4. HPLC/DAD for phenolic compounds analysis

The extract (0.02 g) was redissolved in 1 ml of water. Twenty microlitres of this aqueous solution was analysed on an analytical HPLC unit (Gilson) and a C18 Spherisorb ODS2 column (25.0 × 0.46 cm; 5 μm, particle size) from Waters (Ireland). The solvent system used was a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min and 80% B at 60 min, at a solvent flow rate of 0.9 ml/min, as reported previously (Carvalho et al., 2010). Detection was achieved with a Gilson Diode Array Detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm and chromatograms were recorded at 350 nm. Chromatographic data was processed by Unipoint® System software from Gilson Medical Electronics (Villiers le Bel, France). The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with the library of spectra previously compiled by the authors. Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3,5-*O*-Dicaffeoylquinic acid was quantified as 5-*O*-caffeoylquinic acid. The other compounds were quantified as themselves.

2.5. HPLC/UV for organic acids analysis

The extract (0.02 g) was redissolved in 1 ml of sulphuric acid 0.01 N. Separation was achieved as reported previously (Oliveira et al., 2008), with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device at 30 °C. Elution was carried out at a solvent flow rate of 0.2 ml/min, isocratically with sulphuric acid 0.01 N as the mobile phase. Detection was performed with a Gilson UV detector at 214 nm. Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

2.6. Oxidative haemolysis inhibition assay

Blood (5–10 ml) was obtained from healthy non-smoking volunteers by venipuncture, after written informed consent was obtained. Human erythrocytes from citrated blood were immediately isolated by centrifugation at 1500 rpm for 10 min at 4 °C. After removal of plasma and buffy coat, the erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4) and then resuspended using the same buffer to the desired haematocrit level. In order to induce free-radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (dissolved in PBS; final concentration 50 mM). To study the protective effects of *D. draco* leaf extracts against AAPH-induced oxidative haemolysis, an erythrocyte suspension at 2% haematocrit was preincubated with the aqueous extracts (50–200 μg/ml final concentrations, dissolved in PBS) at 37 °C for 30 min, followed by incubation with and without 50 mM AAPH. This reaction mixture was shaken gently whilst being incubated for 3 h at 37 °C. In all experiments, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with each extract) were used.

The extent of haemolysis was determined spectrophotometrically as described before (Costa et al., 2009). Briefly, aliquots of the reaction mixture were taken out at each hour of the 3 h of incubation, diluted with saline and centrifuged at 4000 rpm for 10 min to separate the erythrocytes. The percentage of haemolysis was determined by measuring the absorbance of the supernatant (A) at 545 nm and compared with that of complete haemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The haemolysis percentage was calculated using the formula: A/B × 100. The inhibitory concentration 50% (IC_{50}) at time 3 h was also calculated from dose–response curve obtained by plotting the percentage of haemolysis inhibition *versus* the extract concentration. Four independent experiments were used for these calculations.

2.7. Statistical analysis

Statistic analysis was performed using the Statistical Package for Social Sciences (SPSS, version 16.0) for Windows. Comparisons between two groups were performed by unpaired *t*-test. Multiple comparisons between more than two groups were performed by one-way ANOVA supplemented with Tukeýs HSD post hoc test. Significance was accepted at P lower than 0.05.

3. Results and discussion

3.1. Volatile profile of D. draco leaf extract

Volatiles in plants have defensive functions or are attractants, repellents, grazing inhibitors and insecticides (Guedes de Pinho et al., 2009b). Plant research often report biologically active volatile compounds. To our knowledge *D. Draco* leaf volatile profile was achieved for the first time in this work and 31 volatile and semi-volatile compounds were identified by HS-SPME/GC-IT-MS being distributed by distinct chemical classes: 5 aldehydes, 2 alcohols, 8 terpenic compounds and 16 carotenoid derivatives compounds (Figs. 1 and 2 and Table 1). The most important chemical family present in leaves is the carotenoid derivatives, which represent more the 45% of the total volatiles. These compounds are known to be oxidative byproducts or degradation products of carotenoids, such as carotene and lutein (Goff & Klee, 2006). Ionone (2) (12) was found to be the major compound in leaves (representing 17% of the total volatiles) (Table 1). A number of biological activities have been described for β-ionone, which is an isomer of ionone (2) compound, namely anticancer capacity (Yang, 2008). Janakiram, Cooma, Mohammed, Steele, and Rao (2008) demonstrated that β-ionone inhibits colonic aberrant crypt foci formation in rats, suppresses cell growth and induces retinoid X receptor-alpha in human colon cancer cells.

Some works have suggested that biological activity of carotenoids might be attributed to its cleavage products formed by biochemical or auto-oxidation pathways (Macías, Lacret, Varela, Nogueiras, & Molinillo, 2008), owing to the fact that carotenoids are not easily bioavailable as they are molecules with high molecular weights (Faulks & Southon, 2005). It seems that the chemoprotective effects of carotenoids are due to their polar oxidative cleavage products (Hu, White, Jacobsen, Mangelsdorf, & Canfield, 1998; Zhang, Kotatake-Nara, Ono, & Nagao, 2003). Isoprenoids are plant compounds that have tumour suppressing activity in experimental animals. It is also reported that isoprenoids have been shown to modulate cell growth, induce cell cycle arrest, initi-

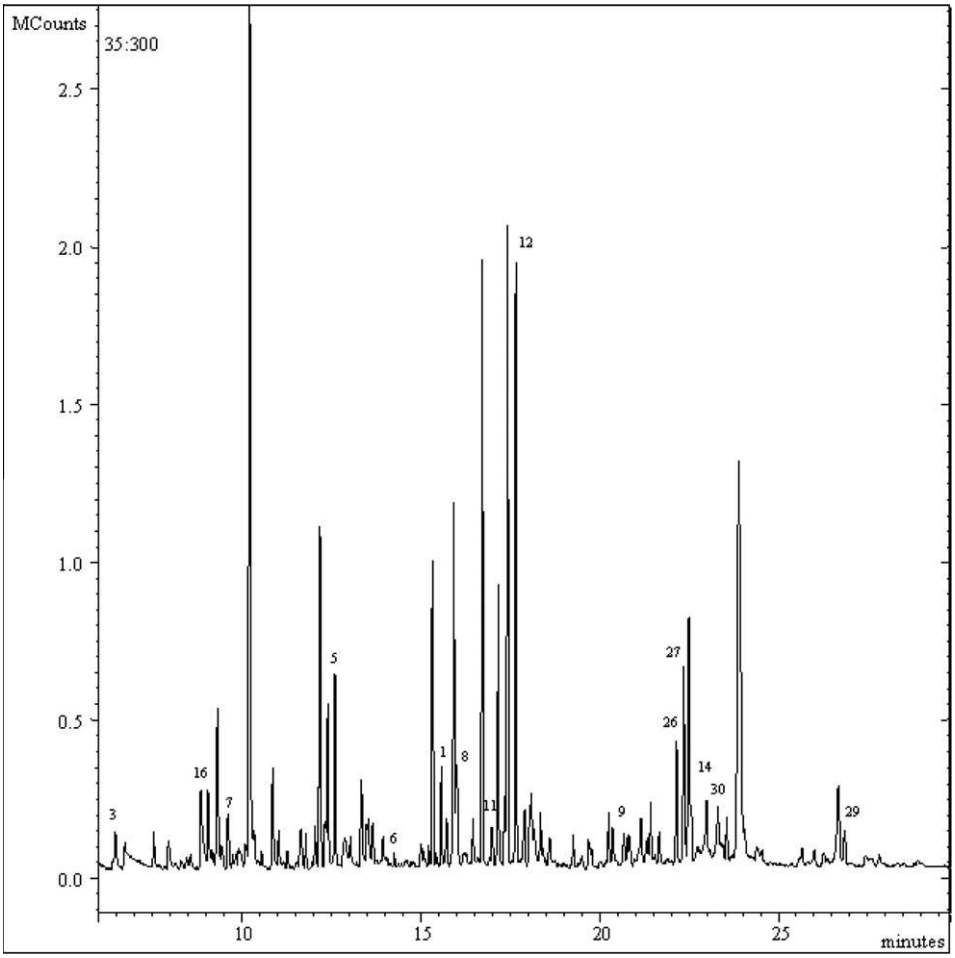

Fig. 1. Chromatograms of the HS-SPME of *D. draco* leaf extract in full scan acquisition. The corresponding compound names are shown in Table 1.

ate apoptosis and suppress cellular signalling activities (Janakiram et al., 2008).

Terpenic compounds are the following most representative family identified in *D. draco* leaves (Table 1). Many terpenoids have medicinal properties, such as anti-carcinogenic, antimalarial, anti-ulcer, antimicrobial, antiseptic, nematicidal, larvicidal, anti-inflammatory and diuretic activities (Schwab, Davidovich-Rikanati, & Lewinsohn, 2008), therefore being a most valuable class of compounds. The α-caryophyllene was the major sesquiterpene compound identified in this matrix. Biological activities have been ascribed to β-caryophyllene, it seems to possess anti-carcinogenic properties, due to its capability to induce detoxifying enzymes or to enhance, *in vitro* and *in vivo*, the natural killer cell-induced cytotoxicity against tumours (Di Sotto, Mazzanti, Carbone, Hrelia, & Maffei, 2010).

Finally, aldehydes and alcohols were found as minor compounds in leaves, representing 8.5% and 4.7% of the total volatiles, respectively. Gardner, Dornbos, and Desjardins (1990) have found that aldehydes, such as hexanal, exhibit strong antifungal activities.

3.2. Phenolic profile of D. draco leaf extract

Recent studies conducted both in cell cultures and animal models seem to indicate that polyphenols are the main phytochemicals with antioxidant and antiproliferative properties of higher plants (Fattouch et al., 2007; Fresco, Borges, Diniz, & Marques, 2006; Khan & Mukhtar, 2008; Mertens-Talcott, Lee, Percival, & Talcott, 2006; Proença da Cunha, 2005; Zhang, Zhao, & Wang, 2008). The aqueous extract of *D. draco* leaf is a rich source of phenolic compounds (12.1 g/kg). Its phenolic profile is composed by nine constituents (Fig. 3 and Table 2): two hydroxycinnamic acid derivatives – 5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids; four hydroxycinnamic acids – caffeic, *p*-coumaric, ferulic and sinapic acids; three flavonol glycosides – quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside. As far as we know, this is the first time that these phenolic compounds are reported in *D. draco* leaf. The most representative class of phenolic compounds is the flavonol family (80.8% of the total phenolic content) and the most abundant is quercetin-3-*O*-rutinoside (6.1 g/kg, representing 50.2% of the total polyphenols). Noteworthy, the antioxidant, anti-inflammatory and anticancer effects of flavonols, as quercetin and kaempferol, has been recently reported (Fresco et al., 2006). Flavonols and their derivatives, like quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside, are able to act as antioxidants in a number of ways. These antioxidants act as reducing agents, hydrogen donators, free radicals scavengers and singlet oxygen quenchers and, therefore, as cell saviours (Fattouch et al., 2007).

3.3. Organic acids profile of D. draco leaf extract

Organic acids are primary metabolites found in great amounts in all plants, which may well exert a protective role against various diseases, due to their antioxidant activity. *D. draco* leaf extract is an specially rich source of organic acids (168.6 g/kg), presenting an

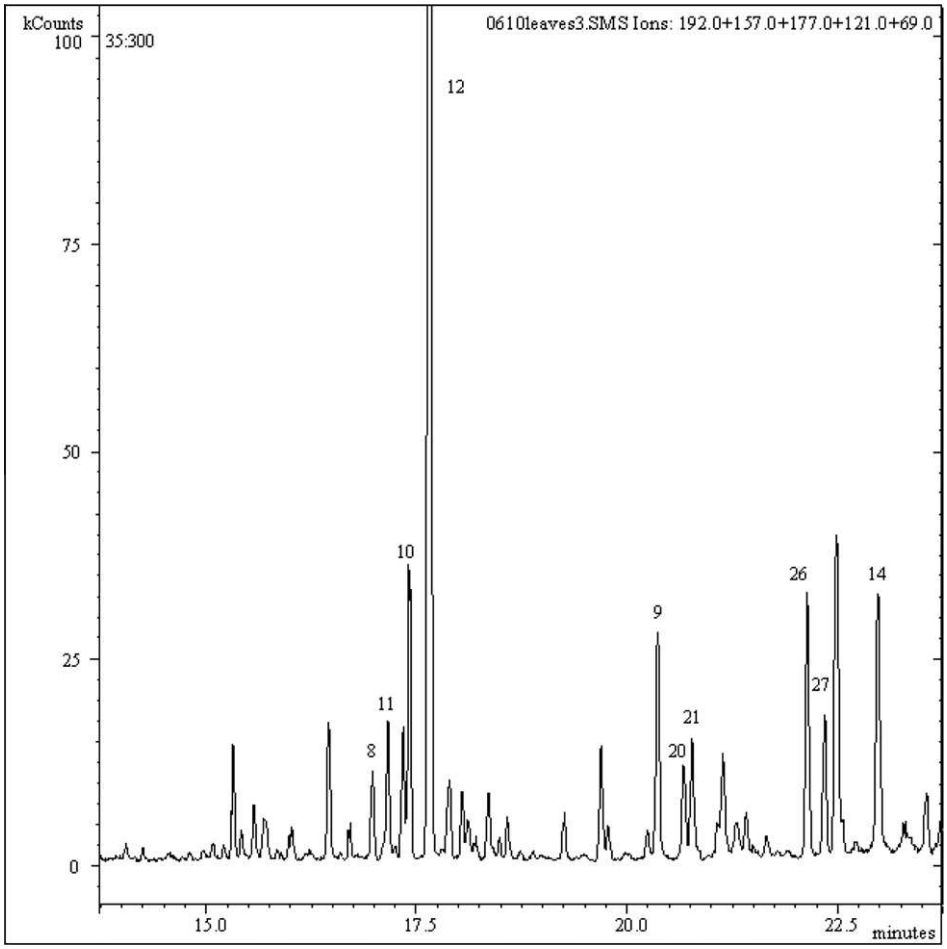

Fig. 2. Reconstructed full scan chromatogram using some characteristic *m/z* ions of volatile carotenoid derivatives (C11 and C13) and terpenic compounds. The corresponding compound names are shown in Table 1.

organic acid profile composed by four compounds (Fig. 4 and Table 3): oxalic, citric, malic and fumaric acids. As far as we know, this is the first time that organic acid profile is described in *D. draco* leaf. The main organic acids are oxalic and citric acids (representing 47.3% and 47.4% of the total organic acid content, respectively). Oxalic acid is the simplest dicarboxylic acid and its most striking chemical impact is its strong chelating ability for multivalent cations. Citric acid behaves as an antioxidant since it also has the ability to chelate metals. They are classified as "preventive" or synergistic antioxidants (Oliveira et al., 2008; Seabra et al., 2006).

3.4. Protective effect of D. draco leaf extract against free radical-induced haemolysis

Erythrocytes are considered as major targets for free radical attack owing to the presence of high membrane concentration of polyunsaturated fatty acids and to their specific role as oxygen carriers (Ajila & Rao, 2008). Thus, *in vitro* oxidative haemolysis of human erythrocytes was used herein as a model to study the antioxidant effect of *D. draco* leaf extract on free radical-induced damage of biological membranes. For this study, AAPH was used as the free-radical initiator to induce oxidative damage in erythrocytes. Thermal decomposition at physiological temperature of AAPH generates peroxyl radicals ($ROO^{\cdot}$) in the aqueous phase (Niki, 1990), which can attack the erythrocyte membrane to induce lipid peroxidation and ultimately haemolysis.

Fig. 5 shows the antioxidant effect of *D. draco* leaf extract (20–80 μg/ml) on human erythrocytes exposed to the water-soluble radical initiator AAPH. Erythrocytes incubated at 37 °C in PBS (control samples) were stable, with little haemolysis observed within 3 h. In addition, cells incubated with extracts of *D. draco* leaf alone (without AAPH) at the highest concentration tested (80 μg/ml) presented haemolysis background level similar to that of control samples (data not shown). When AAPH was added to the erythrocyte suspension, haemolysis induction was time-dependent. *D. draco* leaf extract significantly protected the erythrocyte membrane from haemolysis induced by AAPH in a concentration- and time-dependent manner. The IC_{50} value calculated after 3 h of incubation was 39 ± 11 μg/ml. Noteworthy, a similar IC_{50} value was reported by Costa et al. (2009) regarding the green tea extract (24.3 ± 9.6 μg/ml) in the same antioxidant test conditions, which emphasise the strong antioxidant activity obtained in this study for *D.draco* leaf extract. Several studies have demonstrated that polyphenolic compounds enhance erythrocyte resistance to oxidative stress (Costa et al., 2009; Magalhães et al., 2009; Youdim, Shukitt-Hale, MacKinnon, Kalt, & Joseph, 2000). The strong antioxidant effects of polyphenols have been highlighted by several studies, with underlying mechanisms involving both free radical scavenging (Bors, Heller, Michel, & Saran, 1990) and redox-active metal chelation (van Acker, van Balen, van den Berg, Bast, & van der Vijgh, 1998). In our model, these phytochemicals present in the incubation medium may quench peroxyl radicals in the aqueous phase before these radicals attack the lipid molecules of the erythrocyte

Table 1
Volatile composition of *D. draco* leaf extract.

No	Compound	RI[a]	ID[b]	QI[c] (*m/z*)	Area[d] (RA)
Alcohols					
1	2-Decenol	1295	S, MS	81/95	2.07
2	Maltol	1202	S, MS	126	2.65
	Total of alcohols				4.73
Aldehydes					
3	Hexanal	890	S, MS	56/67/82	1.12
4	2,4-Nonadienal	1081	S, MS	81	1.13
5	Nonanal	1194	S, MS	81/95	4.28
6	trans-2-nonenal	1252	S, MS	83/96	0.29
7	Octanal	1094	S, MS	69/82	1.65
	Total of aldehydes				8.46
Carotenoid derivative compounds					
8	β-Cyclocitral	1305	S, MS	109/137/152	4.73
9	β-Damascenone	1376	S, MS	69/121	3.08
10	β-Damascone	1326	MS (74.9/75.5)	123/177/192	0.97
11	2,6,6-Trimethyl-(1-cyclohexen-2-yl)-3-buten-2-one (ionone 1)	1315	MS (76.0/80.7)	121/177/192	1.65
12	2,6,6-Trimethyl-(1-cyclohexen-2-yl)-3-buten-2-one (ionone 2)	1339	MS (78.5/82.8)	121/177/192	17.12
13	1,1,6-Trimethyl-2,3,4-tetrahydronaphtalene	1325	MS (67.4/78.3)	159/174	0.35
14	2,6,6-Trimethyl-(1-cyclohexen-2-yl)-3-buten-2-one (β-ionone)	1474	S, MS	177	3.64
15	2-Methyl-2-cyclohexen-1-one	1152	MS (76.9/81.4)	82/110	1.33
16	6-Methyl-5-hepten-2-one	1077	S, MS	69/108	2.72
17	Megastigmatrienone	1385	MS (74.8/76.6)	105/175/190	1.93
18	Safranal	1290	S, MS	107/121/150	0.75
19	2,2,6-Trimethylcyclohexanone	1127	S, MS	82/140	1.37
20	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN1)	1387	S*, MS	142/157/172	2.80
21	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN2)	1392	S*, MS	157/172	1.34
22	Dihydroactinidiolide	1534	MS (76.6/87.7)	111/137/180	1.77
23	α-Calacorene (C15-H20)	1538	MS (76.0/91.0)	142/157/200	1.22
	Total of carotenoid derivatives compounds				45.41
Terpenic compounds					
24	3-Methylene-1,5,5-trimethylcyclohexene	1085	MS (81.1/86.4)	93/121	13.75
25	Limonene	1119	S, MS	67/93/121	1.63
26	Geranylacetone	1441	S, MS	69/107	5.73
27	α-Caryophyllene	1449	S, MS	93/105/121	12.01
28	β-Caryophyllene	1411	S, MS	93/161/189	1.94
29	α-Cedrene	1638	MS (75.3/76.6)	91/119/161/204	2.95
30	Valencene	1486	S, MS	161/189/204	2.50
31	Caryophyllene oxide	1611	MS (68.1/73.1)	67/96/109	0.91
	Total of terpenic compounds				41.42

[a] RI = Retention index.
[b] ID = Identification method (fit/retrofit values, %). S = identified by comparison with standard, MS = tentatively identified by NIST05, S* = synthesised compound.
[c] QI = quantification ions.
[d] Area expressed as arbitrary units. RA (%) = relative area in percentage.

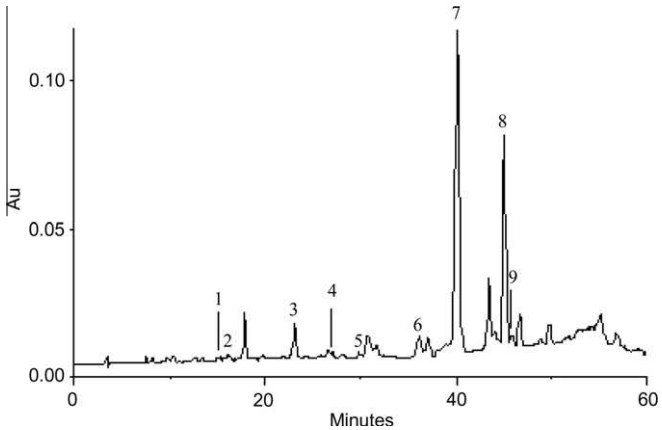

Fig. 3. HPLC phenolic profile of *D. draco* leaf extract. Detection at 350 nm. Peaks: (1) 5-*O*-caffeoylquinic acid, (2) caffeic acid, (3) *p*-coumaric acid, (4) ferulic acid, (5) sinapic acid, (6) 3,5-*O*-dicaffeoylquinic acid, (7) quercetin-3-*O*-rutinoside, (8) kaempferol-3-*O*-glucoside, (9) kaempferol-3-*O*-rutinoside.

Table 2
Phenolic composition of *D. draco* leaf extract.

Phenolic compound	Content[a]
5-*O*-caffeoylquinic acid	23.7 ± 1.5
Caffeic acid	26.1 ± 0.2
p-Coumaric acid	1395.8 ± 10.1
Ferulic acid	175.2 ± 11.0
Sinapic acid	328.7 ± 6.8
3,5-*O*-dicaffeoylquinic acid	366.1 ± 5.5
Quercetin-3-*O*-rutinoside	6053.7 ± 80.9
Kaempferol-3-*O*- glucoside	3492.8 ± 64.3
Kaempferol-3-*O*-rutinoside	207.7 ± 1.5
Σ	12069.8

[a] Values are expressed as mean ± standard deviation of three assays for each sample (mg/kg of aqueous extract). Abbreviations: Σ – sum of the determined phenolics.

membrane, which breaks the free-radical chain reaction and inhibits its subsequent oxidative haemolysis.

Besides polyphenols, other antioxidant compounds present in leaves may also contribute for its antihemolytic activity. In fact, the major organic acids identified in leaves – oxalic and citric acids – are also effective antioxidants. As previously referred, the antioxidant activity of these compounds is attributed to their strong ability to chelate metal ions involved in the production of free radicals (Seabra et al., 2006). Antioxidant activities have also been described for some volatile compounds, including maltol and limonene (Grassmann, Hippeli, Vollmann, & Elstner, 2003; Wei, Mura, & Shibamoto, 2001; Wei & Shibamoto, 2007). Additionally, syner-

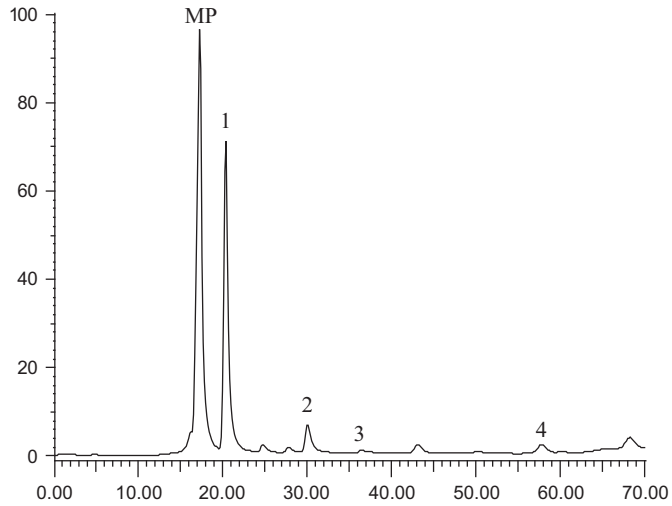

Fig. 4. HPLC organic acid profile of *D. draco* leaf extract. Detection at 214 nm. Peaks: (MP) mobile phase, (1) oxalic acid, (2) citric acid, (3) malic acid, (4) fumaric acid.

Table 3
Organic acid composition of *D. draco* leaf extract.

Organic acid	Content[a]
Oxalic acid	79.7 ± 0.4
Citric acid	79.9 ± 2.2
Malic acid	8.7 ± 0.1
Fumaric acid	0.3 ± 0.0
Σ	168.6

[a] Values are expressed as mean ± standard deviation of three assays for each sample (g/kg of aqueous extract). Abbreviations: Σ – sum of the determined organic acids.

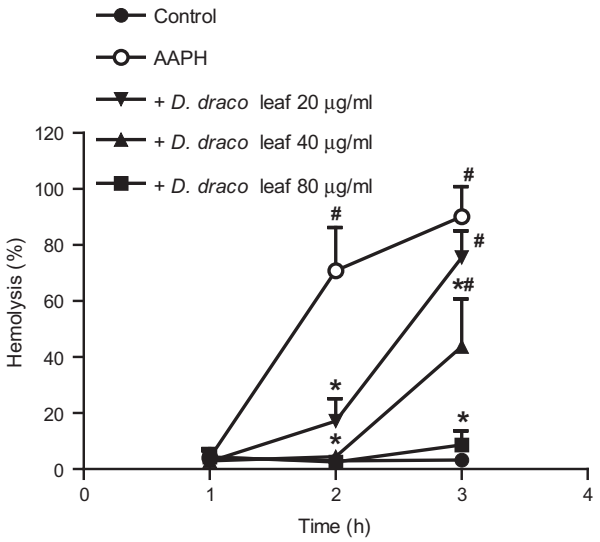

Fig. 5. Effects of *D. draco* leaf extract on AAPH-induced haemolysis in erythrocytes. An erythrocyte suspension at 2% haematocrit was preincubated with extracts at the indicated concentrations for 30 min at 37 °C. The cell suspension was then incubated with 50 mM AAPH for 3 h at 37 °C. In all experiments, control erythrocytes (incubated with PBS only) and AAPH-treated erythrocytes (incubated with 50 mM AAPH) were used. Values are expressed as the mean ± SEM of four independent experiments. $^{*}P < 0.05$, as compared with AAPH at respective time, $^{\#}P < 0.05$, as compared with control at respective time.

gistic effects of phenolics with other antioxidants have been described (Croft, 1998; Liao & Yin, 2000) and hence the protective effect showed by *D. draco* leaf against free radical-induced oxidative injury in erythrocytes may reflects their combined action.

4. Conclusion

This study reports the volatile, phenolic and organic acid constituents present in *D. draco* leaf, which increase our knowledge on the antioxidant potential of this species. In addition, our results demonstrate for the first time that *D. draco* leaf extract confers protection against free radical-induced oxidative damage on biological membranes. It is therefore suggested that *D. draco* leaf is a novel and promising natural antioxidant agent with high potential to prevent or slow the progress of human diseases mediated by oxidative stress. Considering its chemical composition and excellent antioxidant properties, further assays are being undertaken in this matrix to assess other biological activities, namely its anticancer potential.

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Dracaena draco L. fruit: Phytochemical and antioxidant activity assessment

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ABSTRACT

The present study reports for the first time the metabolite profile and antioxidant activity of aqueous extract obtained from *Dracaena draco* L. fruit. Volatiles profile was determined by HS-SPME/GC-IT-MS, with 9 compounds being identified, distributed by several distinct chemical classes: 1 alcohol, 3 aldehydes, 2 carotenoid derivatives, and 3 terpenic compounds. Aldehydes constituted the most abundant class in this exotic berry, representing 59% of total identified volatile compounds. Phenolics profile was determined by HPLC/DAD and 5 constituents were identified: 5-*O*-caffeoylquinic, 3,5-*O*-dicaffeoylquinic, ferulic and sinapic acids, and quercetin-3-*O*-rutinoside. The major phenolic compound is quercetin-3-*O*-rutinoside, comprising 42% of the total phenolic content. Organic acids composition was also characterized, by HPLC-UV, and oxalic, citric, L-ascorbic, malic, quinic and shikimic acids were determined. The most abundant is quinic acid, representing 39% of the total organic acid content. The antioxidant potential of this matrix was assessed by (i) reducing power of Fe^{3+}/ferricyanide complex, (ii) scavenging effect on DPPH free radicals, and (iii) ability to inhibit the 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative hemolysis in human erythrocytes. Strawberry (*Fragaria* × *ananassa* Duch. cv. Camarosa) extract was used for comparison purposes. All assay models showed remarkable concentration dependent antioxidant activity, reducing power and radical scavenging efficiency for *D. draco* fruit, being invariably higher than that of strawberry extract. This is the first report showing that *D. draco* fruit is a promising new antioxidant agent.

1. Introduction

Overwhelming scientific evidence suggests that significant health risks and benefits are associated with the dietary food choices (García-Alonso et al., 2004; Seeram, 2008; Zhang et al., 2008). Increased consumption of fruits and vegetables has been associated with protection against various chronic diseases, including cardiovascular diseases and cancer (Fattouch et al., 2007; García-Alonso et al., 2004; Seeram, 2008; Zhang et al., 2008). This association is often attributed to the antioxidant compounds present in these plant foods, such as vitamins C and E, carotenoids, phenolic acids and flavonoids, which prevent free radical damage (Cao et al., 1996; du Toit et al., 2001).

The dragon tree (*Dracaena draco* L.; Dracaenaceae family) is a subtropical plant species which is endemic to Macaronesia. The Macaronesian region is a biogeographic area, combining the geological characteristics with fauna and, especially, flora specificities. This region comprises the Archipelagos of Madeira and Azores, Canaries and Cape Verde, and a small enclave of the Moroccan coast (opposite the Canaries Islands).

Several studies demonstrated that the different morphological parts of *D. draco* species are rich sources of cytostatic and/or cytotoxic steroidal saponins (González et al., 2003; Gupta et al., 2008; Hernández et al., 2004, 2006; Mimaki et al., 1999; Sparg et al., 2004). Despite these studies concerning the *D. draco* saponins, little research has been done on this plant species. Recently, our group has reported the volatiles, phenolics and organic acids constituents present in *D. draco* leaves water extract and has demonstrated that it possess significant antioxidant activity on biological membranes and therefore may be recommended as a novel source of natural antioxidants (Santos et al., 2011). However, as far as we know, the chemical composition and biological activities of *D. draco* fruit are still unknown. So, continuing the research on *D. draco* species, we have carried out a phytochemical screening of the aqueous extract obtained from this exotic "berry-type" fruit. Volatiles, polyphenols and organic acids profiles were determined by GC/MS, HPLC/DAD and HPLC/UV, respectively. In addition, in this work, the antioxidant properties of *D. draco* fruit were evaluated by different *in vitro* antioxidant assays such as DPPH radical scavenging, reducing power, and protection against

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free radical-induced human erythrocyte hemolysis. Since *D. draco* berry presents orange/red color and strawberry (*Fragaria×ananassa* Duch.) is a well documented antioxidant red "berry-type" fruit with recognized biological effects (García-Alonso et al., 2004; Seeram, 2008; Zhang et al., 2008), a strawberry aqueous extract was used as a control for comparison purposes.

2. Materials and methods

2.1. Standards and reagents

All chemicals used were of analytical grade. The standard volatile compounds were purchased from various suppliers: 2-decenol, 6-methyl-5-hepten-2-one, valencene, β-caryophyllene, nonanal, geranylacetone, were obtained from Sigma-Aldrich (St. Louis, MO); hexanal, and (*E,E*)-2,4-nonadienal were from SAFC (Steinheim, Germany). The 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) synthesis was attempted according to the method of Schneider et al. (2001). Methanol and formic acid were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Plant material and extraction

D. draco fruits were collected in Tenerife (Canaries, Spain), in August of 2008. The samples were immediately frozen and freeze-dried (Ly-8-FM-ULE, Snijders) prior to extraction. Three powdered subsamples (~5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min. The resulting extract was then lyophilized and kept in a dessicator (in the dark), until analysis.

Strawberries (*Fragaria×ananassa* Duch. cv. Camarosa) were purchased in the Portuguese market, in June of 2009, and the extract was prepared as described for *D. draco* fruit.

The yields for extraction process were 28.77 ± 1.19% and 45.53 ± 4.90% for *D. draco* fruit and strawberry, respectively.

2.3. Headspace solid-phase microextraction (HS-SPME) for volatile compounds analysis

2.3.1. SPME fibres

Several commercial fibres can be used to extract volatiles. According to bibliography, recommendations of supplier (Supelco, Bellefonte, PA, USA) and to our own knowledge (Guedes de Pinho et al., 2009; Santos et al., 2011) the fibre used was coated with divinylbenzene/polydimethylsiloxane (DVB/PDMS), 65 μm.

2.3.2. Volatiles extraction

Approximately 0.1 g of freeze-dried powered sample was dissolved in 5 mL of water in a 15 mL vial, and 0.5 g of anhydrous sodium sulphate was added to favour the release of analytes from the matrix. It was then sealed with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA). The mixture was then magnetically stirred at 760 rpm, at 45 °C, for 5 min. The fibre was then exposed to the headspace for 20 min, with agitation (800 rpm). Afterwards, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 2 min the fibre was removed and conditioned in another GC injection port for 15 min at 250 °C.

2.3.3. Gas chromatography-ion trap-mass spectrometry analysis

GC-IT-MS analysis was performed with a Varian CP-3800 gas chromatograph (USA) coupled to a VARIAN Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. A VF-5 ms 30 m × 0.25 mm × 0.25 μm (FactorFour) column from VARIAN was used in the analysis. The injector port was heated to 220 °C and injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. Oven temperature was set at 40 °C (for 1 min), then increasing 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first minute. Transfer line, manifold and trap temperatures of the ion trap detector were set at 280, 50 and 180 °C, respectively. Covered mass ranged from 35 to 300 m/z, with a scan rate of 6 scans/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionization time was 25,000 ms, with an ionization storage level of 35 m/z. The analysis was performed in FullScan mode. Compounds were identified by comparing their retention times with those of authentic compounds analyzed under the same conditions, and by comparison of the retention indices (as Kovats indices) with literature data (Guedes de Pinho et al., 2009; Santos et al., 2011). The comparison of MS fragmentation pattern with those of pure compounds and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Confirmation was also accomplished using laboratory built MS spectral database, obtained from chromatographic runs of pure compounds performed with the same equipment and conditions. Peaks' areas were determined by reconstructed Full Scan chromatogram using for each compound some specific ions, quantification ions. By this way some peaks which were co-eluted in Full Scan mode (resolution value less than 1) could be integrated with a value of resolution higher than 1.

2.4. HPLC/DAD for phenolic compounds analysis

The extract (0.03 g) was redissolved in 1 mL of water. Twenty microliters of this aqueous solution was analyzed using an analytical HPLC unit (Gilson) and a C18 Spherisorb ODS2 column (25.0 × 0.46 cm; 5 μm, particle size) from Waters (Ireland). The solvent system used was a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min and 80% B at 60 min, at a solvent flow rate of 0.9 mL/min, as reported previously (Silva et al., 2008; Carvalho et al., 2010; Santos et al., 2011). Detection was achieved with a Gilson Diode Array Detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 350 nm. Chromatographic data was processed by Unipoint® System software from Gilson Medical Electronics (Villiers le Bel, France). The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with the library of spectra previously compiled by the authors (Silva et al., 2008; Carvalho et al., 2010; Santos et al., 2011). Quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3,5-*O*-dicaffeoylquinic acid was quantified as 5-*O*-caffeoylquinic acid. The other compounds were quantified as themselves.

2.5. HPLC/UV for organic acids analysis

The extract (0.04 g) was redissolved in 1 mL of 0.01 N sulphuric acid. Separation was achieved as reported previously (Silva et al., 2008; Santos et al., 2011), with an analytical HPLC unit (Gilson), using an ion exclusion column Nucleogel® Ion 300 OA (300 × 7.7 mm), in conjunction with a column heating device at 30 °C. Elution was carried out at a solvent flow rate of 0.2 mL/min, isocratically with sulphuric acid 0.01 N as the mobile phase. Detection was performed with a Gilson UV detector at 214 nm. Organic acids quantification was

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achieved by the absorbance recorded in the chromatograms relative to external standards.

2.6. Antioxidant activity assessment

2.6.1. Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of sample extracts (1 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10% tricloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm for 8 min in a refrigerated centrifuge (Centorion K24OR—2003). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against extract concentration.

2.6.2. DPPH radical scavenging assay

The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a method reported before (Hatano et al., 1988). Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect $=[(A_{DPPH}-A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration.

2.6.3. Oxidative hemolysis inhibition assay

Blood (5–10 mL) was obtained from healthy non-smoking volunteers by venipuncture, after written informed consent was obtained. Human erythrocytes from citrated blood were immediately isolated by centrifugation at 1500 rpm for 10 min at 4 °C. After removal of plasma and buffy coat, the erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4), and then resuspended using the same buffer to the desired hematocrit level. In order to induce free-radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (dissolved in PBS; final concentration 50 mM). To study the protective effects of *D. draco* fruit extract against AAPH-induced oxidative hemolysis, an erythrocyte suspension at 2% hematocrit was preincubated with the aqueous extracts (5–20 μg/mL final concentrations, dissolved in PBS) at 37 °C for 30 min, followed by incubation with and without 50 mM AAPH. This reaction mixture was shaken gently while being incubated for 4 h at 37 °C. Strawberry aqueous extract (100–400 μg/mL final concentrations, dissolved in PBS) was used for comparison purposes, since it is a well documented antioxidant berry fruit with recognized biologically significance effects (García-Alonso et al., 2004; Seeram, 2008; Zhang et al., 2008). In all experiments, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with each extract) were used.

The extent of hemolysis was determined spectrophotometrically as described before (Costa et al., 2009; Magalhães et al., 2009; Santos et al., 2011). Briefly, aliquots of the reaction mixture were taken out at each hour of the 4 h of incubation, diluted with saline, and centrifuged at 4000 rpm for 10 min to separate the erythrocytes. The percentage of hemolysis was determined by measuring the absorbance of the supernatant (A) at 545 nm and compared with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The hemolysis percentage was calculated using the formula: $A/B\times100$. The inhibitory concentration 50% (IC_{50}) at time 3 h was also calculated from dose–response curve obtained by plotting the percentage of hemolysis inhibition *versus* the extract concentration. Four independent experiments were used for these calculations.

2.7. Statistical analysis

Statistic analysis was performed using the Statistical Package for Social Sciences (SPSS, version 16.0) for Windows. Comparisons between two groups were performed by unpaired t-test. Multiple comparisons between more than two groups were performed by one-way ANOVA supplemented with Tukey´s HSD post hoc test. Significance was accepted at P lower than 0.05.

3. Results and discussion

3.1. Volatile profile of D. draco fruit extract

Present in minor concentrations, volatile compounds are secondary metabolites of plants which greatly influence their sensorial quality. Research often reports the biological activities of essential oils and of many pure volatile components, notably antibacterial, antifungal and antioxidant properties (Baratta et al., 1998; Ruberto & Baratta, 2000; Sacchetti et al., 2005). To our knowledge, *D. draco* fruit volatile profile was achieved for the first time in this work and nine volatile and semi-volatile compounds were identified by HS-SPME/GC-IT-MS being distributed by distinct chemical classes: one alcohol, three aldehydes, two carotenoid derivatives, and three terpenic compounds (Fig. 1 and Table 1). The most important chemical family present in this fruit is aldehydes, which represent 58.8% of the total volatiles. Nonanal and (*E*,*E*)-2,4-nonadienal were found to be the major compounds in *D. draco* fruit, representing respectively 34.9% and 13.6% of the total volatiles (Table 1). Nonanal has a strong fruity or floral odor and is often used in the industrial production of flavors and perfumes. This aldehyde, as hexanal (10.4% of the total volatiles of this matrix), is also known by its antifungal activity (Kobaisy et al., 2001). The antidiarrhoeal activity of nonanal has also been reported by Zavala-Sanchez et al. (2002). Ruberto and Baratta (2000) have analyzed the antioxidant effectiveness of about one hundred pure components of essential oils, including nonanal, which was considered by this authors as a weak antioxidant (when compared to other classes of volatile compounds and to α-tocopherol). The antimicrobial activity against several Gram-positive and Gram-negative bacteria of the essential oil produced from the flowers of *Tamarix boveana* has been reported by Saïdana et al. (2008). Of note is that aldehydes were the prevalence volatiles (as in *D. draco* berry) and that (*E*,*E*)-2,4-nonadienal was the major compound in this oil (Saïdana et al., 2008).

Terpenic compounds are the following most representative family identified in this exotic fruit (Table 1). Many terpenoids have medicinal properties, such as anti-carcinogenic, antimalarial, anti-ulcer, antimicrobial, antiseptic, nematicidal, larvicidal, anti-inflammatory and diuretic activities (Schwab et al., 2008), therefore being a most valuable class of compounds. The valencene was the major sesquiterpene hydrocarbon identified in this matrix. It is an aroma component of citrus fruit and citrus-derived odorants. Antimycobacterial activities have been ascribed to essential oils rich in sesquiterpenes, namely in valencene (Julião et al., 2009). Ruberto and Baratta (2000) have reported the low antioxidant effect of sesquiterpene hydrocarbons such as valencene and β-caryophyllene (a minor

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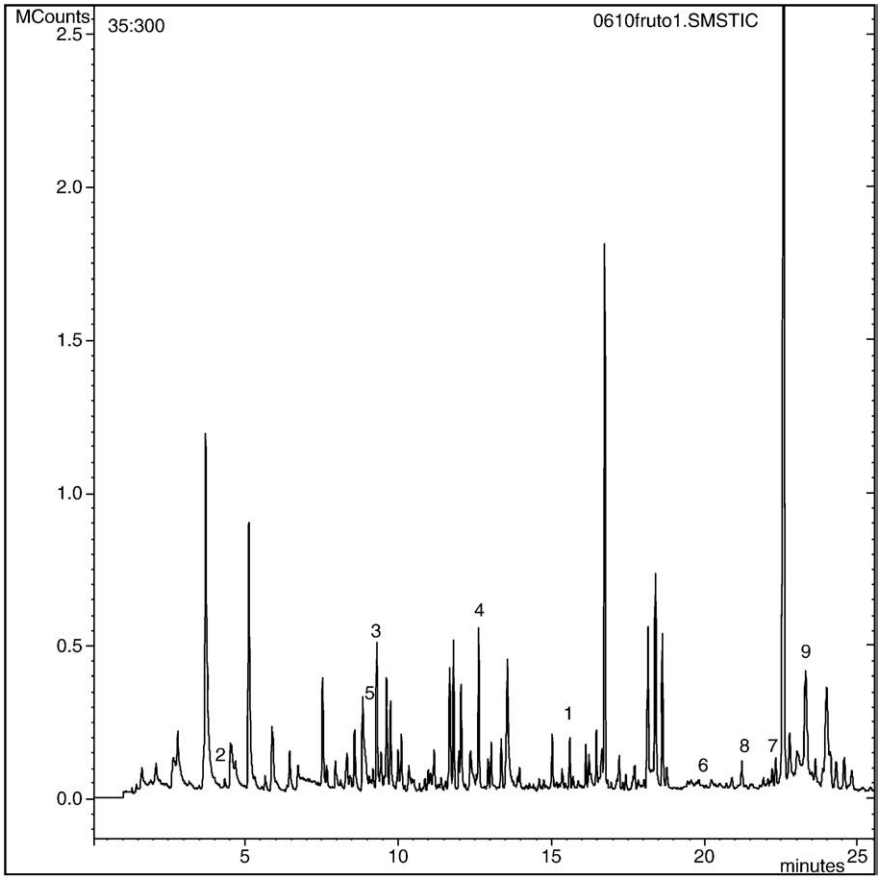

Fig. 1. Chromatogram of the HS-SPME of *Dracaena draco* fruit extract in full scan acquisition. The corresponding compound names are shown in Table 1.

terpenic compound of *D. draco* fruit). Although, the antioxidant and antimicrobial properties of *Cananga odorata* and *Psidium guayava* essential oils, which contain considerable amounts of β-caryophyllene, have been reported by Baratta et al. (1998) and Sacchetti et al. (2005), respectively. In addition, β-caryophyllene seems to possess anti-carcinogenic properties (Di Sotto et al., 2010).

Finally, alcohols and carotenoids were found as minor compounds in fruit, representing 11.4% and 5.6% of the total volatiles, respectively. On the contrary, in *D. draco* leaf aqueous extract carotenoid derivative compounds constituted the most abundant class, comprising about 45% of the total identified volatile compounds (Santos et al., 2011).

3.2. Phenolic profile of D. draco fruit extract

Phenolic compounds are plant secondary compounds that are quite widespread in nature. Their antimicrobial, antioxidant and

Table 1
Volatile composition of *Dracaena draco* fruit extract.

No.	Compound	RT[a]	ID[b]	QI[c] (m/z)	Area [d] (RA (%))
Alcohols					
1	2-decenol	15.6	S, MS	81/95	11.37
	Total of alcohols				11.37
Aldehydes					
2	Hexanal	4.3	S, MS	56/67/82	10.40
3	(E,E)-2,4-nonadienal	9.2	S, MS	81	13.57
4	Nonanal	12.6	S, MS	81/95	34.87
	Total of aldehydes				58.83
Carotenoid derivative compounds					
5	6-methyl-5-hepten-2-one	9.0	S, MS	69/108	4.11
6	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	19.7	S*, MS	142/157/172	1.50
	Total of carotenoid derivative compounds				5.61
Terpenic compounds					
7	Geranylacetone	22.2	S, MS	69/107	8.40
8	β-caryophyllene	21.4	S, MS	93/161/189	2.38
9	Valencene	23.3	S, MS	161/189/204	13.40
	Total of terpenic compounds				24.18

[a] RT = retention time (min).
[b] ID = Identification method (fit/retrofit values, %). S = identified by comparison with standard, MS = tentatively identified by NIST05, S* = synthesised compound.
[c] QI = quantification ions.
[d] Area expressed as arbitrary units. RA (%) = relative area in percentage (n = 3).

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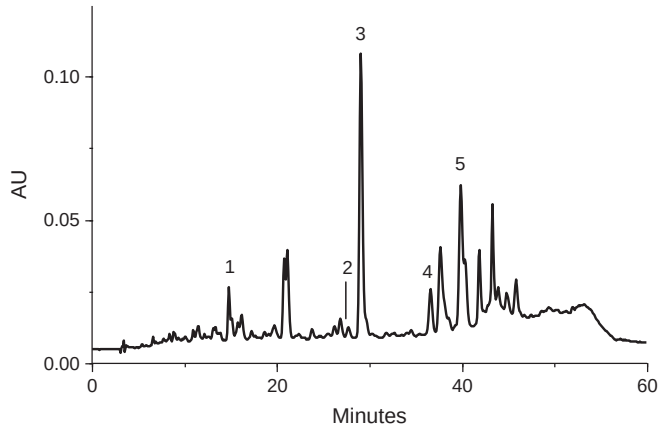

Fig. 2. HPLC phenolic profile of *Dracaena draco* fruit extract. Detection at 350 nm. Peaks: (1) 5-*O*-caffeoylquinic acid; (2) ferulic acid; (3) sinapic acid; (4) 3,5-*O*-dicaffeoylquinic acid; (5) quercetin-3-*O*-rutinoside.

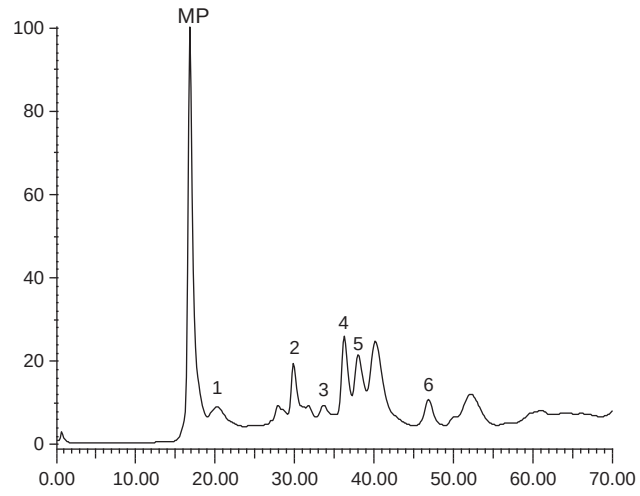

Fig. 3. HPLC organic acid profile of *Dracaena draco* fruit extract. Detection at 214 nm. Peaks: (MP) mobile phase; (1) oxalic acid; (2) citric acid; (3) L-ascorbic acid; (4) malic acid; (5) quinic acid; (6) shikimic acid.

anticancer activities, between many others, are well known and widely documented (Cheng et al., 2007; Giada & Filho, 2006; Fresco et al., 2006; Russo, 2007; Seabra et al., 2006). *D. draco* fruit extract presents a characteristic phenolic profile composed by five compounds (Fig. 2 and Table 2): two hydroxycinnamic acid derivatives—5-*O*-caffeoylquinic and 3,5-*O*-dicaffeoylquinic acids, two hydroxycinnamic acids—ferulic and sinapic acids, and one flavonol glycoside—quercetin-3-*O*-rutinoside. As far as we know, this is the first time that these phenolic compounds are reported in *D. draco* fruit.

The total phenolic content is high (3.5 g/kg of aqueous extract) and the most abundant phenolics are quercetin-3-*O*-rutinoside and sinapic acid (representing 41.7 and 38.2% of the total phenolic content, respectively). The antioxidant, antiinflammatory and anticancer effects of flavonols (as quercetin) has been recently reported (Fresco et al., 2006; Granado-Serrano et al., 2006; Kandaswami et al., 2005; Murakami et al., 2008). Flavonols and their derivatives, like quercetin-3-*O*-rutinoside, are able to act as antioxidants in a number of ways. These antioxidants act as reducing agents, hydrogen donors, free radicals scavengers, and singlet oxygen quenchers (Fattouch et al., 2007).

In addition, hydroxycinnamic acids and their derivatives are also well known antioxidant and anticancer agents (Cheng et al., 2007; Marques & Farah, 2009; Seabra et al., 2006; Silva et al., 2008). The effective protection conferred by some of them, including 5-*O*-caffeoylquinic, sinapic and ferulic acids, against free radical-induced damage of biological membranes has already been reported by Cheng et al. (2007).

The aqueous extract of *D. draco* leaf is a even richer source of phenolic compounds (12.1 g/kg of aqueous extract) but the most abundant one is also quercetin-3-*O*-rutinoside (representing about 50% of the total phenolic content) (Santos et al., 2011).

3.3. Organic acids profile of *D. draco* fruit extract

Organic acids are primary metabolites, which can be found in great amounts in all plants, especially in fruits. The aqueous extract of this berry is a particularly rich source of organic acids (162.7 g/kg of aqueous extract). Its organic acid profile is composed by six constituents (Fig. 3 and Table 3): oxalic, citric, L-ascorbic, malic, quinic, and shikimic acids. As far as we know, this is the first time that organic acid profile is described in this fruit. The most abundant is quinic acid, representing 39.1% of the total organic acid content. Malic and citric acids are also present in considerable amount, representing respectively 25.8% and 22.4% of the total organic acids. These three carboxylic acids behave as antioxidants since they have the ability to chelate metals (Seabra et al., 2006).

Of note is the presence of L-ascorbic acid in this matrix (10.9% of the total organic acid content) due to its well-known scavenging capacity on a wide range of oxygen and nitrogen reactive species, including hydroxyl radical, alkoxyl radicals, peroxyl radicals, superoxide anion, hypochlorous acid, singlet oxygen, among others (Seabra et al., 2006). Oxalic and shikimic acids were found as minor compounds in this fruit.

D. draco leaf water extract is also a rich source of organic acids (168.6 g/kg of aqueous extract) but L-ascorbic acid is absent in that matrix (Santos et al., 2011). The main organic acids present in leaf extract are oxalic and citric acids (representing 47% of the total organic acid content, each) (Santos et al., 2011).

Table 2
Phenolic composition of *Dracaena draco* fruit extract (mg / kg of aqueous extract).

Phenolic compound	Content[a]
5-*O*-caffeoylquinic acid	307.5 ± 22.1
Ferulic acid	62.5 ± 0.1
Sinapic acid	1338.8 ± 73.2
3,5-*O*-dicaffeoylquinic acid	333.9 ± 7.5
quercetin-3-*O*-rutinoside	1462.6 ± 174.6
Σ	3505.3

[a] Values are expressed as mean ± SD (n = 3). Abbreviation: Σ—sum of the determined phenolics.

Table 3
Organic acid composition of *Dracaena draco* fruit extract (g/kg of aqueous extract).

Organic acid	Content[a]
Oxalic acid	2.8 ± 0.3
Citric acid	36.4 ± 3.4
L-ascorbic acid	17.7 ± 1.6
Malic acid	41.9 ± 0.9
Quinic acid	63.6 ± 0.4
Shikimic acid	0.3 ± 0.0
Σ	162.7

[a] Values are expressed as mean ± SD (n = 3). Abbreviation: Σ—sum of the determined organic acids.

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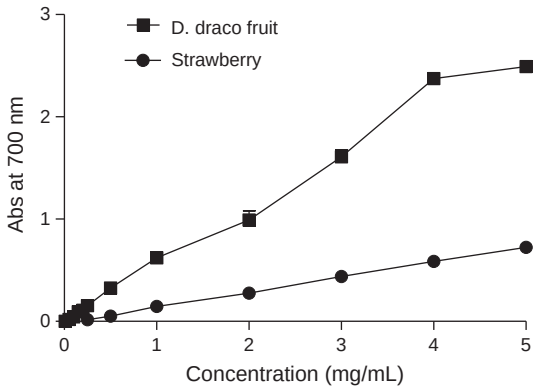

Fig. 4. Reducing power of *Dracaena draco* fruit and strawberry extracts. Each value is expressed as mean ± SD (n = 3).

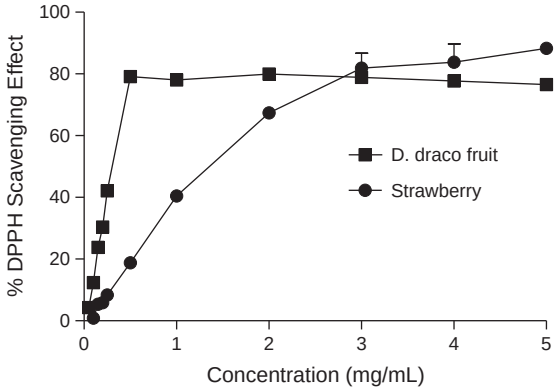

Fig. 5. Scavenging effect of *Dracaena draco* fruit and strawberry extracts on the DPPH radical. Each value represents the mean ± SD (n = 3).

3.4. Antioxidant properties of D. draco fruit extract

In this work, the antioxidant properties of *D. draco* fruit were evaluated by different *in vitro* antioxidant assays such as reducing power, DPPH radical scavenging activity, and protection against free radical-induced human erythrocyte hemolysis. The first two are chemical-based assays, while the last one is biologically more relevant as erythrocyte is a cell model. As expected, owing to its antioxidant composition, all assayed models demonstrated antioxidant and scavenging efficiency for *D. draco* fruit extract. Strawberry extract was used as a control for comparison purposes, since it is a well documented antioxidant red fruit with recognized biologically significant effects (García-Alonso et al., 2004; Seeram, 2008; Zhang et al., 2008).

In the reducing power assay, both *D. draco* fruit and strawberry extracts displayed a concentration-dependent antioxidant potential (Fig. 4). In this assay, the presence of reducing agents in the extracts causes the conversion of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form. Fe^{2+} is monitorized by measuring the formation of Perl's Prussian blue at 700 nm, with rising absorbances indicating an increase in reducing power. The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity (Meir et al., 1995). Statistically significant differences ($P<0.05$) were observed in the EC_{50} values calculated for *D. draco* fruit and strawberry extracts (Table 4). *D. draco* fruit exhibited the strongest capacity (EC_{50} value of 0.80 ± 0.02 mg/mL), while the strawberry extract was less active (EC_{50} value of 3.42 ± 0.06 mg/mL). These results showed that *D. draco* fruit extract may act as an electron donor and therefore react with free radicals, convert them to more stable products and terminate radical chain reaction.

The scavenging activity on DPPH radicals assay is generally used as a basic screening method for testing the antiradical activity of a large variety of compounds (Sharma & Bhat, 2009). DPPH is a stable free radical that possesses a characteristic absorption maximum between 515 and 517 nm, which is diminished in the presence of a compound capable of reducing it to its hydrazine form by a hydrogen/electron transfer reaction (Huang et al., 2005). Free radical scavenging is one of the recognized mechanisms by which antioxidants inhibit lipid peroxidation (Halliwell & Gutteridge, 1999). The scavenging activity of *D. draco* fruit and strawberry extracts on DPPH radicals is shown in Fig. 5. In this assay, DPPH radicals were scavenged by both extracts in a concentration-dependent manner within the range of the given concentrations. The radical scavenging capacity *D. draco* fruit extract was considerably higher than that of reference extract. As in the reducing power assay, significantly differences ($P<0.05$) were observed in the EC_{50} values calculated for the extracts on DPPH assay and follows similar behavior. The lower value that corresponds to the highest scavenging activity on DPPH radicals was obtained by *D. draco* fruit extract (0.30 ± 0.01 and 1.36 ± 0.03 mg/mL, for *D. draco* fruit and strawberry, respectively) (Table 4).

Table 4
Extraction yield, EC_{50} values determined for the reducing power and DPPH radical scavenging capacity and IC_{50} values calculated for the antihemolytic activity of *Dracaena draco* fruit and strawberry extracts after 3 h of incubation with AAPH. Each value represents mean ± SD (n = 3). Means marked with different letters, within each column, are significantly different ($P<0.05$).

Sample	Extraction yield (%)	Reducing power EC_{50} (mg/mL)	DPPH scavenging activity EC_{50} (mg/mL)	Antihemolytic activity IC_{50} (μg/mL)
D. draco fruit	28.77 ± 1.19 a	0.80 ± 0.02 a	0.30 ± 0.01 a	2.56 ± 0.97 a
Strawberry	45.53 ± 4.90 b	3.42 ± 0.06 b	1.36 ± 0.03 b	273.84 ± 49.38 b

To further elucidate the antioxidant properties of *D. draco* fruit extract in human cells, human erythrocytes were selected as a metabolically simplified model system. Erythrocytes are considered as major targets for free radical attack owing to the presence of high membrane concentration of polyunsaturated fatty acids and to their specific role as oxygen carriers (Ajila & Rao, 2008). The erythrocyte membrane is rich in polyunsaturated fatty acids which are susceptible to free radical-mediated lipid peroxidation. Such damage causes hemolysis of the erythrocytes. To the best of our knowledge, this is the first study evaluating the antioxidant potential of *D. draco* fruit in this cell model.

For this work, AAPH was used as the free-radical initiator to induce oxidative damage in erythrocytes. Thermal decomposition at physiological temperature of AAPH generates peroxyl radicals ($ROO^{\cdot}$) in the aqueous phase (Niki, 1990), which can attack the erythrocyte membrane to induce lipid peroxidation, and ultimately hemolysis.

Fig. 6A shows the antioxidant effect of *D. draco* fruit extract (5–20 μg/mL) on human erythrocytes exposed to AAPH. Erythrocytes incubated at 37 °C in PBS (control samples) were stable, with little hemolysis observed within 4 h. In addition, cells incubated with extracts of *D. draco* fruit alone (without AAPH) at the highest concentration tested (20 μg/mL) presented hemolysis background level similar to that of control samples. When AAPH was added to the erythrocyte suspension, hemolysis induction was time-dependent. *D. draco* fruit extract significantly protected the erythrocyte membrane from hemolysis induced by AAPH in a concentration- and time-dependent manner. This effect was greater than that of strawberry extract (Fig. 6B). The IC_{50} values calculated after 3 h of incubation for *D. draco* fruit was significantly higher than of strawberry extract (2.56 ± 0.97 μg/mL and 273.84 ± 49.38 μg/mL, respectively; $P<0.05$) in the same antioxidant test conditions, which emphasize the strong antiradical activity obtained in this study for *D.draco* fruit extract.

In a previous study, we have reported the strong protection conferred by leaf water extract against oxidative hemolysis (Santos et al., 2011).

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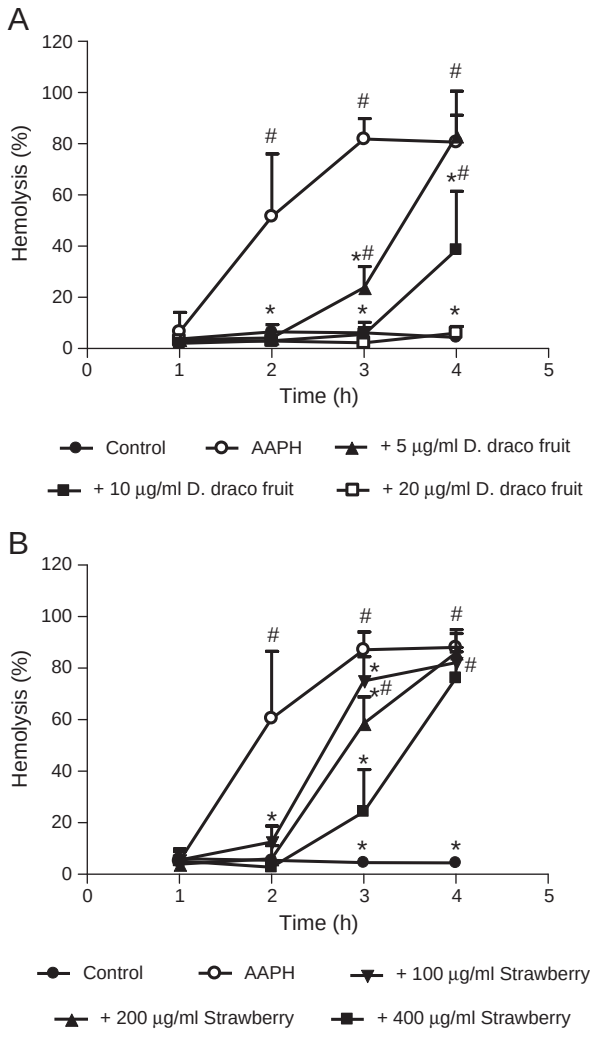

Fig. 6. Effects of (A) *Dracaena draco* fruit and (B) strawberry extracts on AAPH-induced hemolysis in human erythrocytes. An erythrocyte suspension at 2% hematocrit was preincubated with extracts at the indicated concentrations for 30 min at 37 °C. The cell suspension was then incubated with 50 mM AAPH for 4 h at 37 °C. In all experiments, control erythrocytes (incubated with PBS only) and AAPH-treated erythrocytes (incubated with 50 mM AAPH) were used. Values are expressed as the mean ± SD (n = 4). $^{*}P<0.05$, as compared with AAPH at respective time, $^{\#}P<0.05$, as compared with control at respective time.

However, the IC_{50} value obtained (39 ± 11 μg/mL) is much higher than that of the fruit, which means that this last matrix is even more interesting as an antioxidant agent.

Antioxidants, especially polyphenols, have been found to protect erythrocytes from oxidative stress or increase their resistance to damage caused by oxidants (Cheng et al., 2007; Costa et al., 2009; Magalhães et al., 2009; Youdim et al., 2000). The strong antioxidant effects of polyphenols have been highlighted by several studies, with underlying mechanisms involving both free radical scavenging (Bors et al., 1990) and redox-active metal chelation (van Acker et al., 1998). In our model, these phytochemicals present in the incubation medium can protect against lipid peroxidation by trapping the peroxyl radicals in the aqueous phase before these radicals attack the lipid molecules of the erythrocyte membrane. This breaks the free radical chain reaction and inhibits subsequent oxidative hemolysis.

Besides polyphenols, other antioxidant compounds present in fruits may also contribute for its antihemolytic activity. In fact, the major organic acids identified in fruits—quinic, malic and citric acids—are also effective antioxidants. The antioxidant activity of these compounds is attributed to their strong ability to chelate metal ions involved in the production of free radicals (Seabra et al., 2006). Antioxidant activities have also been described for some volatile compounds, especially for those with basic structure of isoprene (Mau et al., 2003). Additionally, synergistic effects of phenolics with other antioxidants have been described (Croft, 1998; Liao & Yin, 2000) and therefore the protective effect showed by *D. draco* fruit against free radical-induced oxidative injury in erythrocytes may reflects their combined action.

4. Conclusion

The involvement of oxidative stress appears to be a common feature to most human diseases, including cardiovascular disease, neurodegeneration and cancer (García-Alonso et al., 2004; Magalhães et al., 2009; Seeram, 2008; Zhang et al., 2008). Dietary antioxidants seem to be particularly important tools to fight against these diseases, by affording protection towards free radical damage in cellular DNA, lipids and proteins (Cao et al., 1996; Costa et al., 2009; du Toit et al., 2001; García-Alonso et al., 2004; Magalhães et al., 2009; Marques & Farah, 2009; Seeram, 2008; Zhang et al., 2008). Our results demonstrate for the first time that *D. draco* fruit extract possesses remarkable antioxidant and free radical scavenging properties and confers protection against free radical-induced oxidative damage on biological membranes. These bioactivities may reflect the combined action of volatiles, semi-volatiles, phenolics and organic acids present in this exotic fruit. It is therefore suggested that *D. draco* fruit is a novel unexploited natural antioxidant agent with high potential to prevent or slow the progress of human diseases mediated by oxidative stress.

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