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Gabriela Azofeifa. Study of biological activities of the tropical highland blackberry polyphenols and the impact of digestion and pasteurization processes. Food and Nutrition. Université Montpellier II - Sciences et Techniques du Languedoc, 2013. English. NNT : 2013MON20064 . tel-01002531

HAL Id: tel-01002531

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THÈSE

Pour obtenir le grade de
Docteur

Délivré par **UNIVERSITE MONTPELLIER 2**

Préparée au sein de l'école doctorale
Sciences des Procédés, Sciences des Aliments

Et de l'unité de recherche **QUALISUD**

Spécialité : Biochimie, chimie et technologie des aliments

Présentée par **Gabriela AZOFEIFA**

**Study of biological activities of the tropical highland
blackberry polyphenols and the impact of digestion and
pasteurization processes**

Soutenue le 16/12/2013 devant le jury composé de

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Abstract

This study evaluated biological activities of blackberries (*Rubus adenotrichos* Schldl.) and the effects of digestion or pasteurization processes on these activities. The blackberries phenolic extract showed an ORAC value of 4.34 ± 0.14 mmol TE/g, higher than those of quercetin and ellagic acid. It also protected liposomes and liver homogenates against lipid peroxidation; with IC_{50} of 7.0 ± 0.5 and 20.3 ± 4.2 $\mu\text{g/mL}$, respectively. The blackberry polyphenols inhibited nitrite production in J774A.1 cells stimulated with LPS+IFN γ due to down-regulation of iNOS protein expression, suggesting an anti-inflammatory potential.

The biological activities of the fruits could be affected by the industrial processes. The pasteurization of a blackberry juice caused a 7% decrease in the anthocyanins concentration of the highest temperature tested, 92 °C. For the same sample, a significant reduction of 27% and 15% were showed in the DPPH and NO scavenging capacity, respectively. However, no significant differences were evident neither for the peroxidation inhibitory capacity nor for the intracellular antioxidant activity after the pasteurization step.

The antioxidant capacity is largely dependent of their bioavailability and because of this a blackberry juice was digested *in vitro* to obtain samples that represent the major stages of the digestion process. The gastric digestion had slight impact on polyphenolic composition while the pancreatin/bile digestion had marked effects on polyphenolic composition, particularly in the ratio anthocyanins-ellagitannins. These changes in phenolic composition caused in the dialyzed fraction a reduction of 33% and 35% in the activity against nitrogen derived radicals DPPH and NO respectively. As well the capacity to inhibit intracellular ROS was decrease by an 85% in the dialyzed fraction. Nevertheless, no significant differences were evident in the capacity to inhibit lipid peroxidation.

Finally, this study used an *in vivo* model with diabetic rats to evaluate the effect of the consumption of a blackberry beverage. The blackberry intake provoked a decrease in plasmatic glucose (-48.6%), triglycerides (-43.5%) and cholesterol (-28.6%) levels in the diabetic rats with respect to the diabetic controls animals. The diabetic rats which consumed blackberry, improved (+7%) the plasma antioxidant capacity (PAC) values and reduced the levels of lipid peroxidation in plasma (-19%) and in kidney tissue (-23%).

This study suggests that blackberry could be considered as a candidate for the group of functional foods even when considering the bioavailability aspects of the phenolic compounds and the pasteurization effects.

Key words:

Blackberry, *Rubus adenotrichos*, antioxidant activity, anti-inflammatory activity, diabetes mellitus, pasteurization, bioavailability.

Résumé

Dans cette étude nous avons évalué les activités biologiques de la mûre tropicale de montagne (*Rubus adenotrichos* Schltl.) et les effets de la digestion ou des procédés de pasteurisation sur ces activités. L'extrait polyphénolique de mûre présente un pouvoir antioxydant déterminé par la méthode ORAC de 4.34 ± 0.14 mmol TE/g, plus élevé que celui de la quercétine et de l'acide ellagique. L'extrait de mûre présente des propriétés protectrices des liposomes et des homogénats de foie contre la peroxydation lipidique avec des IC_{50} de 7.0 ± 0.5 et 20.3 ± 4.2 mg/ml, respectivement. Les polyphénols de mûre inhibent la production de nitrite dans par les macrophages murins J774A.1 stimulées par le mélange LPS, IFN. Cette activité est confirmée par la diminution du taux de NO synthase inductible dans ces mêmes cellules.

Les activités biologiques des fruits pourraient être affectés par les procédés industriels. Le processus de pasteurisation du jus de mûre a provoqué une diminution d'environ 7% de la concentration en anthocyanes pour la température la plus élevée évalué, 92°C. Pour le même échantillon, une réduction significative de 27% de l'activité anti-oxydante mesurée par la méthode DPPH et de 16% de la capacité de complexations des radicaux NO. Toutefois, aucune différence significative n'a été mise en évidence autant pour la capacité d'inhibition de la peroxydation que pour l'activité anti-oxydante intracellulaire. Les effets *in vivo* sont largement dépendants de la biodisponibilité des composés phénoliques et de ce fait un jus de mûre a été digéré *in vitro* pour obtenir des échantillons qui représentent les grandes étapes du processus de digestion. La digestion gastrique a peu d'influence au contraire de la digestion intestinale sur la composition polyphénolique de l'extrait de mûre en particulier au niveau du rapport anthocyanines/elagitannins. La fraction dialysable présente une réduction de 33% et de 35% de l'activité contre les radicaux dérivés de l'azote DPPH et NO, respectivement. De même, la capacité d'inhiber au niveau intracellulaire les radicaux libres (ROS) a diminué d'environ 85%. Toutefois, aucune différence significative n'a été mise en évidence au niveau de la capacité d'inhiber la peroxydation lipidique.

Finalement, cette étude a utilisé un modèle *in vivo* pour évaluer l'effet de la consommation d'une boisson de mûre chez des rats diabétiques. L'apport de mûre a provoqué une diminution du glucose (-48.6%), des triglycérides (-43.5%) et du taux de cholestérol (-28.6%) plasmatique des rats diabétiques par rapport aux témoins diabétiques qui n'ont pas consommé la boisson de ce fruit. De plus, les rats diabétiques qui consomment de la mûre ont vus améliorées les valeurs de la capacité antioxydante du plasma (+7%), tandis que les niveaux de peroxydation lipidique dans le plasma (-19%) et dans les tissus rénaux (-23%) ont été réduits. Cette étude suggère que la mûre pourrait être considérée comme un candidat pour le groupe des aliments fonctionnels, même si l'on considère les aspects de biodisponibilité et les effets du procédé de pasteurisation.

Mots clés:

Mûre, *Rubus adenotrichos*, activité anti-oxydante, activité anti-inflammatoire, diabète sucré, pasteurisation, biodisponibilité.

Gratitude

I thank Pr. Michel, Dr. Vaillant and Pr. Cristol for all the knowledge shared with me during all this doctoral processes.

I thank Silvia Quesada, Marion Morena and Ana Mercedes Pérez for their scientific advice and even more for their friendship.

I thank Karine Portet and Ana Lorena Torres for their excellent technical support.

I thank all the staff of the Faculté de Pharmacie (UMI), IURC and CIRAD for all their technical advice and for making me feel welcome, particularly Frederic Boudard, Eric Badia, Christian Mertz, Florence Laurent, Patrick Poucheret, Jean Giaimis, Aurelie Goux, Nadine Lopez...

I thank all my colleges on the Biochemistry department at University of Costa Rica for their support and friendship.

I thank the University of Costa Rica, the Institute Francais d'Amérique Central and CIRAD for their financial support for my doctoral studies

Last but not least,

I thank my parents for all their support and motivation during my whole life, my sister for all her language support in the writing of this document and my brother for his prayers for me.

I thank Carlos for his absolute support during all this process. Thanks for following me to France, for listening all the details of my experiments, for helping me in all the technological issues and for your unconditional support in all the aspects of my life.

I thank God for all the doors He opened during this process and for giving me Alejandro, who is the inspiration of my life.

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List of Abbreviations

AA	Ascorbic acid
AAPH	2,2-azobis-2-methyl-propion-amidine-dihydrochloride
ALT	Alanine transaminase
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
AST	Aspartate transaminase
ATCC	American Type Culture Collection
AUC	Area under the curve
BB	Blackberry
C3G	Cyanidin-3-glucoside
CICUA	Institutional Committee for Care and Handling of Experimental Animals (Universidad de Costa Rica)
C_{max}	Plasma peak concentration
COX	Cyclooxygenase
Cy	Cyanidin
DAD	Diode array detector
DCFDA	2,7-dichlorodihydrofluorescein diacetate
DHA	Dehydroascorbic
Dp	Delphinidin
DPPH	2,2 diphenyl-1-picrylhydrazyl
DW	Dry weight
EA	Ellagic acid
EMEM	Eagle's minimal essential medium
ERYCA	Erythrocyte cellular antioxidant activity
ESI-TRAP-MS/MS	Electrospray Ion Trap Mass spectrometry detector
FW	Fresh weight
GA	Gallic acid
GAE	Gallic acid equivalents
GIT	Gastro intestinal tract
GLUT	Glucose transporter
GST	Glutathione-S-transferase
HDL	High density lipoproteins
HHDP	Hexahydroxydiphenic acid
HPLC	High-performance liquid chromatography
IC₅₀	Half maximal inhibitory concentration
IFN-	Interferon
IL-6	Interleukin 6
iNOS	Inducible Nitric oxide synthase
JP75	Juice pasteurized at 75°C
JP92	Juice pasteurized at 92°C

LDL	Low density lipoproteins
LEBi®	Laboratorio de Ensayos Biológicos
LPOs	Lipid peroxides
LPS	Lipopolysaccharides
MCP-1	Monocyte chemotactic protein 1
MDA	Malondialdehyde
MTS	Tetrazolium salt
Mv	Malvidin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPJ	Non-pasteurized juice
ORAC	Oxygen radical absorbance capacity
PAC	Plasma antioxidant capacity
PBS	Phosphate-buffered saline
Pg	Pelargonidin
PMA	Phorbol 12-myristate 13-acetate
PMS	phenazine methosulfate
Pn	Peonidin
Pt	Petunidin
QR	Quinone reductase
RDA	Recommended Daily Allowances
ROS	Radical oxygen species
RPMI	Roswell Park Memorial Institute medium
SE	Standard error
SGLT1	Sodium-dependent glucose transporter
SNP	Sodium nitroprusside
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHP	tert-butyl hydroperoxide
TCA	Trichloroacetic acid
TE	Trolox equivalents
T_{max}	Time to reach C _{max}
TNF	Tumor necrosis factor
TRAF-2	Tumor necrosis receptor-associated factor 2
USDA	United States Department of Agriculture

Résumé

Étude des activités biologiques des polyphénols de la mûre tropicale de montagne et détermination de l'impact des processus de digestion et de pasteurisation.

Les baies comestibles sont largement consommées au niveau mondial comme fruits frais ou sous forme de produits transformés tels que crème glacée, yaourts, confitures, gelées, jus de fruits, produits de boulangerie et céréales (Hummer, 2010). Les baies sont reconnues comme une bonne source de glucides, de certaines vitamines, de minéraux et elles constituent une excellente source de fibres alimentaires. Récemment, un intérêt particulier s'est porté sur les baies en raison de leur exceptionnelle teneur en composés polyphénoliques, la grande variété de ce type de composants et leurs effets bénéfiques possibles au niveau de la santé. Des études épidémiologiques ont suggéré une association inverse entre la consommation élevée de polyphénols et la baisse de l'incidence de certaines maladies chroniques, telles que la prévention de l'inflammation et du stress oxydant, les maladies cardiovasculaires, certains types de cancer, le diabète, l'obésité et les maladies neurodégénératives (Basu *et al.*, 2010; Basu & Lyons, 2012; Giamperi *et al.*, 2012).

Pour appréhender l'effet des polyphénols dans l'amélioration de la santé humaine, il est important de prendre en compte de nombreux facteurs tels que la biodiversité des apports alimentaires, l'absorption et le métabolisme des micronutriments. Il est également important d'évaluer d'autres facteurs qui influent les composés polyphénoliques des baies, tels que les conditions et pratiques agricoles ainsi que les procédés de transformation des aliments (D'Archivio *et al.*, 2010).

L'objectif de cette étude est d'évaluer les activités biologiques des mûres tropicales de montagne (*Rubus adenotrichos* Schltld.), ainsi que d'étudier l'impact des processus de digestion et de pasteurisation. Pour toutes les expériences de cette thèse, des fruits bien mûrs du Costa Rica ont été récoltés dans la localité de Cartago. Certaines des études menées dans le cadre de cette thèse ont été réalisées avec le fruit complet, et d'autres avec du jus de mûre microfiltré (Vaillant *et al.*, 2008). Les résultats de ce travail ont été organisés en quatre articles scientifiques: un article déjà publié, un soumis et deux en cours de préparation).

Publication 1. Activités antioxydantes et anti-inflammatoires *in vitro* des composés polyphénoliques de la mûre tropicale de montagne (*Rubus adenotrichos*) (Publié dans le Journal of Agricultural and Food Chemistry, 2013, 61: 5798-5804).

Cet article a évalué *in vitro* les activités antioxydantes et anti-inflammatoires d'un extrait de polyphénols de la variété de mûre cultivée au Costa Rica, *Rubus adenotrichos*.

Le profil HPLC de l'extrait de mûre étudié est similaire à celui publié précédemment (Mertz *et al.*, 2007). Ces auteurs décrivent un profil polyphénolique constitué de quatre composés principaux, les anthocyanes: cyanidine-3-glucoside et cyanidine-3-malonyl glucoside et les ellagitannins: lambertianin C et sanguin H-6. Ces types de polyphénols sont des contributeurs essentiels de la capacité antioxydante, les anthocyanidines, en raison de leur groupes hydroxyles dans la structure de l'anneau B (Seeram & Nair, 2002), et les ellagitannins, en raison des structures de haut poids moléculaire avec plusieurs atomes d'hydrogène qui pourraient être donnés et piéger les électrons non appariés (Bakkalbasi *et al.*, 2009).

L'activité de l'extrait de polyphénols de mûre contre les radicaux libres mesurée par la méthode DPPH ($IC_{50} 2.57 \pm 0.11 \mu\text{g/mL}$) a été significativement inférieure à celle de la quercétine et de l'acide ellagique ($IC_{50} 2.17 \pm 0.08$ et $2.22 \pm 0.07 \mu\text{g/mL}$, respectivement). L'activité antioxydante vis-à-vis l'oxide nitrique (NO) de l'extrait de mûre a été significativement supérieure ($IC_{50} 24.5 \pm 1.3 \mu\text{g/mL}$) à celle de l'acide ellagique ($IC_{50} 298 \pm 14 \mu\text{g/mL}$) et inférieure à celle de la quercétine ($IC_{50} 6.6 \pm 0.8 \mu\text{g/mL}$). Néanmoins, dans le cas de l'activité antioxydante contre les radicaux peroxyde mesurée par la méthode ORAC, l'extrait de polyphénols de mûre ($4.34 \pm 0.14 \text{ mmol TE/g}$) a été significativement plus élevé que celles de la quercétine et de l'acide ellagique (2.07 ± 0.08 et $0.53 \pm 0.02 \text{ mmol TE/g}$, respectivement). La variabilité observée entre les résultats des différentes techniques peut être expliquée par le fait que chaque méthode évalue l'activité antioxydante contre différents type de radicaux et que différents mécanismes sont impliqués (Huang *et al.*, 2005; Niki, 2010; Niki, 2011). Les essais DPPH et NO concernent des radicaux dérivés de l'azote, alors que la méthode ORAC utilise un radical peroxyde. En outre, les techniques DPPH et NO sont des méthodes directes, alors que le test ORAC une méthode de compétition nécessitant une sonde, un radical libre et le composé antioxydant (Niki, 2011).

Notre étude a évalué l'inhibition de la peroxydation lipidique en utilisant des liposomes de lécithine artificiels et des broyats de foie de rat. Les polyphénols de la mûre ont diminué les niveaux de peroxydation lipidique dans les liposomes d'une manière dose-dépendante, avec une IC_{50} de $7.0 \pm 0.5 \mu\text{g/mL}$. Un effet inhibiteur aussi a été observé pour les homogénats de foie de rat, avec une IC_{50} de $20.3 \pm 4.2 \mu\text{g/mL}$. Les valeurs IC_{50} dans les deux tests étaient significativement ($p < 0.05$) supérieures à celle du produit de référence, le Trolox, $13.3 \pm 0.9 \mu\text{g/mL}$ et $43.1 \pm 0.3 \mu\text{g/mL}$ respectivement.

Outre l'évaluation des dommages causés à l'extérieur de la cellule, cette étude a également évalué la capacité d'inhibition intracellulaire des radicaux libres oxygénés (ROS), en particulier le NO et les radicaux superoxydes qui jouent un rôle important dans la modulation de l'inflammation et dans la régulation immunitaire (Guzik *et al.*, 2003). Les extraits de polyphénols de mûre inhibent de manière significative la production d'anion superoxyde par la NADPH oxydase ($p < 0.05$), de $50 \mu\text{g/mL}$ à $150 \mu\text{g/mL}$ d'extrait, avec une valeur IC_{50} de $80.3 \pm 4.1 \mu\text{g/mL}$. La cinétique d'inhibition de la production d'anion superoxyde dû à l'extrait mûre a montré une réduction significative ($p < 0.05$) de 35%, même après un temps court d'incubation de 15 min. L'inhibition maximale (76%) a été observée après 8 h d'incubation.

Le pré-traitement des macrophages avec un extrait de polyphénols de mûre inhibe la production de nitrites induite par le LPS+IFN γ . Un pré-traitement de 4 h réduit la production de NO uniquement lorsque le LPS+IFN γ a été incubé simultanément avec l'extrait (IC_{50} $24.4 \pm 3.2 \mu\text{g/mL}$) et non pas si l'extrait est éliminé avant l'addition de LPS+IFN γ (IC_{50} $99.1 \pm 7.3 \mu\text{g/mL}$). Ceci suggère que l'extrait exerce seulement une activité de piégeage. Cependant, une pré-incubation de l'extrait 24 h avant la stimulation avec le LPS+IFN γ , diminue la production de NO lorsque l'extrait est incubé simultanément avec LPS+IFN γ (IC_{50} $30.4 \pm 3.7 \mu\text{g/mL}$), et même quand il a été éliminé avant l'addition de LPS+IFN γ (IC_{50} $45.6 \pm 1.2 \mu\text{g/mL}$). Ceci permet de proposer que l'extrait régule l'expression de l'expression de la NO synthase inductible (iNOS) dans les cellules, car l'inhibition a été constatée, même si l'extrait a été éliminé. Cette régulation a été confirmée par la méthode *Western blot* qui a montré une diminution dose-dépendante de iNOS, de 29, 40, 74, et 79%, lorsque les cellules ont été incubées avec des extraits de mûre à des concentrations de 12.5, 25, 50 et 100 $\mu\text{g/mL}$, respectivement.

L'effet inhibiteur des polyphénols de mûre sur la production d'anion superoxydes et d'oxyde nitrique modifie le statut redox des molécules de signalisation et des facteurs de transcription impliqués dans la progression de l'inflammation. Par exemple, le facteur de transcription NF- κ B, qui est impliqué dans l'expression du gène iNOS, a été signalé comme étant sensible aux ROS (Guzik *et al.*, 2003; Valko *et al.*, 2007; Hatanaka *et al.*, 1998). Nous suggérons que l'activité antiradicalaire démontrée par les extraits de mûre pourrait diminuer les concentrations de différents types de ROS et donc empêcher, au moins en partie, l'activation de NF- κ B, provoquant ainsi une diminution de l'expression d'iNOS.

Notre étude a démontré l'influence potentielle des polyphénols de mûre sur le processus inflammatoire au moyen de leurs propriétés inhibitrices contre l'anion superoxyde, l'oxyde nitrique et de l'IL-6. Cependant, cet effet peut être augmenté, car ces molécules sont impliqués aussi dans la régulation des processus cytotoxiques, comme les lésions de l'ADN, l'oxydation des LDL, la nitration du résidu de la tyrosine, et l'inhibition de la respiration mitochondriale (Valko *et al.*, 2007) (Guzik *et al.*, 2003). Par conséquent, une étude détaillée des mécanismes ainsi que expériences *in vivo* sont nécessaires pour déterminer si la mûre pourrait être promue comme un aliment fonctionnel.

Publication 2. La flash-pasteurisation du jus de mûre permet de conserver une capacité d'inhibition élevée de la peroxydation lipidique évaluée par différentes méthodes (article soumis à Food Research International)

Le deuxième article vise à évaluer l'impact des traitements thermiques dans les activités biologiques des polyphénols de mûre. Pour cela, deux jus pasteurisés ont été préparés à partir d'un jus microfiltré non pasteurisé (NPJ) en utilisant un système de traitement UHT à l'échelle pilote. L'un des jus a été pasteurisé à 75 °C pendant 15 s (JP75) et l'autre à 92 °C pendant 10 s (JP92). Les jus pasteurisés ont été soumis à une purification des polyphénols au moyen d'une colonne XAD-7 Amberlite et les composés phénoliques ont été élués et lyophilisés pour une évaluation de la capacité antioxydante.

Aucune des deux conditions de pasteurisation évaluées dans cette étude n'a modifié de manière significative les concentrations en polyphénols totaux ou en ellagitannins. La littérature scientifique a démontré que l'effet des traitements thermiques sur les composés phénoliques est variable, en fonction de la sévérité des conditions de traitement et de la

sensibilité des différents composés phytochimiques à la température (Rawson *et al.*, 2011). Les ellagitannins sont caractérisés comme des composés stables à la chaleur (Hager *et al.*, 2010; Mena *et al.*, 2013). Au contraire, les anthocyanes ont été décrites comme des molécules sensibles à la décomposition pendant les procédés thermiques (Piasek *et al.*, 2011; Rawson *et al.*, 2011; Mena *et al.*, 2013). Les anthocyanes du jus de mûre pasteurisé dans cette étude ont montré une diminution significative (7%) par rapport au jus de fruits non pasteurisé (NPJ).

La capacité de piégeage des radicaux libres des jus de mûre pasteurisés a été comparée en utilisant différentes méthodes. L'activité de piégeage contre le DPPH et le NO a été significativement plus faible pour les jus de fruits pasteurisés par rapport au jus non pasteurisé. La réduction mesurée par la méthode DPPH pour les jus JP75 et JP92 a été de 26% et 27%, respectivement, et pour la technique NO, les jus JP75 et JP92 ont perdu 15% et 16% de leur activité, respectivement. Aucune différence significative n'a été trouvée entre les jus JP75 et JP92 pour les deux techniques DPPH et NO. D'autre part, la capacité de piégeage mesurée par la méthode ORAC n'a pas montré des différences significatives dues à la pasteurisation.

Les variations observées entre les résultats obtenus par les techniques DPPH, NO et ORAC pourraient s'expliquer en raison du fait que chaque composé phénolique est différemment affecté par les traitements thermiques, et également que chaque composé phénolique contribue d'une manière différente à l'activité antioxydante mesurée par rapport à un certain radical (Oliveira *et al.*, 2012). La combinaison de ces facteurs provoque un manque de corrélation dans les résultats des dosages en antioxydants. D'autre part, il a été rapporté que la perte de certains composés après un traitement thermique pourrait être compensée par la formation simultanée de molécules nouvelles dont les propriétés antioxydantes sont améliorées (Nicoli *et al.*, 1999).

L'évaluation de la capacité à inhiber la peroxydation lipidique implique des facteurs tels que l'interaction avec les autres composantes biologiques et la mobilité de l'antioxydant dans un microenvironnement spécifique (Niki, 2011). Les extraits de polyphénols des jus, pasteurisés et non pasteurisé, protègent les niveaux de la peroxydation lipidiques dans les liposomes, la valeur IC₅₀ étant de 3.9 ± 0.7 µg/mL pour NPJ, 3.9 ± 0.1 µg/mL pour JP75 et 4.0 ± 0.5 µg/mL pour JP92. Un effet inhibiteur similaire a été observé pour les homogénats de foie de rat, avec une valeur IC₅₀ de 49.4 ± 2.7 µg/mL pour NPJ, 49.9 ±

5.1 $\mu\text{g/mL}$ pour JP75 et $57.7 \pm 5.3 \mu\text{g/mL}$ pour JP92. Également, un effet protecteur a été démontré contre la lyse des érythrocytes causée par la peroxydation lipidique. Les échantillons NPJ, JP75 et JP92 ont montré respectivement des valeurs de 3.65 ± 0.15 , 3.74 ± 0.18 et 3.70 ± 0.18 mmol équivalent quercétine (QE)/g d'extrait. Aucune différence significative n'a été retrouvée pour la capacité d'inhibition de la peroxydation des trois jus dans les suivants essais: liposomes, homogénats de foie et érythrocytes (ERYCA).

Les ROS modifient les voies de signalisation intracellulaires et ils jouent un rôle crucial dans la physiopathologie de plusieurs maladies (Finkel, 2011; Valko *et al.*, 2007). Les polyphénols de mûre réduisent les ROS intracellulaires d'une manière dose-dépendante et l'on a démontré que la capacité antioxydante n'a pas diminué significativement lors des différents traitements thermiques. La concentration en polyphénols nécessaire pour piéger 50% de la production de ROS a été de $204 \pm 9 \mu\text{g/mL}$ pour NPJ, $219 \pm 10 \mu\text{g/mL}$ pour JP75 et $220 \pm 9 \mu\text{g/mL}$ pour JP92.

Les résultats de cette étude permettent de penser que, indépendamment de la réduction en anthocyanes, les métabolites formés par le traitement thermique pourraient présenter des réarrangements des groupes hydroxyles ou des groupements glycosides (Rawson *et al.*, 2011; Srivastava *et al.*, 2007) et que ces modifications n'ont pas nécessairement affecté leur capacité à inhiber les ROS ou leur capacité à protéger contre la peroxydation lipidique (Gancel *et al.*, 2011). Comme il a été cité auparavant, la capacité antioxydante des polymères formés pendant le chauffage, peut compenser la perte de la capacité antioxydante due à la dégradation des anthocyanes (Hager *et al.*, 2008). Par exemple, les résidus de sucre des anthocyanes (glucosides) diminuent la capacité d'inhibition de la peroxydation lipidique par rapport aux anthocyanidines (aglycones), de sorte que la formation des aglycones pendant le traitement thermique pourrait être bénéfique pour l'activité d'inhibition de la peroxydation lipidique (Seeram & Nair, 2002).

La plupart des publications qui ont évalué la rétention des composés bioactifs après la pasteurisation ont porté sur les changements dans la composition phénolique des fruits et sur les effets de piégeage des radicaux libres. Notre étude comprend des résultats ayant un impact biologique plus pertinent et l'on montre que malgré les changements dans la composition phénolique et dans la capacité de piégeage des radicaux, le jus de mûre pasteurisé maintient les propriétés biologiques *in vitro* liées à la capacité de protéger

contre la peroxydation lipidique et la capacité d'inhibition des ROS au niveau intracellulaire.

Publication 3. Effet d'une digestion *in vitro* sur la capacité antioxydante des polyphénols de la mûre tropicale de montagne (*Rubus adenotrichos*)

Ce troisième article analyse des échantillons représentatifs des principales étapes de la digestion des mûres et compare la capacité antioxydante des échantillons digérés. Les échantillons ont été obtenus à partir d'une technique de digestion *in vitro* (Gil-Izquierdo *et al.*, 2002; Coates *et al.*, 2007). En résumé, le jus de mûre microfiltré (échantillon 1 - **S1**) a été soumis à une digestion à la pepsine-HCl, à pH 2, à 37 ° C pendant 2 h (échantillon 2 - **S2**). Après, l'échantillon issu de la digestion gastrique a été transféré dans un bécher contenant deux tubes de dialyse de cellulose additionnés d'une quantité suffisante de NaHCO₃ pour atteindre un pH de 7. Ensuite un mélange de pancréatine (4 g/L) et d'un extrait biliaire (25 g/L) a été ajouté et incubé pendant 2.5 heures. Finalement, le jus retrouvé à l'extérieur du tube de dialyse a été récupéré (échantillon 3 - **S3**), représentant les composés qui atteindraient le côlon. D'autre part, la solution à l'intérieur du tube de dialyse a été récupérée (échantillon 4 - **S4**) et représente les composés de la mûre disponibles dans le sérum sanguin. Les quatre échantillons ont été soumis à une purification pour la séparation des polyphénols en utilisant une colonne XAD-7 Amberlite et les composés phénoliques élués ont été lyophilisés et utilisés pour l'analyse de la capacité antioxydante.

L'analyse HPLC des échantillons digérés de mûre a montré que la digestion gastrique (S2) n'a pas modifié de manière significative la concentration des anthocyanes et des ellagitannins. Au contraire, d'importants changements dans les concentrations des composés phénoliques ont été observés dans la fraction non dialysée (S3) qui a conservé 46% d'anthocyanes et 66% des ellagitannins, alors que la fraction dialysée (S4) a gardé 19% des anthocyanes et de 6.8% des ellagitannins. Ces pourcentages montrent un ratio anthocyanes-ellagitannins différent dans les fractions S3 et S4 par rapport au jus d'origine; pour la fraction S3, les ellagitannins sont devenus les composés majoritaires et pour la fraction S4, les anthocyanes sont les principaux composés phénoliques. Cela suggère que les anthocyanes de mûres seront plus disponibles *in vivo* au niveau du sérum sanguin et que les ellagitannins seront principalement maintenus au niveau du côlon. Les ellagitannins présents dans la fraction non dialysée (S3) seront transformés dans des

conditions physiologiques par la flore microbienne en acide ellagique, puis en urolithins, ce qui va favoriser l'absorption intestinale (Espín, *et al.*, 2007).

Les échantillons digérés ont été évalués par rapport à différents radicaux libres. La fraction dialysée (S4) a montré une diminution significative de l'activité de piégeage des radicaux dérivés d'azote (techniques NO et DPPH) comparé aux autres échantillons (S1, S2, S3). La même fraction (S4) n'a pas présenté des différences significatives quand sa capacité antioxydante a été évaluée contre les radicaux peroxyde en utilisant la méthode ORAC. Selon Niki, il est plus approprié de mesurer l'activité antioxydante contre les radicaux peroxyde, avec par exemple la technique ORAC, parce que ce radical joue un rôle important dans les réactions en chaîne de la peroxydation lipidique (Niki, 2011).

Les antioxydants pourraient piéger les radicaux libres et empêcher l'oxydation des lipides, et de cette façon ils pourraient prévenir des dommages dans la structure, l'intégrité et la fluidité des membranes biologiques. Aussi, les antioxydants pourraient prévenir les modifications des lipoprotéines de basse et de haute densité, ainsi que la génération de structures pro-athérogènes et pro-inflammatoires (Niki, 2012). Dans notre étude, les polyphénols digérés ont inhibé la peroxydation lipidique dans les trois substrats évalués: liposomes artificiels de lécithine, homogénats de foie de rat et érythrocytes humains (ERYCA). Aucune différence significative n'a été observée dans les valeurs IC_{50} des échantillons digérés, mais il existe une tendance à réduire la capacité d'inhibition de la peroxydation lipidique dans les cas de la fraction non dialysée (S3) et de la fraction dialysée (S4), jusqu'à atteindre une perte maximale de 19%.

L'incubation des polyphénols dans un environnement cellulaire provoque des modifications chimiques supplémentaires des composants bioactifs. En outre, les transporteurs cellulaires et l'interaction avec les métabolites internes pourraient modifier la capacité antioxydante des polyphénols. Pour cette raison, la capacité à inhiber les ROS au niveau intracellulaire a été évaluée dans les échantillons de polyphénols digérés. La concentration en polyphénols nécessaire pour piéger 50% des ROS (IC_{50}) a été de 106 ± 2 $\mu\text{g/mL}$ pour S1, 124 ± 2 $\mu\text{g/mL}$ pour S2, 132 ± 9 $\mu\text{g/mL}$ pour S3 et 196 ± 8 $\mu\text{g/mL}$ pour S4. La valeur IC_{50} de l'échantillon dialysé (S4) a montré une diminution significative de 85% de son activité inhibitrice, par rapport à l'échantillon non digéré (S1).

Les résultats obtenus avec les différentes méthodes utilisées dans cette étude indiquent que les polyphénols de mûre soumis aux conditions du tractus gastro-intestinal (GIT) conservent en grande partie leur capacité antioxydante. Seule la fraction de polyphénols dialysée, qui représente les polyphénols disponibles au niveau du sérum sanguin, montre une perte partielle de sa capacité antioxydante, ce qui pourrait être attribué à l'absence d'ellagitannins. Toutefois, il est encore nécessaire d'évaluer l'effet de la flore dans le tractus gastro-intestinal sur la dégradation des polyphénols en composés phénoliques plus petits qui pourraient être réabsorbés et ainsi contribuer à la capacité antioxydante du sérum. Néanmoins, la procédure *in vitro* réalisée dans cette étude imite les modifications physico-chimiques au cours de la digestion et permet d'obtenir une composition similaire dans les échantillons à celle trouvée *in vivo*, en fournissant des extraits plus appropriés pour évaluer les activités biologiques. Toutefois, une vérification est nécessaire dans une étude *in vivo*.

Publication 4. Effets hypoglycémiants, hypolipémiants et antioxydants de la consommation d'une boisson de mûre chez des rats rendus diabétiques par la streptozotocine (STZ)

Enfin, le quatrième article présente une étude *in vivo* sur des rats diabétiques dont le régime a été complété avec une boisson de mûre. Des rats Sprague-Dawley avec un poids de $200 \text{ g} \pm 20 \text{ g}$ ont été utilisés et le diabète a été induit par une injection intrapéritonéale unique de STZ ($60 \text{ mg} / \text{kg}$ de poids corporel). Après 72 h, les rats ayant une glycémie à jeun de $250 \text{ mg} / \text{dL}$ ou plus ont été considérés comme diabétiques et utilisés pour l'étude. Les rats ont été divisés en 5 groupes ($n = 7$), 3 diabétiques et 2 avec des animaux non diabétiques. L'un des groupes de rats diabétiques a reçu à boire de l'eau (STZ-H₂O) et les deux autres ont reçu des boissons à base de jus de mûre diluées à 12.5% et 25% (STZ-BB12.5% et STZ-BB25%). L'un des groupes non-diabétiques a reçu de l'eau et l'autre une boisson de mûre à 25%. Dans tous les groupes, l'apport de liquide a été *ad libitum* et le traitement a duré 40 jours. À la fin de l'étude, les rats ont été sacrifiés et le sang a été collecté pour les analyses biochimiques et les tissus ont été prélevés et congelés pour l'analyse ultérieure.

La composition de la boisson de mûre donnée aux rats a révélé une faible concentration de vitamine C et d'acide ascorbique ($<2 \text{ mg} / 1000 \text{ g}$), mais une concentration importante de polyphénols totaux (1245 mg d'équivalents d'acide gallique / L) et une valeur ORAC

élevée (14.6 ± 0.3 mmol d'équivalents de trolox/ L). L'analyse HPLC indique que la boisson de mûre contient quatre principaux composés phénoliques: les anthocyanes, cyanidine-3-glucoside et cyanidine-3-malonyl glucoside, et les ellagitannins lambertianin C et sanguin H-6 (Mertz *et al.*, 2007).

Tous les groupes des rats diabétiques ont présenté à la fin du traitement une perte significative de poids corporel, par rapport aux groupes témoins non diabétiques. La consommation en nourriture et en liquide a été significativement plus élevée dans les groupes STZ-H₂O et groupes STZ- BB12.5%, par rapport aux témoins non diabétiques. Cependant, la dose maximale de mûre testée dans cette expérience (STZ- BB25%) a montré une diminution significative de la consommation de nourriture et de liquide par rapport au groupe STZ-H₂O, se rapprochant des niveaux de consommation des groupes témoins non diabétiques.

Une glycémie et un profil lipidique élevés ont été induits par la STZ, en raison d'un déséquilibre dans certaines voies métaboliques. Toutefois, l'administration orale de la dose la plus faible de la boisson de mûre (STZ-BB12.5%) a provoqué une diminution non statistiquement significative de la glycémie (-10.4%), des triglycérides (-4.6%) et du taux de cholestérol (-21.0%) par rapport au groupe STZ - H₂O. La dose la plus élevée de la boisson de mûre (STZ- BB25%) a montré une diminution significative de la glycémie (-48.6%), des triglycérides (-43.5%) et du taux de cholestérol (-28.6%) par rapport au groupe STZ- H₂O. Dans le cas des triglycérides et du cholestérol, les valeurs du groupe STZ- BB25% ont des niveaux comparables à ceux des groupes non -diabétiques.

L'effet hypoglycémiant et hypolipémiant montré par la mûre a également été signalé pour d'autres types de baies. Les mécanismes proposés par d'autres auteurs incluent l'inhibition de la α -amylase, des activités lipase pancréatique et α -glucosidase (Basu & Lyons, 2012; Battino *et al.*, 2009). La stimulation de l'utilisation périphérique du glucose et une augmentation du retrait de glucose du sang a été suggéré (Sefi *et al.*, 2011; Wacho *et al.*, 2012). Néanmoins, le mécanisme spécifique de l'effet hypolipémiant et hypoglycémiant des composés polyphénoliques de la mûre doit être approfondi.

La consommation de la mûre a amélioré la capacité antioxydante du plasma (PAC) par rapport au groupe STZ-H₂O. Alors que le groupe STZ-H₂O a perdu 12% de la valeur PAC en comparaison aux groupes témoins non diabétiques, les deux groupes ayant

consommés une boisson de mûre ont perdu seulement 4 et 5% de la valeur PAC. L'amélioration des valeurs PAC lors de la consommation de baies a été documentée principalement dans des essais cliniques (Paredes-López *et al.*, 2010; Basu *et al.*, 2010).

L'augmentation des concentrations de MDA a été utilisée comme un indicateur de la peroxydation lipidique causée par le stress oxydatif. La dose inférieure de la boisson de mûre (STZ 12.5%) fait diminuer les niveaux de MDA dans le plasma (-7.5%) et dans les reins (-19%) par rapport au groupe STZ-H₂O. Par ailleurs, la plus forte dose de la boisson de mûre (STZ 25%) a réduit les niveaux de MDA à des valeurs similaires à celles des rats non diabétiques dans le plasma (-19%) et à des niveaux intermédiaires dans le cas des tissus rénaux (-23%). Cela démontre que les boissons de mûre testées dans cette étude contiennent des antioxydants qui ont une bonne capacité de piégeage des radicaux libres, mais qu'ils protègent également de la peroxydation lipidique chez les animaux diabétiques. Cette activité a été associée par la littérature, à prévenir les altérations du gradient de la fluidité transmembranaire, des activités des enzymes et des récepteurs membranaires qui pourraient être associés avec le développement des complications du diabète (Ma, 2012).

La capacité à augmenter le pouvoir antioxydant au niveau du sérum et de diminuer la peroxydation de lipides en association avec l'effet hypoglycémiant et hypolipidémiant provoqués par la consommation de mûre a démontré un potentiel dans la prévention de certaines des complications secondaires du diabète sévère. Malgré cela, le mécanisme spécifique pour expliquer ces propriétés bénéfiques pour la santé doivent être étudiées d'avantage.

Conclusion générale

Les études menées au cours de cette thèse suggèrent que l'inclusion de la mûre dans le groupe des aliments fonctionnels pourrait avoir des effets bénéfiques, même si l'on considère les aspects de la biodisponibilité des composés polyphénoliques et les effets des procédés de transformation, tels que la pasteurisation. Cependant, il est nécessaire de clarifier les mécanismes d'action des polyphénols de la mûre parmi les activités biologiques retrouvées et aussi d'élucider les composants bioactifs spécifiques de ce fruit, associées aux activités *in vitro* évalués dans cette étude.

Chapter 1: General Introduction

1.1 Berries taxonomy and varieties

Edible berries are commonly consumed in the world diet as fresh fruit or as part of processed forms, such as ice cream, yogurts, jams, jellies, juices, baked goods and cereals. The cultivation of berries became common in Europe after the 16th century, and today berries are cultivated in all the six continents. Nowadays, breeders have developed plants with large succulent fruits having delicate sugar-acid complex flavors (Hummer, 2010). In 2005, more than 730.000 hectares of land were cultivated with berry crops in the world, producing around 6.3 million tons of fruit. The most important berry crops are strawberries, black currants, blueberries, red raspberries, gooseberries, cranberries and blackberries, but other minor berry crops are also available in the market (Strik, 2007).

Edible berries usually group species from 3 botanical families: Ericaceae, Grossulariaceae and Rosaceae. The most common Ericaceae berries includes cranberries (*Vaccinium oxycoccos*), lingonberries (*V. vitis-idaea*), blueberries (*V. corymbosum*) and bilberries (*V. myrtillus*). The Grossulariaceae family includes the blackcurrant, red currant, white currant and green currant, all from the *Ribes* genus. Finally, the Rosaceae family includes the chokeberry (*Aronia mitschurinii*), strawberries (*Fragaria ananassa*) and the *Rubus* genus, which consist of ≈ 740 species (Hummer, 2010), including blackberries (*R. adenotrichos*, *R. glaucus*, *R. jamaicensis*, *R. fruticosus*), raspberries (*R. idaeus*, *R. occidentalis*) and cloudberry (*R. chamaemorus*) (Häkinnen *et al.*, 1999).

1.1.1 Blackberries

In 2005, about 20.000 hectares were cultivated with blackberries around the world and the total production reached 154.578 tons of fruit. The largest blackberry production region was North America, with a 42.1% of the global production, and the major producer was United States. Interestingly, around 65% of the North America production came from the state of Oregon. Europe is the second producer of blackberries, with a 30.7% of the global production. Serbia and Hungary were the principal producers, but other countries as United Kingdom, Romania, Poland, Germany and Croatia also presented smaller productions. China is the third blackberry producer in the world, with an 18.8%. South America, Ecuador and Chile were important producers of blackberries, mainly of the

Rubus glaucus species and produced around the 4.5% of the global fruit. The countries of Central America cultivated 1.13% of blackberry world production. In 2005, in Costa Rica, around 1550 hectares were cultivated with blackberry crops, specially of the *Rubus adenotrichos* species, and the production was around 1653 tons. However, less than 15% of the production was exported, because most of the fruit is used for local processing and fresh consumption. Other continents as Oceania and Africa reported smaller productions of 2.6% and 0.14% respectively (Strik, 2007).

1.2 Nutrient composition

Berries are known as rich source of carbohydrates, some essential vitamins, minerals and an excellent source of dietary fiber (Table 1.1). One hundred grams of raw berries contains between 9-14 g of carbohydrates, mainly glucose and fructose (USDA standard reference database). Consumption of berries offer some contribution of vitamin C, A and K and folate, and the principal minerals offered by berries are potassium, calcium, phosphorus and magnesium (USDA 2013).

Table 1.1 Nutrient values for the most popular edible berries

	units / 100g FW	Ericaceae		Rosaceae			Grossulariaceae
		Blueberry	Cranberry	Blackberry	Strawberry	Raspberry	Black currant
Proximates							
Water	g	84.21	87.13	88.15	90.95	85.75	81.96
Energy	kcal	57	46	43	32	52	63
Protein	g	0.74	0.39	1.39	0.67	1.20	1.40
Total Lipids	g	0.33	0.13	0.49	0.30	0.65	0.41
Total Carbohydrates	g	14.49	12.20	9.61	7.68	11.94	15.38
Sucrose	g	0.11	0.13	0.07	0.47	0.20	nd
Glucose	g	4.88	3.28	2.31	1.99	1.86	nd
Fructose	g	4.97	0.63	2.40	2.44	2.35	nd
Fiber Total Dietary	g	2.4	4.6	5.3	2.0	6.5	nd
Vitamins							
Vitamin C, total ascorbic acid	mg	9.7	13.3	21.0	58.8	26.2	181.0
Vitamin A	IU	54	60	214	12	33	230
Vitamin K	µg	19.3	5.1	19.8	2.2	7.8	1.00
Folate, total	µg	6	1	25	24	21	nd
Minerals							
Ca	mg	6	8	29	16	25	55
K	mg	77	85	162	153	151	322
P	mg	12	13	22	24	29	59
Mg	mg	6	6	20	13	22	24

Reference: United States Department of Agriculture (USDA), Agricultural Research Service.
<http://ndb.nal.usda.gov/ndb/search/list>

nd: no data available

Berry fruits have become of particular interest because of they are exceptionally-rich sources of a wide variety of phenolic compounds associated with health benefits. The total phenolic content of berries range between 102 to 1342 mg /100 g of fresh weight (Szajdek *et al.*, 2008). However, the content of these phenolic compounds vary significantly due to genetic differences, environmental growing conditions and maturation stages (Howard & Hager, 2007).

The principal phenolic compounds found in berries include flavonoids and hydrolysable tannins, but phenolic acids, isoflavonoids and stilbenes are also present in minor concentrations. The classification of phenolic constituents is illustrated in Figure 1.1

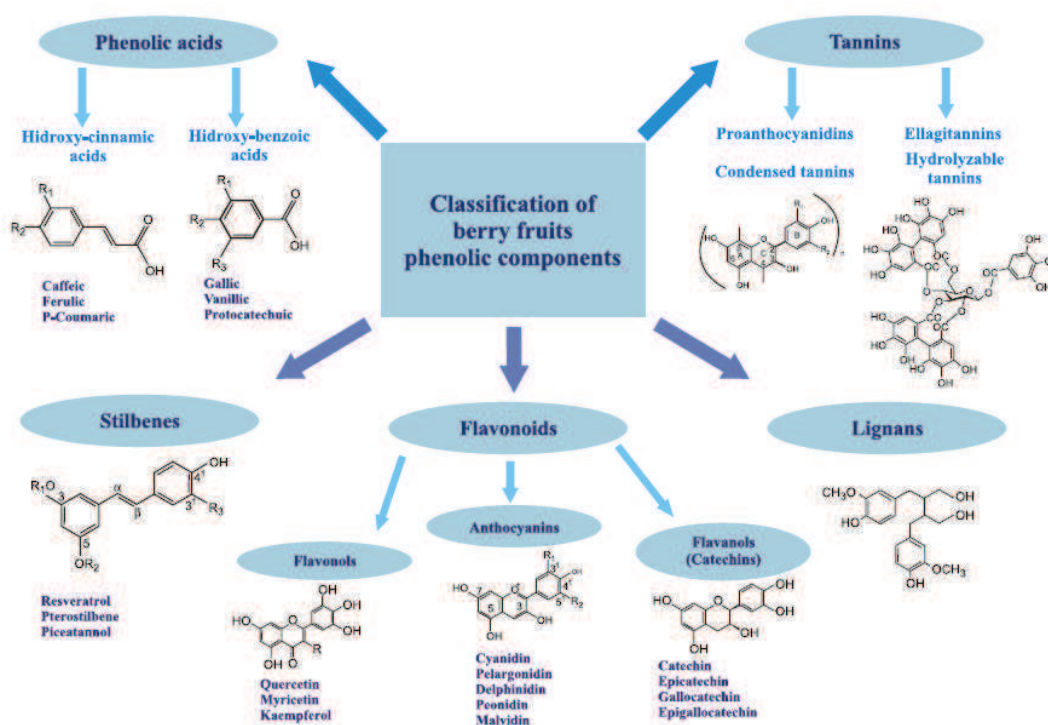


Figure 1.1 Common phenolic components of berries.
From Paredes-López *et al.* 2010. *Plant Foods Hum Nutr* 65(3):301

1.2.1 Flavonoids

Flavonoids are compounds produced by plants and contribute to the brilliant colors in leaves, fruits and flowers, also favor visual signals for pollinating insects, play roles in the defense system against insects and in the catalysis of light during photosynthesis. Plants synthesize flavonoids from malonate and the aromatic amino acids phenylalanine and tyrosine. The basic flavonoid structure is a nucleus of 15 carbon atoms arranged in three rings, C₆-C₃-C₆, labeled A, C and B respectively. Different classes of flavonoids

vary in the level of oxidation and patterns of substitution of the C ring, while each compound within a class presents a different pattern of substitution of the A and B rings. Flavonoids generally occur as glycosylated derivatives (Pietta, 2000). The main classes of flavonoids present in berries are the anthocyanidins, flavonols and flavanols (Paredes-López *et al.*, 2010).

Anthocyanidins

The anthocyanins vary in the patterns of hydroxylation and methylation on the different positions of rings and 25 different aglycones have been identified in nature. However, approximately 95% of all anthocyanins correspond to six main aglycones: cyanidin (Cy), peonidin (Pn), pelargonidin (Pg), malvidin (Mv), delphinidin (Dp) and petunidin (Pt) (Figure 1.2). The aglycones are rarely found in nature because of their poor stability. Usually, a hydroxyl group on the aglycone is substituted by sugar moieties, which could also be linked to other sugars or acylated with organic aromatic or aliphatic acids (acetic, malonic, succinic or oxalic acids). The glycosylation and acylation affect the physical and chemical properties of anthocyanins, modifying the molecular size and the polarity. Glycosylation increases water solubility whereas acylation decreases it. Glucose, rhamnose, galactose and arabinose are the more common sugars moieties attached to the aglycone (He & Giusti, 2010). In berries, around 90% exist as monoglycosides, whereas 10% are found as diglycosides. The principal anthocyanin found in berries and in most fruits is cyanidin-3-glucoside (Kaume *et al.*, 2012) (Del Rio *et al.*, 2010).

Anthocyanins concentrations in berries ranged from 20 to 450 mg/100 g of fresh weight (Basu *et al.*, 2010; Benvenuti *et al.*, 2004; Zheng & Wang, 2003). However, this concentration differ among species, varieties, geographic regions and climate, and is highly influenced as the fruit ripens, because anthocyanins are synthesized during the berry ripening (Paredes-López *et al.*, 2010; Benvenuti *et al.*, 2004; Acosta-Montoya *et al.*, 2010; Cuevas-Rodriguez *et al.*, 2010). For example, juice from 26 black raspberry populations analyzed from a single location, collected in two seasons, showed variability in the concentration of anthocyanins between 245 to 541 mg/100 ml, despite the uniform research field conditions (Dossett *et al.*, 2010). Acosta-Montoya *et al.* studied blackberries in three different maturity stages and demonstrate that major anthocyanin pigments increased from 0.20 mg/g FW in the red fruit to 1.34 mg/g FW in the fully ripe fruit (Acosta-Montoya *et al.*, 2010).

The anthocyanin profile is characteristic of each kind of berry and has been used in taxonomy to differentiate species. While some berries present only one type of anthocyanin, others have complex arrangement of anthocyanins. For example, *Rubus* berries, including blackberries (*Rubus spp*), raspberries (*R. idaeus*) and redcurrants (*R. idaeus*) present cyanidin derivatives as principal major component. Particularly cyanidin-3-glucoside could range from 44% to 95% of the total anthocyanin content, depending of the blackberry variety (Lee *et al.*, 2012; Howard & Hager, 2007). Strawberries (*Fragaria ananasa*), also from the Rosacea family, have as major anthocyanin, pelargonidin 3-glucoside, accounting for more than 70% of total anthocyanins, and this is the compound largely responsible for the red color. Cyanidins in strawberries are only in smaller proportions (Giamperi *et al.*, 2012; Howard & Hager, 2007). Contrarily to blackberries and strawberries, blueberries (*Vaccinium corymbosum*) present an array of different anthocyanins which include as major components malvidin-3-arabinoside, cyanidin-3-galactoside, delphinidin-3-galactoside, petunidin-3-galactoside, malvidin-3-galactoside, and also many other minor anthocyanins (Del Rio *et al.*, 2010). More than 25 anthocyanins have been identified in blueberries. (Howard & Hager, 2007) According to Cho *et al.* the percentage distribution of monomeric anthocyanins in blueberries could be: delphinidin (27-40%), malvidin (22-33%), petunidin (19-26%), cyanidin (6-14%) and peonidin (1-5%), depending of the different genotypes (Cho *et al.*, 2004).

Flavonols

The structure of flavonols consist in the basic flavan structure of three rings, and in the C ring an double bond at the C2 and C3 carbons, a hydroxyl group at C3 and a ketone group at C4. The different flavonols vary on the A and B rings of the flavonoid structure, specifically in the number and position of OH groups at C3 and C5 and the type of sugar attached to the C3 position (Figure 1.2). Quercetin, myricetin and kaempferol are the most commonly identified flavonols found in berries and are usually found glycosylated with glucose and galactose, but also arabinose, rhamanose, rutinose and xylose could be found (Kaume *et al.*, 2012; Cho *et al.*, 2004). Sugar moieties of berry flavonols can be acylated with various acids (acetic, glutaric, glucuronic, oxalic and caffeic) (Howard & Hager, 2007).

Flavonols concentration in berries usually ranges from 0 - 20 mg/100g of FW (Basu *et al.*, 2010; Sellappan *et al.*, 2002), however, they are more abundant in cranberry, 21 - 22

mg/100 mg of FW, and chokeberry, 9 - 89 mg/100 mg of FW (Paredes-López *et al.*, 2010).

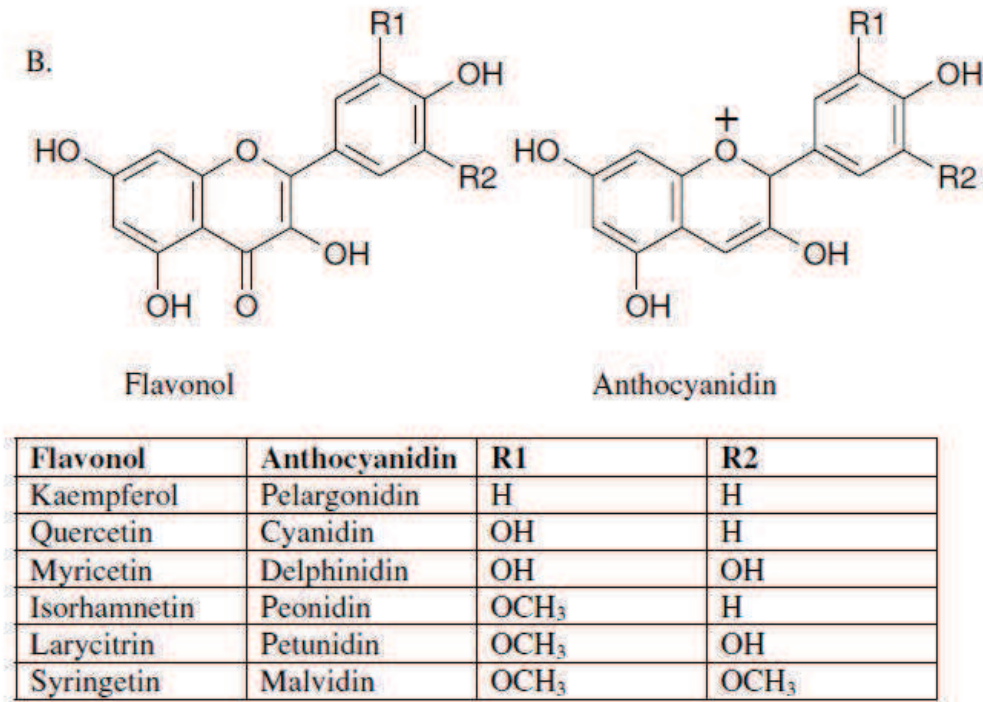


Figure 1.2 Structures of flavonols and anthocyanidins

From Howard & Hager. 2007. Berry fruit phytochemicals. In: Y.Zhao, Berry Fruit: Value-Added Products for Health Promotion (pp.79)

Quercetin, particularly quercetin 3-glucoside and quercetin 3 glucuronide, was found to be the most concentrated flavonol in strawberries (Giamperi *et al.*, 2012), and raspberries (Mullen *et al.*, 2002). Flavonol composition in blackberries and blueberries are more complex. Blackberries include 9 quercetin and 3 kaempferol derivatives, while blueberries contains 14 quercetin and 3 myricetin derivatives. However, there is a large variation among varieties and it is suggested that their synthesis is influenced by genetics (Howard & Hager, 2007).

In *Rubus* fruits, concentration of flavonols typically is much lower than ellagitannins and anthocyanins, and because of this, they are usually quantified together with phenolic acids and flavanols. According to Mertz *et al.* these phenolic monomers are less than 5 - 7% in two different species of *Rubus* whereas anthocyanins are 24 - 39% and ellagitannins 55 - 71% of the phenolic composition (Mertz *et al.*, 2007). Contrarily to the anthocyanins, the levels of total flavonols in blackberry are described to decline as the fruit ripens; Acosta-

Montoya *et al.* reported a drop from 5.1 to 2.0 mg of quercetin equivalents per g of FW in the fully ripe fruit (Acosta-Montoya *et al.*, 2010).

Flavanols and proanthocyanidins

Flavanols are the only class of flavonoids that do not occur naturally as glycosides (Giamperi *et al.*, 2012) Flavanol monomers as catechin and epicatechin are relatively minor constituents in berries (0 to 25 mg/100g FW) and occur in much larger quantities in green tea and cocoa (34-564 mg/ serving) (Lee *et al.*, 2003). Catechin is the major flavanol in strawberries, ranging concentrations from 2 to 9 mg/100g FW. In blackberries, catechin concentrations are less than 1 mg/100g FW, but epicatechin reach higher concentrations from 1 to 18 mg/100g FW. For blueberries the major flavanol is epicatechin, and the concentrations reported are 1 mg/100g FW (Howard & Hager, 2007).

In berries, flavanol dimers, trimers and especially polymeric proanthocyanidins reach higher concentrations ranging 27 to 180 mg/100g of FW (Del Rio *et al.*, 2010; Pietta, 2000). The two major classes of proanthocyanidins found in berries are procyanidins, composed exclusively of epi(catechin) units, and pro-pelargonidins, composed exclusively of (epi)afzelechin units. Blueberry and blackberry contains exclusively procyanidins, whereas strawberry and raspberry contains both procyanidins and propelargonidins (Howard & Hager, 2007).

Proanthocyanidins are particularly concentrated in the *Vaccinium* genus (cranberries, bilberries, blueberries, lingonberries) with 419 mg/100g FW, whereas are less concentrated in *Fragaria* genus (strawberries) which contains 145 mg/100g FW, and *Rubus* genus (blackberry and raspberries) with only 27 mg/100g FW (Gu *et al.*, 2004; Lee *et al.*, 2012).

1.2.2 Ellagitannins

Ellagitannins or hydrolysable tannins are complexes based on ellagic acid units organized in large and diverse structures. The basic structure of ellagitannins found in berries consist of a glucose core esterified with hexahydroxydiphenic acid (HHDP) (Howard & Hager, 2007; Bakkalbasi *et al.*, 2009). When ellagitannins are hydrolysed, hexahydroxydiphenic acids (HHDP) are released and rapidly lactonised to ellagic acid (Lee *et al.*, 2012). As it is shown in Figure 1.3, the large possibilities for the linkage of HHDP residues with the glucose moiety and their strong tendency to form dimeric and oligomeric derivatives

permits an enormous structural variety which comprises molecular weights from 302 g/mol (ellagic acid) to 3738 g/mol (lambertianin D) (Bakkalbasi *et al.*, 2009; Hager *et al.*, 2010).

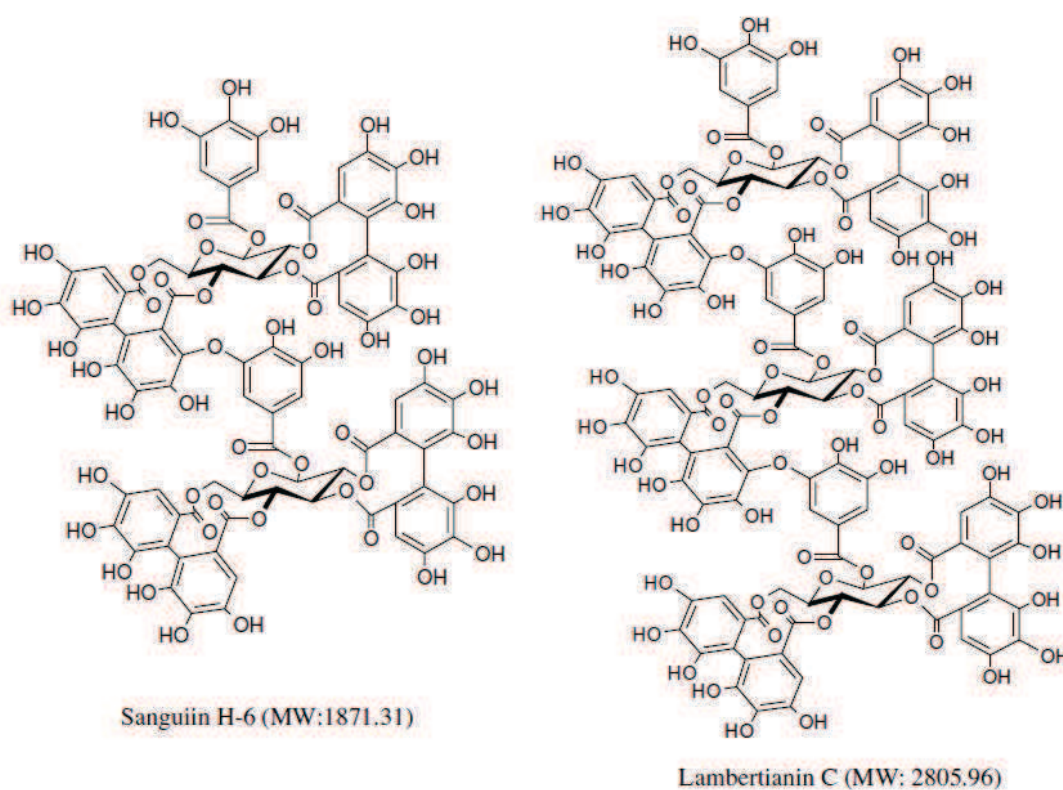


Figure 1.3 Structures of ellagitannins

From Howard & Hager. 2007. Berry fruit phytochemicals. In: Y.Zhao, Berry Fruit: Value-Added Products for Health Promotion (pp.78)

Ellagitannins are particularly concentrated in many economically important trees (oaks, chestnuts), in several nuts (walnuts, chestnuts, oak acorns, pistachios and pecans) and also in some fruits (pomegranates, grapes and berries). In the case of berries, ellagitannins are only detected in species from the Rosaceae family and are particularly concentrated in the species of the *Rubus* genus (Lee *et al.*, 2012; Battino *et al.*, 2009; Giamperi *et al.*, 2012). The most representative ellagitannin in berries is sanguin H-6 (comprises four HHDP, two galloyl and two glucosyl units) (Bakkalbasi *et al.*, 2009; Giamperi *et al.*, 2012), but other ellagitannins are also common, such as lambertianin C, sanguin H-10, lambertianin A and lambertianin D (Lee *et al.*, 2012). Additional ellagitannins reported in minor concentrations and in particular berries are pedunculagin, casuarictin, and potentillin (Bakkalbasi *et al.*, 2009).

Ellagitannins concentration in berries range from 20 to 550 mg/100g FW (Lee *et al.*, 2012) and free ellagic acid derivatives range from 0.7 to 4.3 mg/100g FW (Howard &

Hager, 2007). For cloudberry, raspberry, rose hip, sea buckthorn, blackberry and strawberry, ellagitannins represent between a 51% and 88% of all phenolic compounds (Paredes-López *et al.*, 2010) and these berries are considered to have about three times as much ellagic acid than walnuts and pecans (Bakkalbasi *et al.*, 2009). Ellagitannins and ellagic acid derivatives have not been identified in blueberries, and apparently the *Vaccinium sp* lacks the genetic capacity to synthesize ellagitannins and ellagic acid derivatives (Howard & Hager, 2007).

Contrarily to the anthocyanins, for some berries, such as raspberries, strawberries and blackberries, ellagitannins have been reported to decrease markedly as the fruit ripens (Siriwoharn *et al.*, 2004; Beekwilder *et al.*, 2005; Gasperrotti *et al.*, 2013). Acosta-Montoya *et al.* reported that ellagitannins and ellagic acid derivatives dropped from 3.8 to 2.2 mg of ellagic acid equivalents per gram of FW (Acosta-Montoya *et al.*, 2010).

1.2.3 Other phytochemicals

Some other phytochemicals are found in berries in minor concentrations, such as phenolic acids, lignans, sterols and stilbenes.

Phenolic acids are mainly represented by hydroxybenzoic acid (ellagic acid and gallic acid) and hydroxycinnamic acid (caffeic acid and ferulic acid). These acids rarely occur as free acids, but are commonly found as esters and glycosides conjugated forms (Kaume *et al.*, 2012; Howard & Hager, 2007). However, free acid forms have been reported in raspberries (0.6 mg/100g FW) and strawberries (1.8 mg/100g FW) (Del Rio *et al.*, 2010). Total phenolic acid (conjugated and free forms) in blackberries reaches concentrations of less than 64 mg/100g FW (Kaume *et al.*, 2012), but esters and glycoside forms have been reported to account for 53.1% and 43.6% respectively, and free acids account for only 3.3% (Howard & Hager, 2007). In the case of blueberries, esters and glycosides represent 40.7% and 56.7% respectively, and free acids only a 2.6% of the total phenolic acids. Is important to consider that phenolic acids are commonly studied by HPLC after an acid or alkaline hydrolysis and limited information is available on the native forms present in the fruit (Howard & Hager, 2007).

Compared to other berries, chokeberry (*Aronia melanocarpa*) has particularly high amounts of hydroxycinnamic derivatives as chlorogenic acid, reaching concentrations of 15 mg/100g FW (Paredes-López *et al.*, 2010).

Lignans are biphenolic compounds and play roles as phytoestrogens. Secoisolariciresinol and matairesinol are the major lignans reported in plants. Blackberries, strawberries, red raspberries and blueberries contain 3.72, 1.50, 0.14 and 0.84 mg/100g DW of secoisolariciresinol respectively. Matairesinol is only present in blackberries and strawberries, however concentrations are less than 0.01mg/100g DW (Howard & Hager, 2007).

Stilbenes are small phenolic compounds found in grapes, wines and peanuts. Resveratrol, pterostilbene and piceatannol are stilbenes found in deerberry, cowberry, and lingonberry (Paredes-López, et al., 2010). In blueberries (*Vaccinium* sp) resveratrol concentrations are reported between 0.7 to 588.4 mg/g of DW, but pterostilbene and piceatannol were not detected (Howard & Hager, 2007).

1.3 Metabolism and bioavailability of major phenolics in berries

1.3.1 Flavonoids

The bioavailability of a compound comprises the extent to which it reaches the circulatory system and is available at the site of action, as well as the distribution of the compound into tissues and organs following the absorption (Paredes-López *et al.*, 2010). The bioavailability is determined mainly by the chemical structure of a compound, glycosylation, molecular weight and esterification, but other factors as the food matrix or gut microbiota could also affect the bioavailability (D'Archivio *et al.*, 2010).

Flavonoid intake can range between 50 and 800 mg/day, depending on the consumption of vegetables, fruits and specific beverages, such as red wine or tea (Pietta, 2000). However, absorption of flavonoids from plant foods is minimum. Flavonoid levels detected in plasma and urine after ingestion of flavonoid-rich foods are in generally low, for anthocyanins around 0.02 to 1.8% of the ingested amount (Felgines *et al.*, 2005; Felgines *et al.*, 2009; Kaume *et al.*, 2012). Bioabsorption of anthocyanins are the most studied of the flavonoids and it is reported that, after an anthocyanin-rich food consumption, the flavonoid appears in plasma 15 to 60 min postprandial, and excretion is complete within 6-8 h (Felgines *et al.*, 2002; Felgines *et al.*, 2005; McGhie & Walton, 2007).

Flavonoids are released from the food matrix by chewing and absorption start in the stomach rapidly mediated by bilitranslocases, which are an organic anion membrane

carrier in the gastric mucosa (Passamonti *et al.*, 2002; Passamonti *et al.*, 2003). In spite of the stomach role in flavonoid digestion, the small intestine is the major site for absorption of these molecules.

In the small intestine, endogenic β -glucosidases release aglycones from primary flavonoid-glycoside forms. The released free aglycones are more lipophilic and have a smaller size that permits passive transport across the small intestine brush border. Sugar moiety of flavonoids determines their absorption and bioavailability because endogenic β -glucosidases present a preference for the glucose moiety rather than the galactose, xylose, rutinose or arabinose glycosides (He & Giusti, 2010). Also the aglycone structures influence the anthocyanin bioavailability, pelargonidin-glucoside has been reported to be more bioavailable than cyanidin-based anthocyanins (Giamperi *et al.*, 2012; Felgines *et al.*, 2005). The intact flavonoid-glycosides, not released by the β -glucosidases, could be also absorbed on the small intestine by passive diffusion or by the sodium-dependent glucose transporter (SGLT1) (He & Giusti, 2010).

Usually the acylated flavonoids are non-absorbable in the small intestine because of their larger molecular size and lack of free sugar moiety that facilitates a transporter binding (He & Giusti, 2010). The acylated flavonoids and the flavonoid-glycosides that survive through the small intestine travel down to the colon where glycosidic and ester bonds are cleaved by colonic microflora. Aglycones then undergo spontaneous breakdown of the anthocyanidin heterocycle that is transformed by the colonic bacteria to simple phenolic acids that could be absorbed by the colon and contribute to the bioactivity of ingested flavonoids (Aura *et al.*, 2005). The main phenolic acid produced by colonic microflora after cyanidin-based consumption is protocatechuic acid, and in the case of pelargonidin-glucoside, 4-hydroxybenzoic acid (Giamperi *et al.*, 2012). In spite of microflora transformations, the colon is much less efficient than the small intestine concerning absorption, so lower absorption rates are expected (He & Giusti, 2010).

Flavonoids taken up from gastro intestinal tract (GIT) lumen are subsequently metabolized by phase II drug-metabolizing enzymes to glucuronides, sulfates and methylates in the intestine epithelium, liver and kidney. Several phase II drug detoxification enzymes involved in xenobiotic conjugation are suggested to be important enzymes for flavonoid metabolism after absorption (He & Giusti, 2010, Kaume *et al.*, 2012). For example, catechol-O-methyltransferase may transfer a methyl group to the flavonoid aglycone, UDP-glucuronosyl transferase was proposed to catalyze the

glucuronidation of flavonoid aglycones, and the cytosolic phenol sulfotransferases are responsible to sulfate flavonoids (He & Giusti, 2010). Finally, the unabsorbed flavonoids are excreted in feces and the absorbed flavonoids, glycoside forms, aglycones or conjugated forms, are largely excreted in urine in the next 24 h after consumption (Felgines *et al.*, 2002; Felgines *et al.*, 2005; McGhie *et al.*, 2003). Dietary flavonoid absorption pathways are illustrated in Figure 1.4.

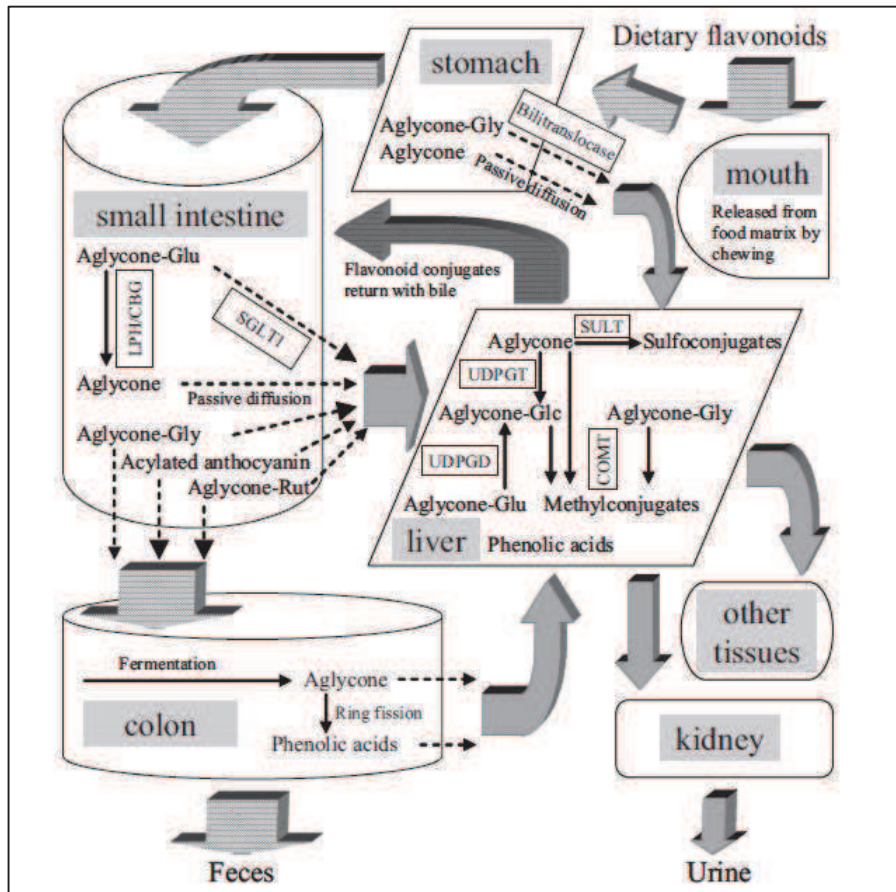


Figure 1.4 Intergrated putative pathways of dietary flavonoids absorption, metabolism, distribution, and excretion.

From He & Giusti *et al.* 2010. *Annu. Rev. Food Sci. Technol.* 1:163–87

The distribution of flavonoids in tissues is also important to evaluate the possible health effects that these molecules could provide. A study performed in rats demonstrated that after 12 days of an anthocyanin-enriched diet, anthocyanins could be detected in tissues as bladder, prostate, adipose tissue and heart, even in its native form as well as conjugated derivatives (Felgines *et al.*, 2005).

1.3.2 Ellagic acid and ellagitannins

The metabolism and bioavailability of ellagic acid and ellagitannins are less studied compared to flavonoids and besides most of the research has been performed with punicalagin of pomegranate. A study in humans who consumed pomegranate juice demonstrate the highest concentration of ellagic acid in plasma after 1 h postprandial, corresponding to a 10% of the total ellagitannins given dose, it was also demonstrated that after 4 h the ellagic acid was eliminated from plasma (Seeram *et al.*, 2004).

It has been demonstrated that free ellagic acid is absorbed in the stomach, however, the transcellular mechanism involved have not been elucidated (Seeram *et al.*, 2004). Once absorbed, ellagic acid is rapidly methyl or dimethyl conjugated by catechol ortho methyl transferase and probably glucuronidated by UDP-glucuronosyltransferases, and it is subject to hepatic phase II biotransformation. However, this metabolic pathway seems to have a very limited contribution to the global metabolisms of ellagitannins (Giamperi *et al.*, 2012).

Ellagitannins are not absorbed as such. Some authors hypothesized that ellagitannins are metabolized by the colon microbiota. Studies in pigs demonstrated that ellagitannins are hydrolyzed to ellagic acid due to the intestinal pH, subsequently the action of the gut microbiota in the small intestine provoke that the ellagic acid is gradually metabolized to urolithins D, C, A and B; in this order from the jejunum to the distal portion of the intestine. The transformation of ellagic acid to urolithins occurs through lactone-ring cleavage via decarboxylation and dehydroxylation reactions. Once absorbed these microbial metabolites are subjected to phase II biotransformations in liver, producing a combination of urolithin metabolites (Espín *et al.*, 2007).

The pharmacokinetics of dietary ellagitannins studied in humans demonstrated that ellagitannins metabolites (urolithins) are present in significant concentrations in plasma and urine, getting the highest concentrations 24-48 h after the consumption, and they could be detected in urine as long as 48-72 h after intake (Cerdá *et al.*, 2004; Cerdá *et al.*, 2005). Studies in pigs demonstrated that ellagitannins metabolites were not detected in liver, lung, kidney, heart, muscle and adipose tissues (Espín *et al.*, 2007). Studies in rats consuming pomegranate reported the presence of punicalagin metabolites in kidney, liver, colon and intestine and prostate tissues (Cerdá *et al.*, 2004; Seeram *et al.*, 2007).

It has been suggested that differences exist in the metabolism of ellagitannins and ellagic acid depending of the food source and the host colonic microflora composition. This results in variation in the timing, quantity and types of urolithins absorbed and excreted in urine (Cerdá *et al.*, 2005; Mertens-Talcott *et al.*, 2006).

1.4 Health benefits

The interest for polyphenols has increased in recent years since numerous studies bring out evidence of their potential beneficial effects on health. Epidemiological studies have suggested a reverse association between high consumption of polyphenols and the incidence of some chronic diseases. The hypothesized health benefits related to berries consumption include: prevention of inflammation, oxidative stress and cardiovascular disease, certain types of cancer, diabetes, obesity and neurodegeneration (Basu *et al.*, 2010; Basu & Lyons, 2012; Giamperi *et al.*, 2012).

1.4.1 Relief of oxidative stress

Reactive oxygen species (ROS) are generated normally by the body and play important roles in the immune system, cell signaling and other normal body functions. However if ROS are overproduced, they could provoke cellular damage, causing degenerative diseases, such as inflammation, cardiovascular diseases, cancer and aging (Niki, 2012). Phenolic compounds are potent antioxidants and it is known that they could quench free radicals and avoid the oxidative damage caused by ROS, primarily in the digestive tract. Phenolic compounds have been assessed as more potent antioxidants than carotenoids, vitamin E and vitamin C (Paredes-López *et al.*, 2010).

The antioxidant effectiveness of polyphenols is essentially due to the ability of the aromatic hydroxyl to donate a hydrogen to the free radical, also the ability to chelate transition metal ions involved in radical forming processes and the induction of endogenous cellular antioxidants (Beattie *et al.*, 2005). Particularly anthocyanins had been reported as strong antioxidants, according to He & Giusti the cyanidin-3-glucoside had highest ORAC value and pelargonidin aglycone the lowest. ORAC value of cyanidin-3-glucoside is 3.5 times more potent than trolox (He & Giusti, 2010). Anthocyanins scavenge the free radicals because of the 3,4-dihydroxy substituent in the B ring, which permits them to react specially with hydroxyl ($\bullet\text{OH}$), azide ($\text{N}_3\bullet$) and peroxy ($\text{ROO}\bullet$) to

form stable flavonoid radicals. However, anthocyanins and other flavonoids have slower scavenging activity for lipid peroxyl (LOO[•]) radicals and superoxide (O₂⁻) due to their polarity that prevents the reaction with LOO[•] radicals (Rice-Evans *et al.*, 1996).

Tannins components are also known as phenolics with high antioxidant potency. The antioxidant capacity is attributed to the multiple phenolic hydroxyl groups present in the hexahydroxydiphenyl (HHDP) units and the galloyl moieties. According to Bialonska *et al.*, the urolithins derived after the ellagitannins metabolism also exhibited an important antioxidant capacity, however other authors that evaluate metabolic derivatives of pomegranate punicalagin, showed a 42 fold lower activity (Bialonska *et al.*, 2009; Cerdá *et al.*, 2004).

In vivo studies had evaluated the increase in plasma antioxidant capacity after berries consumption, showing values that increase in 5 to 30%. This suggests that phenolic compounds are reaching the plasma and could relief the oxidative stress (Paredes-López *et al.*, 2010; Basu *et al.*, 2010).

1.4.2 Cardiovascular disease and metabolic syndrome

There is evidence that inclusion of berries in diet can positively affect risk factors of cardiovascular disease and metabolic syndrome. It has been reported, in human intervention studies, that berries consumption could inhibit inflammation, improve endothelial function, inhibit platelet aggregation, improve plasma lipids profile and glucose concentrations, modulate eicosanoid metabolism and increase resistance of low-density lipoproteins (LDL) to oxidation (Basu *et al.*, 2010; Basu & Lyons, 2012; Giamperi *et al.*, 2012).

It is difficult to demonstrate the mechanism of action that explains the protective effects of phenolic compounds against cardiovascular disease. Some authors have suggested that the relief of oxidative stress is possibly one of the most relevant mechanisms, mainly because oxidation of LDL triggers accumulation of macrophages in the artery walls and oxidation of cholesterol leading to atherosclerosis. Dietary phenols from berries had the potential to increase serum antioxidant capacity and thereby protect against LDL oxidation (Giamperi *et al.*, 2012; He & Giusti, 2010). For anthocyanins, it has been demonstrated, *in vitro* on human endothelial cells, that they suppress the secretion of chemokine monocyte chemoattractant protein 1 (MCP-1). This protein is directly involved in

atherogenesis through its function in recruiting macrophages to sites of infection or inflammation (Basu *et al.*, 2010). Studies in macrophage cell lines had reported that berries anthocyanins inhibited the nitric oxide formed through activation of inducible nitric oxide synthase (iNOS), which has proinflammatory effects, leading to an increased vascular permeability, induction of inflammatory cytokines and the formation of peroxynitrite, a strong oxidizing agent involved in the formation of foam cells (Pergola *et al.*, 2006). Other mechanisms described for berry anthocyanins is the interfering with the recruitment of tumor necrosis receptor-associated factors (TRAF)-2, thereby inhibiting CD40-induced proinflammatory signaling (Basu *et al.*, 2010).

One of the most complete reviews of interventional studies which associate berries consumption with less cardiovascular disease risk, is the one performed by Basu *et al.* (2010). This review summarized the results of 20 trials that have investigated the effects of acai berries, black currants, bilberries, boysenberries, blueberries, chokeberries, cranberries, lingonberries, raspberries, strawberries and wolfberries in healthy human subjects or in subjects with CVD risk factors. Some of the common outcomes of this trials are the increase in plasma and urinary antioxidant capacity, a decrease in LDL oxidation and lipid peroxidation, a decrease in plasma glucose and total cholesterol and an increase in HDL-cholesterol. These data suggests the potential role of edible berries in reducing the CVD factors, moreover in some of these trials, the favorable effects were evident even in subjects with elevated metabolic risk factors, such as diabetes, dyslipidemia, metabolic syndrome and also in smoker subjects. Two of these trials also showed a significant decrease in systolic blood pressure, suggesting a potential dietary therapy for the management of hypertension (Basu *et al.*, 2010).

Diabetes progression and its complications are associated to increased oxidative stress. Excessive glucose oxidation is source of free radicals, which cause damage to cellular proteins, membrane lipids and nucleic acids (Maritim *et al.*, 2003). Many intervention studies have evaluated the effect of polyphenol rich foods on postprandial blood glucose levels. A reduction in the glycemic index was demonstrated for red wine, sugar cane extract, coffee, berries and apple juice. Consumption of berries exhibits an inhibitory potential against α -glucosidase and α -amylase in the digestive tract. Also some reports indicate that polyphenols interact with the sugar transporter GLUT2 inhibiting glucose and fructose transport into brush border membrane vesicles of jejunum (Williamson, 2013; Da Silva Pinto *et al.*, 2008). Cyanidin-3-O-glucoside has demonstrated insulin-like

activity activating the peroxisome proliferator receptor- γ in human adipocytes. Other flavonoids as hesperetin have demonstrated an increase in glucose uptake by AMP-activated protein kinases (AMPK) in cultured skeletal muscle cells and have improved insulin sensitivity by increasing tyrosine phosphorylation (Avignon *et al.*, 2012).

1.4.3 Cancer

Many studies have demonstrated the potential cancer chemopreventive activities of berries, focusing on the inhibiting of different types of cancer cell lines proliferation *in vitro* or in the decreasing of early and late progression of experimental induced tumors in animal models (Seeram *et al.*, 2006; Stoner *et al.*, 2008). *In vitro*, it has been reported that blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry extracts inhibit the growth of human oral, breast, colon and prostate cancer cell lines in a dose-dependent manner. *In vivo* studies had demonstrated that in rodent models juices from blueberries, cranberries and blackberries or extracts from raspberries significantly reduce the formation of preneoplastic lesions induced with azoxymethane or N-nitrosomethylbenzylamine (Seeram *et al.*, 2006).

The role of berries bioactive compounds in the prevention of cancer seems to involve several mechanisms. The antioxidant capacity has been considered the first-line of action by inhibiting mutagenesis and cancer initiation, however, other mechanisms suggested for the chemopreventive activity of berries include decrease the DNA damage, stimulate antioxidant enzymes, inhibit carcinogen-induced DNA adducts formation, enhance DNA repair, modification of signaling pathways associated to cell proliferation, cell differentiation, apoptosis, cell-cycle arrest and inflammation (Stoner *et al.*, 2008; Seeram *et al.*, 2006; Duthie, 2007). Different berries act through different mechanisms in their cancer preventive ability. According to Boivin *et al.* (2007), some berries inhibit the cell proliferation independently of the caspase-cascade apoptosis and appear to involve cell-cycle arrest as evidenced by down-regulation of the expression of cyclin kinases, cdk 4, cdk6, cyclin D1 and cyclin D3. Other berries inhibited the tumor necrosis factor (TNF) induced activation of the COX-2 enzyme expression and activation of the transcription factor, nuclear factor kappa B (NF κ B) (Boivin *et al.*, 2007). For fractions of blueberries, regulation of apoptosis had also been reported through the phase II enzymes glutathione-S-transferase (GST) and quinone reductase (QR) (Srivastava *et al.*, 2007) and for strawberries and raspberries the pro-apoptotic marker Bax was found to be increased

because of the berry extract treatment (Wu *et al.*, 2007). Apart from the potential anticancer mechanism described above, berries have also been shown to inhibit the activities of matrix metalloproteinases, which play a significant role in cancer metastasis. For cranberry, proanthocyanidins have been reported to sensitize cancer cells to the platinum chemotherapy drugs, providing a potential use against drug resistance, and also some berries have been reported to inhibit the absorption of environmental carcinogens (Seeram, 2008).

Some authors consider that among the berry phytochemicals, ellagic acid has been the phenolic component primary associated to chemopreventive effects (Wu *et al.*, 2007; Losso *et al.*, 2004; Seeram *et al.*, 2005).

1.5 Factors affecting the bioavailability and health potential of dietary polyphenols

To establish conclusive evidence for the effectiveness of polyphenols in disease prevention and human health improvement, it is important to take into account different factors that influence the bioavailability of dietary phenolic compounds, such as agricultural factors, food processing practices and food matrix (D'Archivio *et al.*, 2010).

1.5.1 Agricultural factors

The genetic varieties cultivated are one of the most investigated aspects that cause differences in polyphenol concentrations. A study of 10 varieties of raspberries demonstrated differences of more than twice the concentration of anthocyanins and ellagitannins (Sparzak *et al.*, 2010). Similar differences in total ellagitannins concentrations were demonstrated for 6 varieties of raspberries and 5 varieties of blackberries cultivated in Italy (Gasperotti *et al.*, 2010). Another study of 6 blackberries genotypes cultivated in Mexico, demonstrated that wild types present more than twice total anthocyanins content than a commercial cultivars. Also the proportions of different anthocyanins changed between commercial and wild varieties of blackberries, being the commercial type more equal in the percentage of each anthocyanin, whereas the wild types presented a predominant concentration of a specific anthocyanin (Cuevas-Rodriguez *et al.*, 2010). The variation of the cultivar conditions could have an effect on the biological activities. Olson *et al.* describes that organically grown strawberries

showed higher antiproliferative activity in human colon and breast tumor cells than the conventionally grown fruits (Olsson *et al.*, 2006).

The degree of ripeness at the time of harvest affects the concentration and proportion of various polyphenols in different ways. Generally phenolic acids and ellagitannins concentrations decrease during ripening, whereas anthocyanins concentrations increase (D'Archivio *et al.*, 2010). Two different studies of blackberries collected in three different stages of ripeness demonstrate that the total anthocyanin content increased considerably during the ripening process, whereas ellagitannins, ellagic acid and flavonols dropped largely. However in both studies the total phenolic content did not show such pronounced changes (Siriwoharn *et al.*, 2004; Acosta-Montoya *et al.*, 2010). The antioxidant capacity in a study performed with blackberries from Oregon, United States, by Siriwoharn *et al.* did not show differences because of the ripen stage, however the study performed with blackberries from Costa Rica showed an increase in the antioxidant capacity during ripening (Siriwoharn *et al.*, 2004; Acosta-Montoya *et al.*, 2010).

Other agricultural factors as sun exposure, rainfall, soil type, fruit yield for tree, the use of fertilizers, greenhouses, hydroponic cultures or exposed fields could also affect the polyphenol content of the plants (Manach *et al.*, 2004). Studies in blackberries and strawberries have suggested that polyphenol content is higher in organic or sustainable agriculture than those grown in conventional conditions (Asami *et al.*, 2003). These difference is mainly because phenolic acids possess antimicrobial properties and their concentrations increase after infection. According to Manach *et al.*, the conventional conditions, which commonly use pesticides, provide an environment without stress that do not promote phenolic acid synthesis (Manach *et al.*, 2004).

1.5.2 Food processing related factors

Processing methods as simple as peeling of fruits or the elimination of the outer parts and seeds can reduce significantly the portion of polyphenols, because these substances are often present in higher concentrations in the outer parts than in the inner parts. Grinding of plant tissues could cause oxidative degradation of polyphenols and different degrees of polymerization as a result of cellular decompartmentation or because the contact between cytoplasmic polyphenol oxidase and phenolic substrates present in vacuoles (Manach *et al.*, 2004). Contrarily, for other compounds as lycopene and β -carotenes,

homogenization of the fruits tissues improve the bioavailability of the compounds (D'Archivio *et al.*, 2010).

The application of heat is the most widely used process in the food industry, because of its ability to inactivate microorganisms and spoilage enzymes. However, heat processing under severe conditions could induce changes in some bioactive compounds and reduce or increase their content or bioavailability. A review of the impact of thermal treatments in 23 exotic fruits, including mangos, guavas, papayas, acai, mulberry and others, demonstrated that in 20 of the fruits at least one of the bioactive components was reduced in concentration (Rawson *et al.*, 2011).

One group of phytochemicals reported to be easily affected by the heat are anthocyanins, which suffer cleavage of covalent bonds, oxidation and loss of active OH-groups mainly responsible of the antiradical activity (Rawson *et al.*, 2011; Hager *et al.*, 2008; Gancel *et al.*, 2011). Increased glycoside substitution, acylation and methoxylation of anthocyanins tend to improve the stability of these compounds when heated (Srivastava *et al.*, 2007). Other phytochemicals as ellagitannins were not appreciably affected or degraded in less magnitude after a thermal treatment (Hager *et al.*, 2010; Gancel *et al.*, 2011). For these molecules, it is reported that heat could also enhance the ellagitannins hydrolysis to ellagic acid and favor the release from cell walls, helping in its bioavailability (Bakkalbasi *et al.*, 2009). In general, it has been suggested that molecules consisting of unsaturated structures are more sensitive to heat treatments, though, factors as the dissolved oxygen, especially in liquid foods, can increase the rate of degradation during pasteurization (Rawson *et al.*, 2011).

Nowadays other mild sterilization processes are available, such as high pressure, irradiation, pulse electric fields, ultrasound, ozone and oscillation magnetic fields (Rawson *et al.*, 2011). However, it is still necessary to evaluate the impact of these new technologies in the polyphenols beneficial effects on health.

Storage may affect the content of polyphenols that are easily oxidized and this results in the formation of more or less polymerized substances which lead to changes in the health promoting properties of the polyphenols (Manach *et al.*, 2004). However, oxygen, pH, light and temperature conditions during storage influence the degradation levels of polyphenol (D'Archivio *et al.*, 2010). For example, a study in blueberries demonstrated between 17 to 55% loss of total polyphenols after 60 days of storage depending of the

temperature conditions (Srivastava *et al.*, 2007). Hager *et al.* demonstrated that blackberry processed products storage for 6 months lost 75% of the monomeric anthocyanins, but concentration of anthocyanins polymers increased during the storage period and permitted maintenance of the antioxidant capacity (Hager *et al.*, 2008). It is also reported that 55% of blackberry ellagitannins are lost during a six months storage. The losses were mainly because these ellagitannins bind irreversibly to macromolecules causing insoluble materials that sediment during the storage period. This phenomenon has been described for blackberry juices, green tea, muscadine juice and some wines (Hager *et al.*, 2010). For raspberries, it has been demonstrated that ellagic acid concentrations increased during storage, suggesting that a depolymerization of higher ellagitannins is occurring and may result in additional health benefits (Zafrilla *et al.*, 2001). Hager *et al.* demonstrate that juice products were most affected by storage than canned, pureed and frozen products, suggesting a protective effect of other components in the fruit removed with the presscake during the juice processing (Hager *et al.*, 2008; Hager *et al.*, 2010).

Production of fruit juices involving clarification steps and pectinolytic enzymes, hydrolyzes and removes some fruit parts with flavonoids responsible of biological activities (Manach *et al.*, 2004). Particularly for a blackberry juice, the enzymatic treatment resulted in a 34% loss in the total monomeric anthocyanins and an additional 14% was loss during the juice pressing. The ellagitannins were also retained in a 70 to 82% in the presscake, especially because of the removal of seeds, which are a rich ellagitannins source (Hager *et al.*, 2008; Hager *et al.*, 2010). However, some authors reported that pectolytic enzymes used in strawberry and raspberry juices produced higher values of ellagic acid derivatives facilitating their bioavailability (Bakkalbasi *et al.*, 2009).

1.5.3 Food matrix

Interactions between polyphenols with food components, such as proteins, carbohydrates, fiber, fat and alcohol can occur and affect their absorption. The study of the effect of milk, fat and fiber on the bioavailability of polyphenols are some of the most investigated interactions.

The effect of milk had been studied primary for flavanols, specifically epicatechins from cocoa, demonstrating contradictory results that are explained by the interindividual

variability of human flavanols absorption (D'Archivio *et al.*, 2010; Del Rio *et al.*, 2010). Fewer studies have been performed with berries; but a study comparing the antioxidant status in humans after the consumption of a blackberry juice prepared in water or in milk demonstrated that milk reduce the concentrations of anthocyanins and ellagitannins in plasma, suggesting that milk affects their bioavailability. However, the plasma antioxidant capacity and the catalase and glutathione peroxidase activity were not changed because of milk co-digestion (Hassimotto *et al.*, 2008). Contrarily to the blackberry study cited before, humans who consumed blueberries with milk impaired their antioxidant capacity in plasma compared with the blueberries eaten without milk (Serafini *et al.*, 2009). A study performed in pigs which were fed with blackcurrant in addition to milk and cereal demonstrated that these two last ingredients do not affect the plasma C_{max} (peak plasma concentrations) or the area under the curve values, but increased the T_{max} (time to reach C_{max}) from 2 to 4 h (Walton *et al.*, 2006). Similarly, other study in humans which ingest strawberries with cream demonstrated that cream did not alter the plasma C_{max} of a pelargonidin-O-glucuronide, while it increased the T_{max} from 1.1 to 2.4 h. The anthocyanin urinary excretion was also delayed because of the cream (Mullen *et al.*, 2008).

Studies on the effect of fat or dietary fiber showed that both compounds enhance the digestibility and bioavailability of flavonoids, these mainly because either fat or fiber slows the small bowel transit time and delays the absorption, giving more chances for contact of flavonoids with intestinal enzymes or colonic microflora, and improving their absorption (D'Archivio *et al.*, 2010). As well, fat and fiber could enhance the bioaccessibility in the small intestine due to chemical complexes or colloidal structures formed with the antioxidants that improved the bioabsorption (Palafox-Carlos *et al.*, 2011). Finally, the effects of synergistic activities between the different polyphenols of the same fruit played also an important role in the bioactivity or the health promoting potential. For example, a study which compared the *in vitro* antiproliferative, apoptotic and antioxidant activities of a complete pomegranate juice, punicalagin, ellagic acid and a total pomegranate tannin extract, demonstrated that the complete juice had a better antioxidant activity and antiproliferative activity than the other purified compounds (Seeram *et al.*, 2005). This suggests that the effect cannot be ascribed to a single polyphenol.

1.6 Thesis objectives

1.6.1 General objective

To describe biological activities of the tropical highland blackberry (*Rubus adenotrichos*) and to evaluate the impact of an *in vitro* gastrointestinal digestion and an industrial pasteurization process on the antioxidant properties.

1.6.2 Specific objectives

- To describe the antioxidant and anti-inflammatory activities of the polyphenols obtained from the tropical highland blackberry (*Rubus adenotrichos*).
- To evaluate the impact of the pasteurization process on the antioxidant activities of the blackberry phenolic compounds.
- To evaluate the effect of an *in vitro* gastrointestinal digestion in the antioxidant activities of the blackberry phenolic compounds.
- To evaluate the effect of the consumption of a blackberry beverage in some biochemical parameters and the antioxidant status of diabetic rats.

Chapter 2: Materials and Methods

2.1 Samples preparation

2.1.1 Blackberries collection

For all experiments, fully ripe blackberries (*Rubus adenotrichos* Schltdl. cv. 'vino') were used. According to the Germoplasm Resource Information Network, GRIN, this specie from the Rosaceae family is native from Mexico to Ecuador and Venezuela (USDA, 2014). The samples used for this study were collected from different farms in the province of Cartago, Costa Rica (altitude 1864 - 2517 m, latitude 09° 39' 57.1"N - 09°44'40.3"N, longitude 83°53'32.1"W - 84°00'06.3"W).



Figure 2.1 Location of the blackberry collection site (GoogleMaps, 2013)

For studies performed with the complete fruit, blackberries were transported to the laboratory and the samples freeze-dried, packaged in metallic bags, and stored at -20 °C until extract preparation. For studies performed with microfiltrated blackberry juice, the collected fruits were frozen at -20 °C until juice preparation.

2.1.2 Preparation of the microfiltrated blackberry juice

A microfiltrated juice was prepared according to Vaillant *et al.* (Vaillant *et al.*, 2008). Blackberries were pressed and the juice treated with a commercial enzymatic preparation (Klerzyme ®150 from DSM Food Specialties, Heerlen, Netherlands) for 1h at 35 °C with constant agitation. The microfiltration was performed in a tubular ceramic membrane (Membralox® 1 P19-40, Pall Exekia, Bazet, France) with an average pore size diameter of 0.2µm. The obtained juice was packaged in metallic bags, and stored at -20 °C until use for different experiments.

2.1.3 *In vitro* gastrointestinal digestion of the blackberry juice

An *in vitro* digestion method previously described by Gil-Izquierdo *et al.* and Coates *et al.* (Gil-Izquierdo *et al.*, 2002; Coates *et al.*, 2007) was adapted to simulate physiochemical changes undergone by polyphenols in the upper GIT, particularly stomach and small intestine. This protocol was applied to a blackberry microfiltrated juice and samples were collected through the process to represent major stages of the digestion process. Figure 2.2 represents a diagram of the *in vitro* digestion performed and the sampling points.

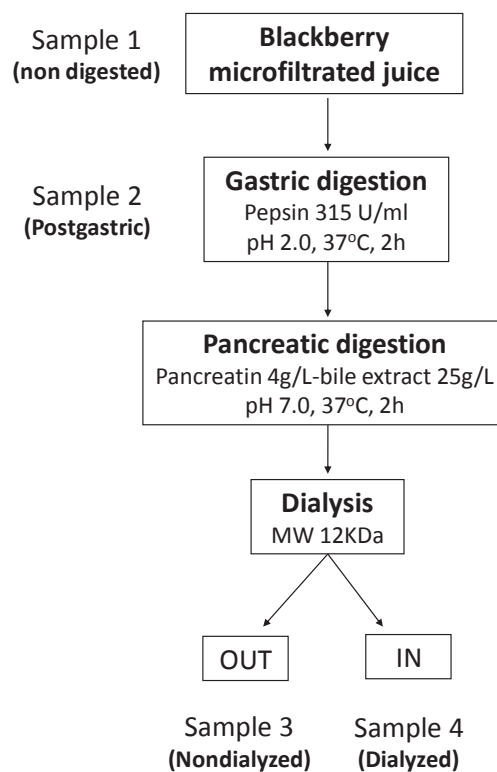


Figure 2.2 *In vitro* gastrointestinal digestion model used for the blackberry juice

One hundred milliliters of a microfiltrated blackberry juice (**Sample 1**) were subjected to a pepsin-HCl digestion, using 31500 units of pepsin and the pH was adjusted to 2 by addition of concentrated HCl. The digestion mixture was incubated for 2 h at 37 °C with continuous agitation in a beaker sealed with parafilm (**Sample 2**). Later, 60ml of the post gastric digestion sample were transferred to a 400ml beaker which contained two cellulose dialysis tubes (molecular cut-off 12 kDa) filled with sufficient NaHCO₃ solution to increase the pH of the gastric digestion sample to 7 (The necessary amount of NaHCO₃ was previously evaluated). The beaker was sealed with parafilm and incubated at 37 °C with continuous agitation until pH reached 7 (30 min approximately). Then a mixture of pancreatin (4 g/L)-bile extract (25 g/L) was added and incubated for 2 h more. At the end of the incubation, the juice solution outside the dialysis tubes was recovered (**Sample 3**), representing compounds that would reach the colon and the solution inside the dialysis tube was recovered (**Sample 4**) and represents the serum available material.

In order to have enough amount of each sample the *in vitro* digestion was repeated and samples obtained each time were pooled. Finally, the pools of each sample were subjected to polyphenol purification. Samples 3 and 4 required to be acidified to pH 2 and centrifuged before polyphenol purification in order to eliminate supernatants formed during the pancreatic digestion.

2.1.4 Pasteurization of the blackberry microfiltrated juice

The blackberry microfiltrated juice was pasteurized in two different conditions using a miniature scale UHT processing system, model FT74X (Armfield Inc., Ringwood, UK). One of the juices was pasteurized at 75 °C for 15 s (**JP75**) and the other one at 92 °C for 10 s (**JP92**). A third sample non-pasteurized (**NPJ**) was included in the experiment as a control. The three juices were subjected to polyphenol purification.

2.1.5 Polyphenol purification of the samples

All samples used in these studies, digested or pasteurized juices and the complete fruit, were subjected to polyphenol purification. This purification had the objective to concentrate the investigation in the polyphenol biological activities and not in the biological activities of other components of blackberries as vitamins, minerals, fiber, etc.

To perform the polyphenol purification from the complete fruit, 35 g of blackberry lyophilized fruit was extracted twice for 15 min with acetone/water/formic acid (70:30:2).

The extract was filtered and concentrated under vacuum to remove the acetone (40 °C). The aqueous layer was mixed with ethyl acetate (2:1), separated, and loaded onto an Amberlite XAD-7 column (150 mm x 20 mm) packed in water. The column was washed with water to remove sugars, and phenolic compounds were eluted with methanol/water (80:20). Polyphenols were concentrated under vacuum and freeze-dried. The sample was stored at -20 °C for further analysis.

For the polyphenol purification of juice samples, the acetone and ethyl acetate extraction were not performed and samples were directly loaded onto the Amberlite XAD-7 column. The rest of the procedure, washings and elution were performed as described before.

2.2 Chemical samples analysis

2.2.1 HPLC analysis

The polyphenol extracts used in different experiments were analyzed by HPLC for anthocyanins and ellagitannins following the protocol described by Mertz *et al.* (Mertz *et al.*, 2007) and Gancel *et al.* (Gancel *et al.*, 2011). Samples were filtered through a 0.45µm filter (Millipore). The HPLC quantitative analysis was performed using a Dionex liquid chromatograph system that was equipped with a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) and an endcapped, reversed-phase Lichrospher ODS-2 column (250 mm x 4.6 mm i.d., 5 µm) (Interchim, Montluçon, France). HPLC solvents consisted of 2% aqueous formic acid (Solvent A) and acetonitrile/water/formic acid (80:18:2, v/v/v; solvent B). The chromatographic conditions were: 30 °C, 0.3 mL/min flow rate, 20 µL injection volume and the detection was carried out between 200 and 600 nm. The gradient used for separation was: 0 min, 5% B; 0-20 min linear gradient from 5% to 25% B; 20-25min linear gradient from 25% to 100% B; 25-30 min linear gradient from 100% to 5% B; 30-35 min, 5% B. Previously, Mertz *et al.* (Mertz, et al., 2007) identified sanguin H6, lambertianin C and cyanidin glucosides following the same HPLC procedure by including an additional hyphenation of the diode array detector (DAD) to an electrospray Ion Trap Mass spectrometry detector (ESI-TRAP-MS/MS). As the same method was followed in this study, and the retention times coincide, a tentative identification of the phenolic compounds was performed based on the work of Mertz *et al.* (Mertz *et al.*, 2007). The quantification of polyphenols in the extract was performed using calibration curves with five concentrations that were

established with standards of ellagic acid for ellagitannins and cyanidin-3-glucoside for anthocyanins. Samples were analyzed in triplicate.

2.2.2 Total polyphenol content

Total phenolic content was determined with Folin-Ciocalteu assay modified by George *et al.* (George *et al.*, 2005). In a final volume of 2.5 ml, the different samples were mixed with the Folin Ciocalteu reagent diluted 1:10 in water. The mixture was incubated for 2 min at room temperature and 2.0 ml of sodium carbonate (75 g/L) was added. After 15 min at 50 °C, the mixtures were cooled in a water-ice bath and the absorbance at 760 nm was measured using a spectrophotometer (Pharmaspec UV-1700 Shimadzu, Kyoto, Japan). Results were expressed as milligrams of gallic acid equivalent (GAE) per liter for liquid samples and per g of extract for lyophilized samples. Each sample was analyzed in triplicate.

2.2.3 Vitamin C and ascorbic acid content

Total vitamin C content of the blackberry beverage used in the *in vivo* model was determined by the quantification of ascorbic acid (AA) and dehydroascorbic acid (DHA) (Hernández *et al.*, 2006; Wechtersbach & Cigic, 2007). Briefly, AA was extracted using a metaphosphoric acid solution (4%) and DHA was reduced to AA with a tris-[2-carboxyethyl]-phosphine solution (40 mM). Both products were analyzed by HPLC with a C18 column, 5 µm, 250 x 4.60 mm (Phenomenex Luna, Torrance, CA), and the AA peak was identified and quantified using commercial standards of AA (Sigma, St. Louis, USA). DHA content was calculated by subtracting the initial AA content from the total AA content after the conversion.

2.2.4 Moisture content and soluble solids (%Brix)

The blackberry beverage used in the *in vivo* model was analyzed for Moisture Content and Soluble solids (% Brix) using standard AOAC methods (AOAC, 2010). To determine the moisture content, 20g of juice were weighted in an aluminum dish (already pre-weighted) and dried in an oven at 70 °C and 30 kPa for 24 h (National Appliance Co., Portland, OR, USA). Later, the dish was transfer to a desiccator to cool and the weight was determined again. The determination was performed in duplicate and for all weight determinations it was used an analytical balance with a precision of 10⁻⁴ g (Sartorius, Gottingen, Germany). The percentage of soluble solids was assessed using a hand held

Abbe refractometer (ATAGO, Tokyo, Japan). Determinations were performed in duplicate and were corrected by temperature and acidity.

2.3 Antioxidant assays

2.3.1 DPPH radical-scavenging activity

The radical-scavenging activity of blackberry extracts was evaluated by assessing the direct DPPH-scavenging activity in the extract (Kim *et al.*, 2004). DPPH (0.25 mM) was prepared in methanol, and 0.5 mL of this solution was incubated with 1 mL of various sample dilutions. The mixtures were incubated at room temperature in the dark for 30 min, and the absorbance of DPPH was measured at 517 nm. Sample blanks were prepared for each dilution. The percentage of the radical-scavenging activity of extracts was plotted against the sample concentration to calculate the IC₅₀, which is the amount of extract necessary to reach the 50% radical-scavenging activity. Samples were analyzed in triplicate.

2.3.2 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Ou *et al.* (Ou *et al.*, 2001). Fluorescein was used as a fluorescent probe, the oxidation was induced with AAPH and the decay in the fluorescence was measured for 45 min, using a spectrofluorimeter equipment (Biotek Instruments, Winooski, VT, USA). ORAC values were expressed as mmol of Trolox equivalents (mmol TE/g of extract).

2.3.3 Nitric oxide-scavenging activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and rapidly converted into the stable product nitrite. The nitrite concentration was measured by the Griess reaction (Balakrishnan *et al.*, 2009).

SNP (5 mM) was mixed with different concentrations of blackberry extracts and incubated for 60 min under direct light to enhance NO production. The Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H₃PO₄) was added, and the absorbance was measured at 540 nm in reference to the absorbance of standard solutions of sodium nitrite. Sample blanks were prepared for each experiment. NO-scavenging activity was calculated as the amount of extract necessary to reduce 50% of the NO generated by SNP (IC₅₀). Samples were analyzed in triplicate.

2.3.4 Inhibition of lipid peroxidation in liposomes

Liposomes were prepared according to Pérez *et al.* (Pérez *et al.*, 2003). Twenty-five milligrams of commercial lecithin was dissolved in 2.15 mL of chloroform, and then 350 μ L of methanol was added. This mixture was dried under a nitrogen atmosphere. The lecithin was resuspended in 4.5 mL of warm phosphate-buffered saline (PBS) and sonicated for 1 h at 4 °C to form liposomes.

To test the capacity of extracts to protect lipid peroxidation, oxidative stress was induced with AAPH. Various dilutions of blackberry extracts (50 μ L) were mixed with 0.45 mL of liposomes and 0.2 mL of AAPH (final concentration of 10 mM), and these solutions were incubated in the dark for 2 h at 37 °C. To determine the malondialdehyde (MDA) concentration, 0.2 mL of 5% TCA (trichloroacetic acid) and 1 mL of 0.75% TBA (thiobarbituric acid) were added, and samples were heated at 96 °C for 30 min. After cooling, 0.25 mL of 3% SDS was added, and the mixture was centrifuged at 2500 x g for 15 min. Absorbance of the supernatant was measured at 532 nm. The thiobarbituric acid reactive substances (TBARS) concentration was assessed using the molar absorption coefficient for malondialdehyde (MDA): $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$. MDA concentrations were plotted against the sample concentration, and results were expressed as the amount of extract that inhibits 50% of lipid peroxidation (IC_{50}). The extract was tested in triplicate.

2.3.5 Inhibition of lipid peroxidation in liver homogenates

This procedure was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 19-06). Sprague-Dawley rats (220 g \pm 20 g), obtained from LEBi® (Laboratorio de ensayos biológicos, Universidad de Costa Rica), were anesthetized with CO₂ and sacrificed by decapitation. The liver tissue of each rat was obtained and homogenized in phosphate-buffered saline (PBS) using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 x g for 15 min to reduce the amount of suspended solids. Different concentrations of blackberry extracts (75 μ L) were added to 0.75 mL of liver supernatant and incubated for 30 min at 37 °C. Oxidative stress was then induced with TBHP (tert-butyl hydroperoxide) at a final concentration of 1.7 mM and incubated for 1 h at 37 °C, and the TBARS were measured as the end product of lipid peroxidation.

TBARS were assayed according to Mihara & Uchiyama (Uchiyama & Mihara, 1978). A 0.25 mL sample of liver homogenate, 0.25 mL of 35% TCA, and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4) were mixed and incubated for 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added, and the mixture was heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, and the sample was mixed and centrifuged at 2500 x g for 15 min. The absorbance of supernatant was measured at 532 nm. The concentration of TBARS was calculated as described previously, and results were expressed as nmol MDA/g liver tissue. MDA concentrations were plotted against the sample concentration to calculate the IC₅₀.

The assay was performed using liver tissue from 5 rats. To establish the basal level of lipid peroxidation, the MDA levels without TBPH were assessed in each liver homogenate. Sample blanks were prepared for each experiment, and the extract was tested in triplicate.

2.3.6 Erythrocyte cellular antioxidant activity (ERYCA)

ERYCA assay was performed according to González *et al.* (González *et al.*, 2010). Human erythrocytes were treated with AAPH (100µM) to enhance lipid peroxidation in the presence of polyphenols extracted from digested samples. Erythrocytes hemolysis triggered a loss in the light scattering ability (turbidity) which was assessed each 5 min for 6 h. The absorbance decay curve (AUC) was calculated for each sample and compared with a standard calibration curve of quercetin. ERYCA values were expressed in mmol of quercetin equivalents per gram of blackberry extract from the different samples.

2.3.7 Inhibition of intracellular ROS

Levels of intracellular ROS were assessed using the DCFDA oxidation-sensitive fluorescent probe. Vero cells, a line of African green monkey kidney (ATCC, Cell No. CCL-81; Rockville, MD, USA) were grown in EMEM+ L-glutamine medium containing 10% inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (20 µg/mL). Cells were cultured in a 24-well plate at a density of 5×10^5 cells/well and were pre-treated with different concentrations of blackberry extracts for 20 h. Later, the cells were washed twice with PBS and oxidative stress was induced with TBHP (0.7 mM) for 2 h. Thirty minutes before the end of TBHP incubation, the probe DCFDA (5 µM) was added to cells, internalized and oxidized by the intracellular ROS to 2'7'-dichlorofluorescein.

After washing with PBS twice, the intensity of intracellular fluorescence was measured using flow cytometer (FACS-Scalibur, Becton-Dickinson). For each analysis, 10000 events were counted.

2.3.8 Inhibition of superoxide anion production

The human promonocytic cell line THP-1 was obtained from American Type Culture Collection (ATCC, Cell No. TIB-202, Rockville, MD, USA). The cells were grown in RPMI 1640-glutamax medium red phenol containing 10% fetal calf serum, 10^5 U/L penicillin/streptomycin, and 10^{-4} g/l fungizon. To differentiate promonocytes, 3.5×10^5 cells/mL were incubated with retinoic acid (10^{-6} M), 1,25 dihydroxycholecalciferol (10^{-7} M), and IFN- γ (10 U/mL). The differentiated monocytes became adherent cells after 72 h.

In the first step, cells were treated with different concentrations of blackberry extracts for 24 h to calculate the IC_{50} of blackberry phenols. The medium and extract were then removed, and cells were washed and collected by scraping into RPMI 1640 medium without red phenol. A cell suspension of 1×10^6 cells/mL was prepared and incubated with lucigenin (10^{-4} M) for 30 min, and the monocyte cell suspension was stimulated with PMA (10^{-7} M) to induce O_2^- production. The O_2^- reduced the lucigenin bioluminescence probe, and the luminescence generated was immediately recorded for 1 h at 37 °C using a Victor Wallac Luminometer (WALLAC Co, Turku, Finland). After the luminescence assay, cells were dissolved in 1 M NaOH to determine the protein concentration (Dc Protein Assay, Bio-Rad, Hercules, CA, USA). The luminescence intensity was normalized to the amount of protein present. The response of PMA-stimulated cells without blackberry extract was used as a control and was considered equal to 100% of the superoxide anion production.

In the second step, to determine the kinetics of O_2^- inhibition activity in blackberry extracts, the same protocol was performed using a unique concentration of 80 μ g/mL (defined as the IC_{50}) that was incubated for 15 min, 30 min, 1 h, 4 h, 8 h, and 24 h. Each experiment was performed in triplicate.

2.4 Anti-inflammatory assays

2.4.1 Cell viability

The murine macrophage cell line J774A.1 was obtained from ATCC. The cells were grown in RPMI 1640-glutamax medium containing 5% inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (20 µg/mL).

To test the cytotoxicity, 10^5 cells/well were seeded in a 96-well plate and incubated for 20 h with different concentrations of blackberry extracts. The cells were washed, and each well was filled with 200 µL of medium and 20 µL of a tetrazolium salt, MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) mixed with an electron-coupling reagent, PMS (phenazine methosulfate), diluted in PBS. The plate was incubated for 4 h, and the absorbance was measured at 550 nm. The percentage of viable cells was calculated using the absorbance of control cells without extract as 100%. The extract was tested in triplicate. The concentrations of extract toxic for the J774 A.1 were not used for further experiments.

2.4.2 Measurement of nitric oxide

J774A.1 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well and were pre-treated with different concentrations of blackberry extracts for 4 h or 24 h. After the treatment, the cells were stimulated for 24 h with a mixture of LPS (10 ng/mL) and murine IFN γ (10 U/mL). The supernatants were collected, and the concentration of nitrite, which reflects the intracellular NO synthase activity, was determined using the Griess reagent, as described above. In some experiments, the extract was eliminated (“Wash”) before stimulation with LPS+IFN γ , and in others, it was not (“No-wash”). Each experiment was performed in triplicate.

2.4.3 Western blotting analysis

To study the effect of blackberry extracts on iNOS expression, J774A.1 macrophages were pre-treated for 24 h with blackberry extract; the cells were washed with medium and stimulated with LPS (10 ng/mL) and murine IFN γ (10 U/mL) for 10 h. The cells were then washed and incubated with a lysis buffer (1X PBS, 1% Triton X-100, and protease inhibitor cocktail, Roche Applied Science, Mannheim, Germany) for 30 min on ice. The lysed cells were collected by scraping and then centrifuged at 10000 x g for 2 min. The supernatant was collected, and the proteins were used for electrophoresis.

A 30- μ g sample of protein was mixed with loading buffer (125 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.5% bromophenol blue) and boiled for 10 min. The samples were loaded onto an 8% SDS-polyacrylamide gel and separated at 0.04 A. The proteins were transferred to a nitrocellulose membrane at 0.07 A overnight. The membrane with the proteins was blocked (5 mM Tris, 0.12 mM NaCl, 0.05% Tween, and 5% non-fat dry milk) for 1 h, washed (0.05% TBS-Tween), and incubated with a rabbit polyclonal anti-iNOS (M-19) antibody diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After incubation, the membrane was washed and incubated with a secondary antibody, anti-rabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 1 h at room temperature. Lastly, the membrane was washed, and the proteins were detected using a chemiluminescence system (ECL, Amersham, Little Chalfont, Buckinghamshire, UK). The band analysis was performed with Image LabTM software (Bio-Rad, Hercules, CA, USA). The densitometric analysis of the iNOS bands was normalized to β -actin.

2.4.4 IL-6 assay

J774A.1 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well. The cells were pre-treated with different concentrations of blackberry extract for 24 h. After the treatment, the cells were washed, and each well was filled with fresh medium and a mixture of LPS (10 ng/mL) and murine IFN γ (10 U/mL). After a 24 h incubation with LPS+IFN γ , the supernatants were collected, and the IL-6 concentration was measured using a murine IL-6 EIA kit (eBioscience, Montrouge, France) following the manufacturer's instructions.

2.5 *In vivo* model of diabetic rats

2.5.1 Animals

The protocol described in this manuscript was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 35-11).

Male Sprague-Dawley rats weighing $200 \text{ g} \pm 20 \text{ g}$ were used in this study. Rats were acclimatized to laboratory conditions for 1 week before diabetes induction. They were fed *ad libitum* with water and regular laboratory pellet diet (22% crude protein, 5.0 crude fat, 4.5% crude fiber, 2.5% minerals and 3300 kcal/kg).

2.5.2 Induction of experimental diabetes

Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, Saint-Louis, MO, US) in fasted rats at a dose of 60 mg/kg body weight, which is a dose commonly used in previous experiments (Pushparaj *et al.*, 2000; Jung *et al.*, 2012; Yin *et al.*, 2010). STZ was dissolved in cold citrate buffer (0.1 M, pH=4.0) immediately before use. After 72 h, rats with fasting blood glucose of 250 mg/dl or more were used for the study and the animals that did not reach the glucose concentrations required were sacrificed. Non-diabetic controls received a cold citrate buffer injection. Treatment with the blackberry juice was started on the third day after STZ injection and continued until day 40.

2.5.3 Experimental design

Rats were divided into 5 groups, with 6 to 8 animals on each group. All groups received regular laboratory pellet diet *ad libitum*. Some groups were given water to drink and others a blackberry beverage, in both cases *ad libitum*. The distribution of the groups was as follows:

Group 1 -STZ-H₂O (n=8): STZ-induced diabetic control rats which were given water to drink.

Group 2 -STZ-BB25% (n=8): STZ-induced diabetic rats which were given a blackberry beverage diluted to 25% with water.

Group 3 -STZ-BB12.5% (n=8): STZ-induced diabetic rats which were given a blackberry beverage diluted to 12.5% with water.

Group 4 -Control-Water (n=6): normal control rats which were given water to drink.

Group 5 -Control-BB 25% (n=6): normal rats which were given a blackberry beverage diluted to 25% with water.

All groups were maintained for 40 days with the same treatment. Liquid and food intake was monitored daily and body weight was monitored weekly. The fasting glucose level was measured weekly by using glucose reagent strips (Accu chek®, Roche diagnostics, Indianapolis, USA) on blood samples obtained from tail. On day 41, after 12 h of fasting,

all rats were sacrificed by decapitation. Blood was collected for biochemical analysis and some portions of liver and kidney were taken out, washed in ice-cold PBS, homogenized and frozen for further analysis. The experimental design described before is illustrated in the Figure 2.3.

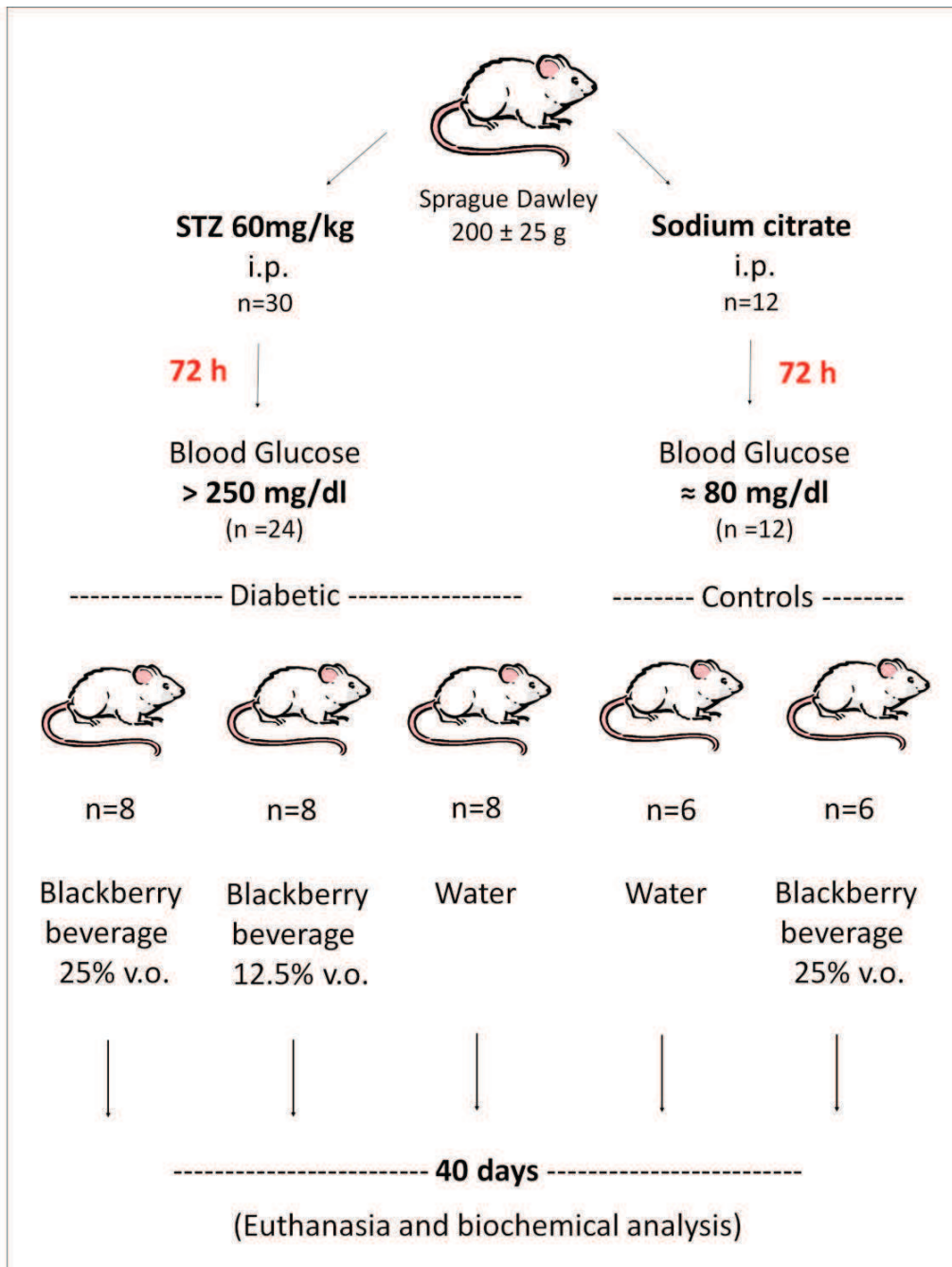


Figure 2.3 Experimental design for an *in vivo* model of diabetic rats treated with a blackberry beverage

2.5.4 Biochemical analysis

Blood chemistry

The estimation of serum glucose, triglycerides, cholesterol, urea, creatinine, uric acid, AST and ALT was done in ROCHE Cobas c501 chemistry analyzer, by using ROCHE kits.

Plasma antioxidant capacity (PAC)

Antioxidant capacity of plasma samples was assessed on the basis of the DPPH free radical-scavenging activity by a modified method of Janaszewska (Janaszewska & Bartosz, 2002). Plasma samples were treated with perchloric acid (3% final concentration) to precipitate the proteins. Samples were centrifuged at 13000 x g for 15 min at 4 °C. The supernatant was recovered, diluted 1:10 in PBS (80µl) and incubated with 500 µl of DPPH 0.1 mM for 20 min. Finally the absorbance was measured at 517 nm and results expressed % of scavenging activity with respect to the control with PBS instead of plasma.

Lipid peroxidation quantification in tissues

Lipid peroxidation in liver, kidney and plasma was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS), as it was described before. For liver and kidney tissue, the sample of each rat was homogenized in phosphate-buffered saline (PBS) using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 x g for 15 min to reduce the suspended solids and then the MDA quantifications were performed with 0.25 mL of the supernatant. The results were expressed in terms of malondialdehyde (MDA) content, µmol of MDA/g of tissue. For plasma, the homogenization was omitted and results are expressed as µmol of MDA/ml of plasma.

Catalase activity

The catalase activity was determined in liver and kidney homogenates following the Aebi method (Aebi, 1984). Briefly, 995 µl of H₂O₂ (10 mM) was mixed with 5 µl of tissue homogenate (20%) and the decomposition of H₂O₂ was followed by monitoring the absorption at 240 nm. The activity was calculated by using a molar absorption coefficient and the enzyme activity was defined as nmol of dissipating H₂O₂ in 30 s per mg of protein.

2.6 Statistical analysis

The results from each experiment represent the means \pm standard error. An analysis of variance (ANOVA) followed by a post hoc test was used to compare differences in biological activities between the digested samples or between the pasteurized samples. A p-value less than 0.05 was considered significant.

Similarly, for the *in vivo* experiment data are expressed in mean \pm SE for each group. The comparison between groups was done using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test. Comparisons with p values < 0.05 were considered to be statistically significant.

Chapter 3: Results

The results of this work are organized in four scientific articles (1 published, 1 submitted and 2 in preparation) that correspond to different aspects to fulfill the general objective of this study. The objective of the first article is to evaluate *in vitro* biological activities (antioxidant and anti-inflammatory) of blackberry species cultivated in Costa Rica, *Rubus adenotrichos*. This article is already published in the Journal of Agricultural and Food Chemistry, 2013, 61: 5798-5804. The objective of the second article of this chapter was to evaluate the impact of thermal treatments (pasteurization) in the antioxidant activities of blackberry polyphenols, for this two different thermal conditions were studied and the antioxidant capacity evaluated through different *in vitro* assays. This article has been already submitted to Food Research International. The third article analyses samples representing the main stages of blackberry digestion and compared the antioxidant capacity of the digested samples through different *in vitro* assays. Finally the fourth article describe an *in vivo* study of diabetic rats supplemented with a blackberry beverage where some biochemical parameters and antioxidant status have been assessed to evaluate the potential of blackberry as a dietary complement to the management of diabetes.

3.1 Antioxidant and anti-inflammatory *in vitro* activities of phenolic compounds from tropical highland blackberry (*Rubus adenotrichos*)

*Activités antioxydantes et anti-inflammatoires in vitro des composés polyphénoliques de la mûre tropicale de montagne (*Rubus adenotrichos*)*

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Article published in: / Article publié dans:

Journal of Agricultural and Food Chemistry, 2013, 61, 5798-5804

ABSTRACT

This study evaluates the antioxidant and anti-inflammatory activities in a polyphenol extract from blackberries. The antioxidant activity measured with ORAC was higher for the blackberry extract (4.34 ± 0.14 mmol TE/g) than for quercetin and ellagic acid. The blackberry phenolic compounds protected liposomes and liver homogenates against lipid peroxidation; in both models, the antioxidant activity ($IC_{50} = 7.0 \pm 0.5$ and 20.3 ± 4.2 $\mu\text{g/mL}$, respectively) was greater than that found with trolox. The extract inhibited superoxide production by NADPH oxidase in THP-1 cells and nitrite production in J774A.1 cells stimulated with LPS+IFN γ , with nitrite production decreasing after 4 h of incubation with the extract, mainly through a strong scavenging activity. However, 24 h of treatment reduced the amount of nitrites ($IC_{50} = 45.6 \pm 1.2$ $\mu\text{g/mL}$) because of a down-regulation of iNOS protein expression, as demonstrated by western blotting. The inhibitory activities found in blackberry phenols suggest a potential beneficial effect against oxidative stress and inflammatory processes.

KEYWORDS

Blackberry, *Rubus adenotrichos*, antioxidant activity, lipid peroxidation, anion superoxide, iNOS, macrophages cell lines

3.1.1 Introduction

Functional foods have received attention from consumers due to increasing evidence regarding the relationship between diet and health (Massaro *et al.*, 2010; Iqbal *et al.*, 2008). Berries have been proposed as fruits that can potentially improve human health due to their high phenolic compound content. These molecules strengthen the antioxidant defenses of the body and reduce reactive oxygen species, molecules that are associated with such pathological processes as cardiovascular diseases, inflammation, and diabetes. (Paredes-López *et al.*, 2010; Battino *et al.*, 2009).

Different biological properties have been reported for the phenolic compounds in berries, and their antioxidant capacities and radical-scavenging activities have been widely demonstrated using *in vitro* techniques and *in vivo* models (Sariburun *et al.*, 2010) (Jiao & Wang, 2000; Hassimotto *et al.*, 2008). The anti-proliferative and anti-cancer activities of these polyphenols have been documented in human oral, breast, colon, cervical, and prostate cancer cell lines (Seeram *et al.*, 2006; Olsson *et al.*, 2004; McDougall *et al.*, 2008). Polyphenols have also been reported to attenuate some inflammation intermediates, including nitric oxide, NF- κ B, and TNF α (Pergola *et al.*, 2006) (Meng *et al.*, 2008).

Moreover, some clinical trials have demonstrated that berry consumption could mitigate the oxidative stressors involved in atherogenesis and cardiovascular diseases (Burton-Freeman *et al.*, 2010; Basu *et al.*, 2009). For example, berry consumption has been associated with decreases in LDL oxidation, lipid peroxidation, serum glucose, and total cholesterol levels and an increase in HDL cholesterol. The mechanisms suggested include the upregulation of endothelial nitric oxide synthase, a decrease in oxidative stress and in the activity of carbohydrate digestive enzymes, and the inhibition of inflammatory gene expression and foam cell formation (Basu *et al.*, 2010).

The major biologically active compounds in berry fruits include such polyphenols as anthocyanins, phenolic acids, tannins (gallo- and ellagitannins), flavonols, favanols, carotenoids, and vitamin C. (Häkkinen *et al.*, 1999; Szajdek & Borowska, 2008). The main polyphenols in blackberry *Rubus adenotrichos* are cyanidin 3- glucoside, cyanidin-3- malonyl glucoside, lambertianin C, and sanguin H-6 (Mertz *et al.*, 2007).

The *Rubus* genus is cultivated worldwide. However, the genotype, species, environment, maturity stage, and cultivation conditions influence the content of anthocyanins and

hydrolyzable tannin compounds in the berries. (Cuevas-Rodriguez *et al.*, 2010), (Acosta-Montoya *et al.*, 2010). For this reason, the objective of this research was to characterize the health-promoting properties of the major blackberry variety consumed in Costa Rica: *Rubus adenotrichos* cv. 'vino'.

3.1.2 Materials and Methods

3.1.2.a Chemicals

All the solvents for HPLC and polyphenol purification were obtained from JT Baker (Griesheim, Germany). Amberlite XAD-7, 2,2 diphenyl-1-picrylhydrazyl (DPPH), 2,2-azobis-2-methyl-propion-amidine-dihydrochloride (AAPH), tert-butyl hydroperoxide (TBHP), thiobarbituric acid (TBA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were acquired from Sigma Aldrich (St. Louis, MO, USA). The standards ellagic acid and quercetin were obtained from Fluka (Buchs, Switzerland), and cyanidin-3-glucoside was obtained from Extrasynthese (Lyon, France). For cell culturing, RPMI 1640 with glutamax, fetal bovine serum, streptomycin, penicillin, and human IFN γ were purchased from Life Technologies (St. Aubin, France). LPS (*Escherichia coli*, serotype 055:B5), retinoic acid, 1,25 dihydroxycholecalciferol, phorbol 12-myristate 13-acetate (PMA), and lucigenin were purchased from Sigma Aldrich (Lyon, France). Recombinant murine IFN γ was purchased from Genzyme (Le Perray en Yvelines, France). For the Griess reagent, sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine dihydrochloride, and sodium nitrite were acquired from Merck (Darmstadt, Germany).

3.1.2.b Blackberry sample

This study used fully ripe blackberries (*Rubus adenotrichos* Schltld. cv. 'vino') harvested in Costa Rica (Cartago). The blackberries were freeze-dried, packaged in metallic bags, and stored at -20 °C until used for extract preparation. The polyphenol purification was according to Mertz *et al.* (Mertz *et al.*, 2007) Briefly, 35 g of blackberry powder was extracted twice for 15 min with acetone/water/formic acid (70:30:2). The extract was filtered and concentrated under vacuum to remove the acetone (40 °C). The aqueous layer was mixed with ethyl acetate (2:1), separated, and loaded onto an Amberlite XAD-7 column (150 mm x 20 mm) packed in water. The column was washed with water to remove the sugars, and the phenolic compounds were eluted with methanol/water (80:20).

The polyphenols were concentrated under vacuum and freeze-dried. The sample was stored at -20 °C for further analysis.

3.1.2.c HPLC analysis

The polyphenol extract was analyzed by HPLC for anthocyanins and ellagitannins following the protocols described by Mertz *et al.* (Mertz *et al.*, 2007) and Acosta-Montoya *et al.* (Acosta-Montoya *et al.*, 2010) Briefly, the HPLC quantitative analysis was performed with a Dionex liquid chromatograph system equipped with a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) and an endcapped reverse-phase Lichrospher ODS-2 column (250 mm x 4.6 mm i.d., 5 µm) (Interchim, Montluçon, France). Mertz *et al.* (Mertz *et al.*, 2007) previously identified sanguin H6, lambertianin C, and cyanidin-malonyl-glucoside following the same HPLC procedure using an additional hyphenation of the diode array detector (DAD) to an electrospray Ion Trap Mass spectrometry detector (ESI-TRAP-MS/MS). As the same method was followed in this paper, a tentative identification of the phenolic compounds was performed based on the work of Mertz *et al.* (Mertz *et al.*, 2007) Quantification of the polyphenols in the extract was performed using calibration curves established with standards of ellagic acid for ellagitannins and cyanidin-3-glucoside for anthocyanins.

3.1.2.d Antioxidant assays

DPPH radical-scavenging activity

The radical-scavenging activity of the blackberry extract was evaluated by assessing the direct DPPH-scavenging activity in the extract. (Kim *et al.*, 2004) DPPH (0.25 mM) was prepared in methanol, and 0.5 mL of this solution was incubated with 1 mL of various sample dilutions from 0.5 µg/ml to 10 µg/ml. The mixtures were incubated at room temperature in the dark for 30 min, and the absorbance of DPPH was measured at 517 nm. Sample blanks were prepared for each dilution. The percentage of the radical-scavenging activity of the extract was plotted against the sample concentration to calculate the IC₅₀, which is the amount of extract necessary to reach the 50% radical-scavenging activity. The samples were analyzed in triplicate. Commercial quercetin and ellagic acid were used as reference standards.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Ou *et al.* (Ou *et al.*, 2001) Fluorescein was used as a fluorescent probe, and oxidation was induced with AAPH. The assays were

performed using spectrofluorimeter equipment (Biotek Instruments, Winooski, VT, USA), and the ORAC values were expressed as mmol of trolox equivalents (mmol TE/g of extract). Commercial quercetin and ellagic acid were used as reference standards.

Nitric oxide-scavenging activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and rapidly converted into the stable product nitrite. The nitrite concentration was measured by the Griess reaction (Balakrishnan *et al.*, 2009).

SNP (5 mM) was mixed with different concentrations of blackberry extract and incubated for 60 min under direct light to enhance NO production. The Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H₃PO₄) was added, and the absorbance was measured at 540 nm in reference to the absorbance of standard solutions of sodium nitrite. Sample blanks were prepared for each experiment. NO-scavenging activity was calculated as the amount of extract necessary to reduce 50% of the NO generated by SNP (IC₅₀). The sample was analyzed in triplicate. Commercial quercetin and ellagic acid were used as reference standards.

Inhibition of lipid peroxidation in liposomes

Liposomes were prepared according to Pérez *et al.* (Pérez *et al.*, 2003). Briefly, 25 mg of commercial lecithin was dissolved in 2.15 mL of chloroform, and then 350 µL of methanol was added. This mixture was dried under a nitrogen atmosphere. The lecithin was resuspended in 4.5 mL of warm phosphate-buffered saline (PBS) and sonicated for 1 h at 4 °C to form liposomes.

To test the capacity of the extract to protect lipid peroxidation, oxidative stress was induced with AAPH. Various dilutions of blackberry extract (50 µL) was mixed with 0.45 mL of liposomes and 0.2 mL of AAPH (final concentration of 10 mM), and these solutions were incubated in the dark for 2 h at 37 °C. To determine the malondialdehyde (MDA) concentration, 0.2 mL of 5% TCA (trichloroacetic acid) and 1 mL of 0.75% TBA (thiobarbituric acid) were added, and the samples were heated at 96 °C for 30 min. After cooling, 0.25 mL of 3% SDS was added, and the mixture was centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The thiobarbituric acid reactive substances (TBARS) concentration was assessed using the molar absorption coefficient for malondialdehyde (MDA): $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$. The MDA concentrations were plotted against the sample concentration, and the results were expressed as the

amount of extract that inhibits 50% of lipid peroxidation (IC_{50}). The extract was tested in triplicate. Commercial trolox was used as a reference standard.

Inhibition of lipid peroxidation in liver homogenates

This procedure was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 19-06). Sprague-Dawley rats (220 g \pm 20 g), obtained from LEBi® (Laboratorio de ensayos biológicos, Universidad de Costa Rica), were anesthetized with CO₂ and sacrificed by decapitation. The liver tissue of each rat was obtained and homogenized in phosphate-buffered saline (PBS) using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 x g for 15 min to reduce the amount of suspended solids. Different concentrations of blackberry extract (75 μ L) were added to 0.75 mL of liver supernatant and incubated for 30 min at 37 °C. Oxidative stress was then induced with TBHP (tert-butyl hydroperoxide) at a final concentration of 1.7 mM and incubated for 1 h at 37 °C, and the TBARS were measured as the end product of lipid peroxidation.

TBARS were assayed according to Mihara & Uchiyama (Uchiyama & Mihara, 1978). Briefly, 0.25 mL of liver homogenate, 0.25 mL of 35% TCA, and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4) were mixed and incubated for 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added, and the mixture was heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, and the sample was mixed and centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated as described previously, and the results were expressed as nmol MDA/g liver tissue. The MDA concentrations were plotted against the sample concentration to calculate the IC_{50} .

The assay was performed using liver tissue from 5 rats. To establish the basal level of lipid peroxidation, the MDA levels without TBPH were assessed in each liver homogenate. Sample blanks were prepared for each experiment, and the extract was tested in triplicate. Commercial trolox was used as a reference standard.

Inhibition of superoxide anion production

The human promonocytic cell line THP-1 was obtained from American Type Culture Collection (ATCC, Cell No. TIB-202, Rockville, MD, USA). The cells were grown in RPMI 1640-glutamax medium red phenol containing 10% fetal calf serum, 10⁵ U/L

penicillin/streptomycin, and 10^{-4} g/l fungizon. To differentiate promonocytes, 3.5×10^5 cells/mL were incubated with retinoic acid (10^{-6} M), 1,25 dihydroxycholecalciferol (10^{-7} M), and IFN- γ (10 U/mL). The differentiated monocytes became adherent cells after 72 h.

In the first step, the cells were treated with different concentrations of the extract for 24 h to calculate the IC₅₀ of the blackberry phenols. The medium and extract were then removed, and the cells were washed and collected by scraping into RPMI 1640 medium without red phenol. A cell suspension of 1×10^6 cells/mL was prepared and incubated with lucigenin (10^{-4} M) for 30 min, and the monocyte cell suspension was stimulated with PMA (10^{-7} M) to induce O₂⁻ production. The O₂⁻ reduced the lucigenin bioluminescence probe, and the luminescence generated was immediately recorded for 1 h at 37 °C using a Victor Wallac Luminometer (WALLAC Co, Turku, Finland). After the luminescence assay, the cells were dissolved in 1 M NaOH to determine the protein concentration (Dc Protein Assay, Bio-Rad, Hercules, CA, USA). The luminescence intensity was normalized to the amount of protein present. The response of PMA-stimulated cells without blackberry extract was used as a control and was considered equal to 100% of the superoxide anion production.

In the second step, to determine the kinetics of O₂⁻ inhibition activity in the blackberry extract, the same protocol was performed using a unique concentration of 80 μ g/mL (defined as the IC₅₀) that was incubated for 15 min, 30 min, 1 h, 4 h, 8 h, and 24 h. Each experiment was performed in triplicate.

3.1.2.e Anti-inflammatory assays

Cell viability

The murine macrophage cell line J774A.1 was obtained from ATCC. The cells were grown in RPMI 1640-glutamax medium containing 5% inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (20 μ g/mL).

To test the cytotoxicity, 10^5 cells/well were seeded in a 96-well plate and incubated for 20 h with different concentrations of the blackberry extract. The cells were washed, and each well was filled with 200 μ L of medium and 20 μ L of a tetrazolium salt, MTS (3-(4,5 dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium), mixed with an electron-coupling reagent, PMS (phenazine methosulfate), diluted in PBS. The plate was incubated for 4 h, and the absorbance was measured at 550 nm.

The percentage of viable cells was calculated using the absorbance of the control cells without extract as 100%. The extract was tested in triplicate.

Measurement of nitric oxide

J774A.1 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well and were pre-treated with different concentrations of the blackberry extract for 4 h or 24 h. After the treatment, the cells were stimulated for 24 h with a mixture of LPS (10 ng/mL) and murine IFN γ (10 U/mL). The supernatants were collected, and the concentration of nitrite, which reflects the intracellular NO synthase activity, was determined using the Griess reagent, as described above. In some experiments, the extract was eliminated (“Wash”) before stimulation with LPS+IFN γ , and in others, it was not (“No-wash”). Each experiment was performed in triplicate.

Western blotting analysis

To study the effect of the blackberry extract on iNOS expression, J774A.1 macrophages were pre-treated for 24 h with the blackberry extract; the cells were washed with medium and stimulated with LPS (10 ng/mL) and murine IFN γ (10 U/mL) for 10 h. The cells were then washed and incubated with a lysis buffer (1X PBS, 1% Triton X-100, and protease inhibitor cocktail, Roche Applied Science, Mannheim, Germany) for 30 min on ice. The lysed cells were collected by scraping and then centrifuged at 10000 x g for 2 min. The supernatant was collected, and the proteins were used for electrophoresis.

A 30- μ g sample of protein was mixed with loading buffer (125 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.5% bromophenol blue) and boiled for 10 min. The samples were loaded onto an 8% SDS-polyacrylamide gel and separated at 0.04 A. The proteins were transferred to a nitrocellulose membrane at 0.07 A overnight. The membrane with the proteins was blocked (5 mM Tris, 0.12 mM NaCl, 0.05% Tween, and 5% non-fat dry milk) for 1 h, washed (0.05% TBS-Tween), and incubated with a rabbit polyclonal anti-iNOS (M-19) antibody diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After the incubation, the membrane was washed and incubated with a secondary antibody, anti-rabbit IgG conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 1 h at room temperature. Lastly, the membrane was washed, and the proteins were detected using a chemiluminescence system (ECL, Amersham, Little Chalfont, Buckinghamshire, UK).

The band analysis was performed with Image Lab™ software (Bio-Rad, Hercules, CA, USA). The densitometric analysis of the iNOS bands was normalized to β -actin.

IL-6 Assay

J774A.1 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well. The cells were pre-treated with different concentrations of blackberry extract for 24 h. After the treatment, the cells were washed, and each well was filled with fresh medium and a mixture of LPS (10 ng/mL) and murine IFN γ (10 U/mL). After a 24 h incubation with LPS+IFN γ , the supernatants were collected, and the IL-6 concentration was measured using a murine IL-6 EIA kit (eBioscience, Montrouge, France) following the manufacturer's instructions.

3.1.2.f Statistical analysis

The results from each experiment represent the means \pm standard error. An analysis of variance (ANOVA) followed by a Tukey post hoc test was used to compare the differences between the antioxidant capacity of blackberry extract, quercetin and ellagic acid. As well, an ANOVA was used to compare between the controls and treatments of the blackberry extract in the lipid peroxidation and anti-inflammatory methods. A p-value less than 0.05 was considered significant.

3.1.3 Results and Discussion

The HPLC chromatogram of the blackberry extract shown in Figure 3.1 was similar to that published previously by Mertz *et al.* (Mertz *et al.*, 2007). These authors performed a tentative identification by HPLC-DAD/ESI-TRAP-MS of the same fruit variety used in this study, and the blackberry samples used in both studies were collected at the same location. Moreover, the present study followed an HPLC protocol that was identical to that of Mertz *et al.*, and the retention times coincide. Correspondingly, our tentative phenolic profile for the blackberry extract includes the anthocyanins cyanidin 3-glucoside and cyanidin-3-malonyl glucoside and the ellagitannins lambertianin C and sanguiin H-6 as the main components. According to the literature, this polyphenol profile is common because cyanidin 3-glucoside is the most widespread anthocyanin in nature and is commonly found in colored fruits (He & Giusti, 2010); sanguiin H-6 is also the most common ellagitannin found in berries (Bakkalbasi, et al., 2009). These types of polyphenols have been described in the literature as key contributors to antioxidant

capacity. Anthocyanidins, such as cyanidin-3-glucoside, are known antioxidants due to their hydroxyl substituent in the structure of the B ring (Seeram & Nair, 2002). Ellagitannins, due to their high molecular weight structures, exhibit an important ability by donating a hydrogen atom and scavenging unpaired electrons (Bakkalbasi *et al.*, 2009).

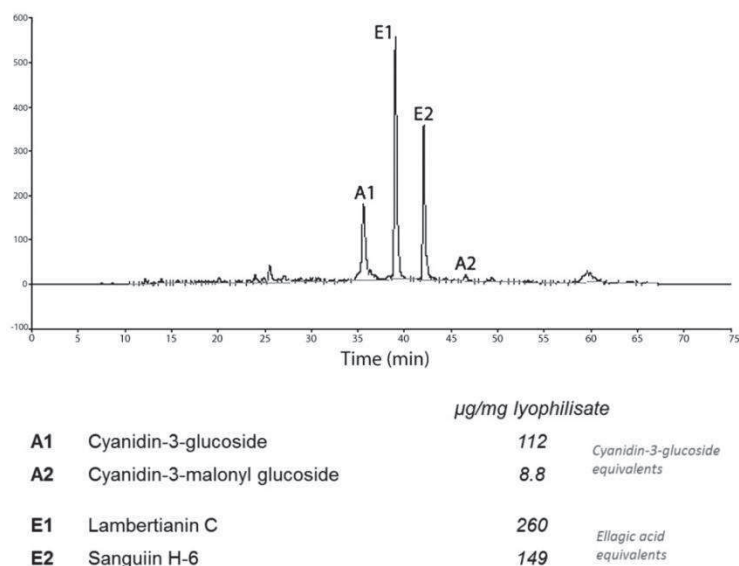


Figure 3.1 HPLC chromatogram of the blackberry polyphenol extract.
(Corresponding putative identification of peaks A1, A2, E1, E2 previously published by Mertz *et al.* (Mertz, *et al.*, 2007))

Table 3.1 shows the free radical-scavenging capacity of blackberry phenols assessed by different *in vitro* methods; the activities were compared to a reference radical scavenger flavonoid, quercetin and to the ellagitannins subunit, ellagic acid. The scavenging activity in the blackberry extract against the free radical DPPH was significantly lower than that of quercetin and ellagic acid. The NO-scavenging activity of the blackberry extract was significantly higher than ellagic acid and lower than quercetin; however, in the case of peroxy radicals measured by ORAC, the antioxidant activity in the extract was significantly higher than that of quercetin and ellagic acid. The differences in the antioxidant capacity evaluated by these assays can be explained by the fact that each method evaluates the scavenging activity against diverse radicals and by different mechanisms (Huang *et al.*, 2005; Niki 2010; Niki, 2011). One of the factors that determines the radical-scavenging capacity is the chemical structure of the free radical used in each antioxidant assay method: the antioxidant performance will differ because the kinetic and stoichiometric factors related to each free radical are different (Niki, 2010;

Niki, 2011). The DPPH and NO assays use nitrogen radicals, whereas ORAC uses a peroxy radical. Additionally, the mechanisms are different: DPPH and NO-scavenging involve direct methods, whereas ORAC uses a competition method that involves a probe in addition to the free radical and antioxidant. In addition, the ORAC method uses a peroxy radical, which is more appropriate to evaluate antioxidants because it has a key role as a chain-carrying radical in lipid peroxidation (Niki, 2011).

Table 3.1 Free radical-scavenging capacity of blackberry phenol extract, quercetin and ellagic acid

	DPPH <i>IC</i> ₅₀ (µg/mL)	ORAC <i>mmol TE/g</i>	NO-scavenging <i>IC</i> ₅₀ (µg/mL)
Blackberry extract	2.57 ± 0.11 ^a	4.34 ± 0.14 ^a	24.5 ± 1.3 ^a
Quercetin	2.17 ± 0.08 ^b	2.07 ± 0.08 ^b	6.6 ± 0.8 ^b
Ellagic acid	2.22 ± 0.07 ^b	0.53 ± 0.02 ^c	298 ± 14 ^c

Each value is the mean ± SE of three replicate experiments. The means in columns followed by different letters differed significantly ($p < 0.05$).

Despite the fact that lipid peroxides (LPOs) exert physiological beneficial effects, there is ample evidence demonstrating that these molecules also provoke membrane damage and tissue injury and are associated with the progression of many diseases. As discussed by Niki (Niki, 2012), cellular enzymatic oxidation is a specific process, as opposed to the random oxidation of lipids by free radicals. Accordingly, lipid peroxidation is one of the most extensively researched processes induced by ROS; indeed, it is important to evaluate the role of antioxidants (Niki, 2009). In this study, the inhibition of lipid peroxidation was first assessed using artificial lecithin liposomes and then using rat liver homogenates. AAPH (in the liposome model) and TBHP (in the liver homogenate model) were used because of their ability to be metabolized into free radical intermediates that cross cellular membranes, leading to the production of highly reactive hydroxyl radicals that initiate lipid peroxidation (Niki, 2010; Hwang *et al.*, 2008).

Figure 3.2A shows that the blackberry phenols decreased the levels of lipid peroxidation in the liposomes in a dose-dependent manner; the concentration of phenols that was necessary to decrease 50% of the MDA concentration in the control liposomes (*IC*₅₀) was 7.0 ± 0.5 µg/mL. As shown in

Figure 3.2B, a similar inhibitory effect was observed for the rat liver homogenates, with an *IC*₅₀ of 20.3 ± 4.2 µg/mL. The *IC*₅₀ values in both assays were significantly ($p < 0.05$) better than that of trolox, a hydrosoluble artificial analog of vitamin E. The trolox *IC*₅₀

value in the liposomes was $13.3 \pm 0.9 \mu\text{g/mL}$, and the IC_{50} value was $43.1 \pm 0.3 \mu\text{g/mL}$ in the liver homogenates. Similar *in vitro* results were reported for other polyphenol-rich extracts, and some *in vivo* models also demonstrated that polyphenol intake could prevent lipid peroxidation (Seeram & Nair, 2002; Hwang *et al.*, 2008; Lee *et al.*, 2005; Dani *et al.*, 2008). Hwang *et al.* suggested that protection against lipid peroxidation is a consequence of free radical quenching (Hwang *et al.*, 2008).

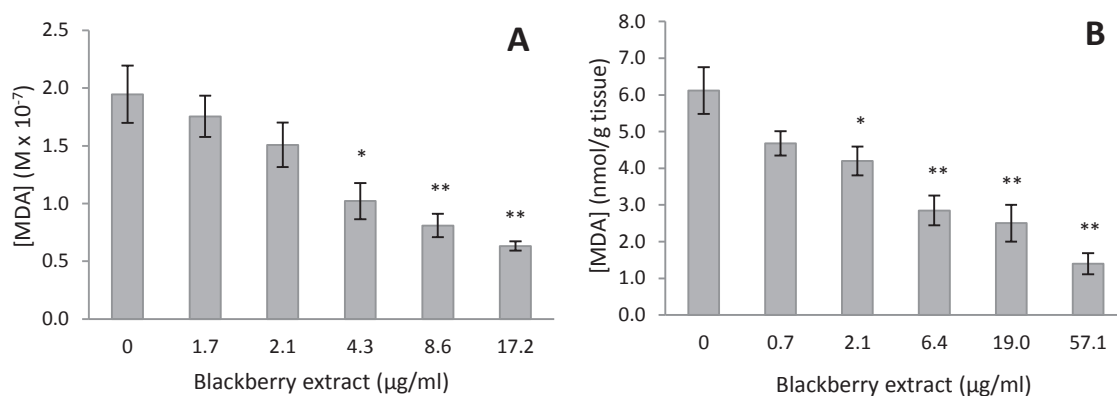


Figure 3.2 Inhibitory capacity of the blackberry extract against lipid peroxidation in liposomes (A) and in liver homogenates (B).

Each value is the mean \pm S.E. (three independent experiments). * $p < 0.05$, ** $p < 0.01$ compared to controls without blackberry treatment.

In addition to mediating damage from outside the cell, such as in lipid peroxidation, ROS also modify the redox status within cells, along with such intracellular ROS as nitric oxide and superoxides, and play important roles in the modulation of inflammation and in immune regulation (Guzik *et al.*, 2003). Figure 3.3A shows that the blackberry phenols significantly inhibited the superoxide anion production by NADPH oxidase ($p < 0.05$), from $50 \mu\text{g/mL}$ up to $150 \mu\text{g/mL}$ of extract, with an IC_{50} value at $80.3 \pm 4.1 \mu\text{g/mL}$. Figure 3.3B shows the kinetics of the superoxide inhibition activity in blackberry extract performed at the IC_{50} value ($80 \mu\text{g/mL}$). These results clearly show a significant inhibition ($p < 0.05$) of 35%, even after a short incubation time of 15 min. The maximum inhibition (76%) was observed after 8 h of incubation. Similar effects were reported in animal models supplemented with raspberry juice or with pure polyphenols (Suh *et al.*, 2011; Sutra *et al.*, 2008).

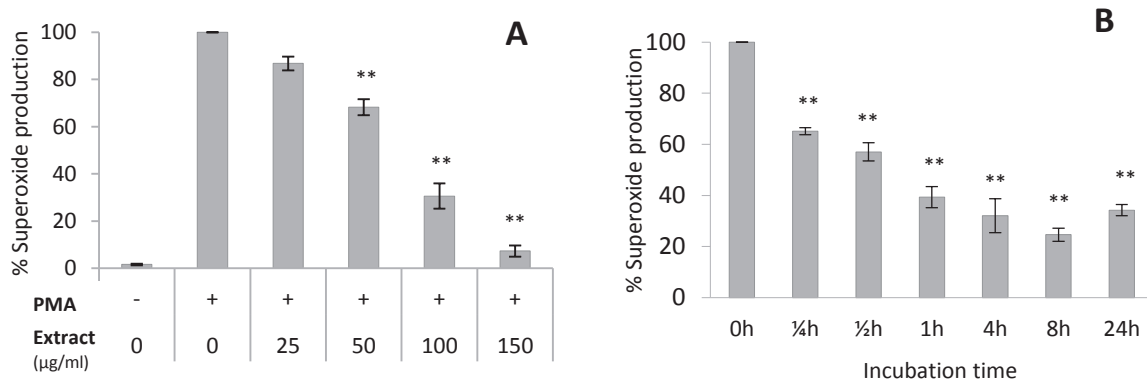


Figure 3.3 Inhibitory effect of the blackberry extract against the superoxide production induced in THP-1 cells.

Dose-effect assay (A) and kinetic assay (B). Each value is the mean \pm S.E. (four independent experiments). * $p < 0.05$, ** $p < 0.01$ compared to controls without blackberry treatment.

In addition to the inhibitory effect on superoxide production, the blackberry phenols inhibited nitrite production when used to pretreat macrophage cells later exposed to LPS+IFN γ . Figure 3.4A shows that 4 h of pre-treatment with blackberry phenols reduced NO production in a dose-dependent manner only when LPS+IFN γ was co-incubated with the extract (no-wash). If the extract was eliminated prior to the addition of LPS+IFN γ (wash), the inhibition of NO production was observed only at the highest concentration tested. The IC₅₀ values after 4 h of incubation were 24.4 ± 3.2 µg/mL and 99.1 ± 7.3 µg/mL under the no-wash and wash conditions, respectively. The different results suggests that the extract only exerts scavenging activity with a 4 h incubation, as was previously confirmed in the NO-scavenging assay.

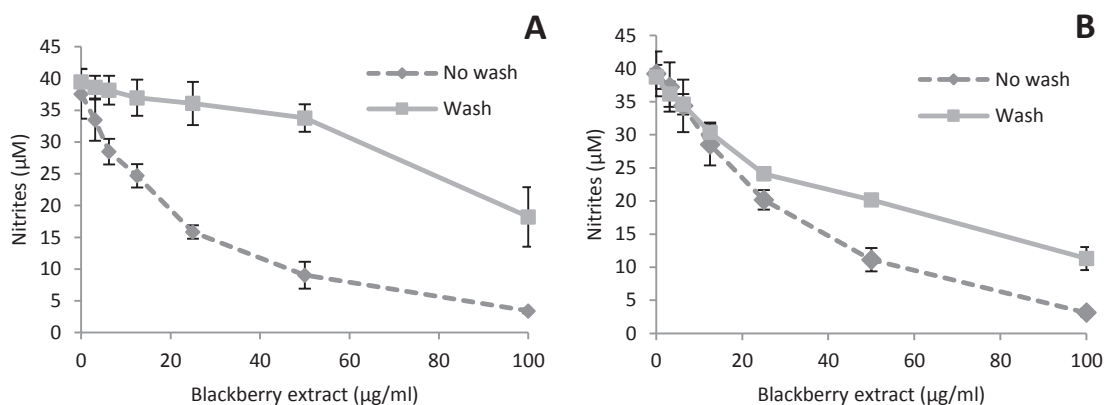


Figure 3.4 Effect of blackberry extract on LPS+IFN γ -induced nitrite production in J774 cells. Blackberry extract, 4 h pre-treatment (A), and blackberry extract, 24 h pre-treatment (B). The data are expressed as the means \pm SEM from three separate experiments performed in triplicate.

Figure 3.4B shows that a 24 h pre-incubation of the blackberry phenol with macrophage cells later stimulated with LPS+IFN γ decreased NO production both when the extract was incubated simultaneously with LPS+IFN γ and when it was eliminated before the addition of LPS+IFN γ . The IC₅₀ values after 24 h of incubation were 30.4 ± 3.7 $\mu\text{g/mL}$ and 45.6 ± 1.2 $\mu\text{g/mL}$ under the no-wash and wash conditions, respectively, suggesting that the extract regulates iNOS expression in cells because the inhibition was found even when the extract was removed. This regulation was confirmed by western blotting (Figure 3.5), which showed a concentration-dependent decrease in iNOS when the cells were incubated with the blackberry extract; the western blot results show reductions of 29, 40, 74, and 79% at 12.5, 25, 50, and 100 $\mu\text{g/mL}$ extract, respectively. Similar results have been shown in the extracts of other berries and with purified polyphenols (McDougall *et al.*, 2008; Wang & Mazza, 2002; Cuevas-Rodríguez *et al.*, 2010; Olszanecki *et al.*, 2002). The blackberry extract alone did not influence nitrite production and iNOS protein expression in the J774A.1 cells relative to the control cells (data not shown), and blackberry extract concentrations under 100 $\mu\text{g/mL}$ did not result in a loss of J774A.1 cell viability over 20 h of incubation, as assessed by an MTS/PMS assay (data not shown).

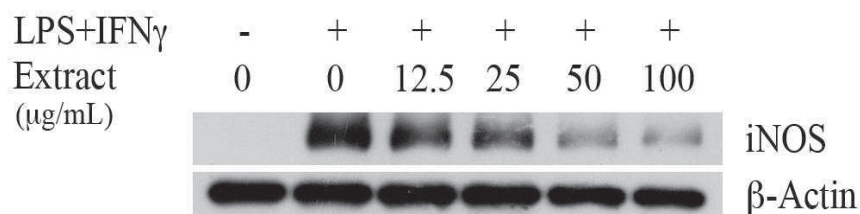


Figure 3.5 Western blot analysis of the effect of blackberry extract on LPS+IFN γ -induced iNOS expression in J774 cells.

Lysed cells were subjected to western blot using an antibody specific for iNOS. β -actin was used as a quality control.

The inhibitory effect of blackberry phenols on the production of superoxides and nitric oxide manifests in a decrease in the oxidative stress in the cell, thereby modifying the redox status of the signaling molecules and transcription factors involved in the progression of inflammation. Specifically, transcriptional regulatory factor NF- κ B, which is involved in iNOS gene expression, has been reported to be sensitive to ROS (Guzik *et al.*, 2003; Valko *et al.*, 2007; Hatanaka *et al.*, 1998). We suggest that the blackberry radical-scavenging activity demonstrated in this study could decrease the concentrations of different types of ROS; this would prevent, at least in part, the activation of NF- κ B,

thus provoking a decrease in iNOS expression, as was observed by western blotting and as has been described for other types of phenolic compounds (Rahman *et al.*, 2006).

Inflammation is a complex process that involves other cytokines in addition to NO, superoxides, and iNOS. For this reason, the capacity of blackberry extract to inhibit IL-6 production was assessed, and the results are shown in Figure 3.6. J774A.1 cells treated with the blackberry extract and then exposed to LPS+IFN γ demonstrated a capacity to inhibit IL-6 production. The treatment reduced IL-6 production in a concentration-dependent manner, with differences that were significant ($p < 0.05$) from the value obtained with the 50 $\mu\text{g/mL}$ concentration. Cho *et al.* reported similar results for the flavonoid quercetin, demonstrating that quercetin blocks the activation of NF- κ B and other mitogen-activated protein kinases that regulate cytokine secretion (Cho *et al.*, 2003). Further studies must be performed to evaluate the effect of the blackberry extract on other cytokines.

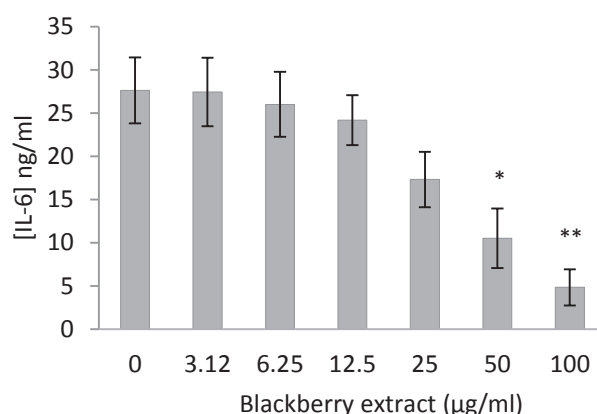


Figure 3.6 Effect of blackberry extract on LPS+IFN γ -induced IL-6 production in J774 cells. The data are expressed as the means \pm SEM from three separate experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ compared to controls without blackberry treatment.

This study demonstrated the potential influence of blackberry phenols on inflammatory processes through their inhibitory properties against superoxides, nitric oxide, and IL-6. However, this influence can be widespread because these molecules also mediate cytotoxic effects, such as DNA damage, LDL oxidation, tyrosine nitration, and the inhibition of mitochondrial respiration (Valko *et al.*, 2007; Guzik *et al.*, 2003).

More research is required to consider both the antioxidant capacity of the blackberry polyphenols and also some alternative mechanisms of action (Chiva-Blanch & Visioli, 2012). Furthermore, such aspects as polyphenol bioavailability, metabolism, tissue

distribution, and deconjugation by gut microbiota, as have been performed for other types of berries, need to be well characterized for blackberries (Chiva-Blanch & Visioli, 2012, Giamperi *et al.*, 2012). Nonetheless, the blackberry extract evaluated in this paper clearly exhibits a potential benefit with regard to its capacity to protect against oxidative stress and inflammatory processes. This protection is mainly due to the very potent antioxidant activities of the major molecules present in the extract. Some authors suggest that ROS might be upstream targets of polyphenols during pathological processes (Wang *et al.*, 2008). Therefore, the inclusion of this fruit in the daily diet could be beneficial, and more studies should be done to evaluate the addition of this fruit in the group of functional foods.

Acknowledgments

We thank Karine Portet and Ana Lorena Torres for their excellent technical support.

3.2 Flash-pasteurization of blackberry juice preserves polyphenol-dependent inhibition for lipid peroxidation and intracellular ROS

La flash-pasteurisation du jus de mûre permet de conserver une capacité d'inhibition élevée de la peroxydation lipidique évaluée par différentes méthodes

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Article submitted to: / Article soumis à:

Food Research International

Abstract

Emerging research has supported the health beneficial effects of berries. However, these fruits are commonly consumed as juice, and the juice-processing conditions could affect the stability of the bioactive compounds. The main objective of this study was to evaluate the effect of thermal treatments on the antioxidant capacity of blackberry polyphenols. Two pasteurized blackberry juices were prepared using two thermal protocols: 75 °C for 15 s (JP75) and 92 °C for 10 s (JP92). The polyphenol analysis showed that for the pasteurized juices, JP75 and JP92, the anthocyanin concentrations decreased significantly by 2% and 7%, respectively, compared to the non-pasteurized juice (NPJ), whereas ellagitannins were not significantly affected. The evaluation of the DPPH scavenging capacity showed a significant decrease of 26% and 27% for the JP75 and JP92 juices, respectively, and, for the NO scavenging capacity, the reduction in the activity was 15% for JP75 and 16% for JP92. There were no significant differences observed for the peroxidation inhibitory capacity of the pasteurized juices for any of the oxidation substrates tested: liposomes, liver homogenates and human erythrocytes. Furthermore, the intracellular antioxidant capacity showed no significant differences due to thermal treatments. The concentration of phenols that were necessary to scavenge 50% of the radical oxygen species (ROS) production was $204 \pm 9 \mu\text{g/mL}$ for NPJ, $219 \pm 10 \mu\text{g/mL}$ for JP75 and $220 \pm 9 \mu\text{g/mL}$ for JP92. This study revealed that, with the exception of the decrease in anthocyanins, the pasteurized blackberry juices maintained their biological properties related to lipid peroxidation inhibitory activity and their capacity to scavenge intracellular ROS.

KEYWORDS

Blackberry juice, *Rubus adenotrichos*, antioxidant activity, lipid peroxidation, pasteurization

3.2.1 Introduction

Emerging research has supported the health beneficial effects of berries. Different trials have demonstrated that berry consumption contributes to protect against cardiovascular disease (Basu *et al.*, 2010) and metabolic syndrome (Basu & Lyons, 2012). Specifically, berry consumption lowers the oxidation of LDL and is associated with lowering blood pressure and improving insulin resistance (Basu & Lyons, 2012). *In vitro* and *in vivo* studies revealed that berry compounds have a potential for cancer prevention through the regulation of cancer cell proliferation, apoptosis and tumor angiogenesis signaling pathways (Seeram, 2008). In addition, anti-inflammatory effects have been described for strawberries, mulberries and blackberries, mainly through immuno-modulatory mechanisms by the inhibition of cytokines (Liu & Lin, 2013; Cuevas-Rodríguez *et al.*, 2010).

Blackberries are one of the most consumed tropical berries. This type of berry, which is from the *Rubus* spp. group, has a high level of polyphenolic compounds that contributes to its high antioxidant capacity. The total phenolics in blackberries range from 114 to 1056 mg/100 g FW and this value is higher than other kinds of berries, as cranberries (120-315 mg/100 g FW) or strawberries (43-443 mg/100 g FW) (Szajdek *et al.*, 2008; Howard & Hager, 2007). The main polyphenols that are present in the blackberries are the anthocyanins and ellagitannins (Kaume *et al.*, 2012).

Tropical highland blackberry (*Rubus adenotrichos*) is commonly processed as a juice or juice concentrate. In major producing countries (Colombia, Ecuador and Costa Rica), blackberry-based beverages are regularly produced by local industries (Gancel *et al.*, 2011). The juice-processing conditions, such as enzymatic reactions, thermal treatments or microfiltration steps, can affect the bioactive compounds and their subsequent antioxidant activity. Thermal decomposition is one of the main causes of bioactive compound loss (Rawson *et al.*, 2011; D'Archivio *et al.*, 2010). Studies evaluating the effect of thermal treatments on fruit phenolic compounds have been reported for mango (Santhirasegaram *et al.*, 2013), grapefruit (Igual *et al.*, 2010), peach (Oliveira *et al.*, 2012), orange (Lo Scalzo *et al.*, 2004) and berries (Aracibia-Avila *et al.*, 2012; Piasek *et al.*, 2011; Gancel *et al.*, 2011; Zhang *et al.*, 2012). However, these studies focused on changes in the phenolic composition of the fruits after thermal treatments rather than on the changes in their potential beneficial activity. The impact on biological activities have also been evaluated for other industrial process, for example, a study on blackberries

demonstrated that despite a reduction of polyphenol concentration by the microfiltration process, the capacity to inhibit lipid peroxidation was not affected (Azofeifa *et al.*, 2011).

The main objective of this study was to evaluate the effect of thermal treatments on the antioxidant capacity of blackberry polyphenols. We evaluated the antioxidant capacities for free radical-scavenging and inhibiting lipid peroxidation and intracellular reactive oxygen species (ROS). We believe that these capacities are close indicators of biological properties.

3.2.2 Materials and Methods

3.2.2.a Chemicals

All organic solvents were obtained from JT Baker (Griesheim, Germany). The HPLC standard ellagic acid was obtained from Fluka (Buchs, Switzerland), and cyanidin-3-glucoside was obtained from Extrasynthese (Lyon, France). Amberlite XAD-7, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azobis-2-methyl-propion-amidine-dihydrochloride (AAPH), *tert*-butyl hydroperoxide (TBHP), thiobarbituric acid (TBA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,7-dichlorodihydrofluorescein diacetate (DCFDA), quercetin, gallic acid and fluorescein were acquired from Sigma Aldrich (St. Louis, MO, USA). For cell culture, EMEM medium, containing L-glutamine, fetal bovine serum, streptomycin, penicillin and trypsin, were purchased from Sigma Aldrich (St. Louis, MO, USA). For the Griess reagent, sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine dihydrochloride and sodium nitrite were acquired from Merck (Darmstadt, Germany).

3.2.2.b Blackberry juice preparation and pasteurization

Full-ripe blackberries (*Rubus adenotrichos* Schltdl. cv. 'vino') were collected from different farms in the province of Cartago, Costa Rica (altitude 1864 – 2517 m, latitude 09° 39' 57.1"N - 09°44'40.3"N, longitude 83°53'32.1"W - 84°00'06.3"W). A microfiltrated juice was prepared as previously indicated (Vaillant *et al.*, 2008). Blackberries were pressed, and the juice was treated with a commercial enzymatic preparation, Ultrazym (Novozymes, Bagsvaerd, Denmark), for 1 h at 35 °C with constant agitation to reduce viscosity and facilitate microfiltration. Cross-flow was performed in a tubular ceramic membrane (Membralox® 1 P19-40, Pall Exekia, Bazet, France) with an average pore size

diameter of 0.2 μm . Microfiltration allowed for the removal of suspended solids that could lead bias.

Two pasteurized juices were prepared from the microfiltrated juice using a pilot-scale, UHT processing system, model FT74X (Armfield Inc., Ringwood, UK). One of the juices was pasteurized at 75 °C for 15 s, and the other was processed at 92 °C for 10 s. As a result, three samples were obtained: one non-pasteurized juice and two pasteurized. The selected temperatures for pasteurization represent mid and high pasteurization conditions commonly used for fruit juices in previous publications (Santhirasegaram *et al.*, 2013; Hager *et al.*, 2010; Mena *et al.*, 2013; Zhang *et al.*, 2012).

3.2.2.c Polyphenol purification

Each of the three blackberry juices samples were subjected to polyphenol purification using an Amberlite XAD-7 column (150 mm x 20 mm) packed in water. Five hundred milliliters of each juice was loaded onto the Amberlite column and washed with 2 L water to remove sugars. Finally, the phenolic compounds were eluted with 200 ml methanol/water (80:20), concentrated under a vacuum (40 °C) and freeze-dried (-40°C, 133×10^{-3} mBar for 48h). The three lyophilized polyphenol extracts: non-pasteurized juice (**NPJ**), juice pasteurized at 75 °C (**JP75**) and juice pasteurized at 92 °C (**JP92**), were stored at -30 °C until further chemical and antioxidant analysis.

3.2.2.d Analytical characterization of the blackberry juices

Total polyphenol content

The total phenolic content of the polyphenol extracts was determined using the Folin-Ciocalteu assay that was modified by George *et al.* (George *et al.*, 2005). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of polyphenol extract. Samples were analyzed in triplicate.

Phenolic compounds by HPLC

The polyphenol extracts were analyzed by HPLC for anthocyanins and ellagitannins following the protocol described by Mertz *et al.* (Mertz *et al.*, 2007) and Gancel *et al.* (Gancel *et al.*, 2011). Briefly, the HPLC quantitative analysis was performed using a Dionex liquid chromatograph system that was equipped with a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) and an endcapped, reversed-

phase Lichrospher ODS-2 column (250 mm x 4.6 mm i.d., 5 µm) (Interchim, Montluçon, France). HPLC solvents consisted of 2% aqueous formic acid (Solvent A) and acetonitrile/water/formic acid (80:18:2, v/v/v; solvent B). The chromatographic conditions were: 30 °C, 0.3 mL/min flow rate, 20 µL injection volume and the gradient used for separation was: 0 min, 5% B; 0-20 min linear gradient from 5% to 25% B; 20-25min linear gradient from 25% to 100% B; 25-30 min linear gradient from 100% to 5% B; 30-35 min, 5% B. Previously, Mertz *et al.* (Mertz, et al., 2007) identified sanguin H6, lambertianin C and cyanidin glucosides following the same HPLC procedure by including an additional hyphenation of the diode array detector (DAD) to an electrospray Ion Trap Mass spectrometry detector (ESI-TRAP-MS/MS). As the same method was followed in this paper and the retention times coincide, a tentative identification of the phenolic compounds was performed based on the work of Mertz *et al.* (Mertz *et al.*, 2007). The quantification of polyphenols in the extract was performed using calibration curves with five concentrations that were established with standards of ellagic acid for ellagitannins and cyanidin-3-glucoside for anthocyanins. Samples were analyzed in triplicate.

3.2.2.e Antioxidant assays

DPPH radical-scavenging activity

The radical-scavenging activities of the polyphenol extracts were evaluated by assessing the direct DPPH-scavenging activity in the samples (Kim *et al.*, 2004). DPPH (0.25 mM) was prepared in methanol, and 0.5 mL of this solution was incubated with 1 mL of various sample dilutions. Sample blanks were prepared for each dilution (0.5 mL methanol + 1ml sample dilutions) and a control tube of DPPH was used to calculate the maximum absorbance (0.5 mL DPPH 0.25 mM + 1ml methanol). All the mixtures were incubated at room temperature in the dark for 30 min, and the absorbance of DPPH was measured at 517 nm. The percentage of the radical-scavenging activity of the extract was calculated with the following equation: % RSA = $100 * (A_{\text{DPPH}} - A_{\text{sample}} / A_{\text{DPPH}})$. The RSA values were plotted against the sample concentration to calculate the IC₅₀, which was the amount of extract that was necessary to reach the 50% radical-scavenging activity. Samples were analyzed in triplicate.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Ou *et al.* (Ou *et al.*, 2002). Fluorescein was used as a fluorescent probe, and oxidation was induced with AAPH. The polyphenol extracts were diluted in water and a calibration curve of trolox was used with concentrations range from 4-31 $\mu\text{mol/L}$. Assays were performed using spectrofluorimeter equipment (Biotek Instruments, Winooski, VT, USA). ORAC values were expressed as the mmol of trolox equivalents per gram of polyphenol extract (mmol TE/g of extract). Samples were analyzed in triplicate.

Nitric Oxide-scavenging activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and rapidly converted into the stable product nitrite. The nitrite concentration was measured using the Griess reaction (Balakrishnan *et al.*, 2009).

SNP (5 mM) was mixed with different concentrations of polyphenol extracts and a control of SNP without polyphenols was also prepared. All the mixtures were incubated for 60 min in direct light to enhance NO production. Later, the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H_3PO_4) was added, and the absorbance was read at 540 nm and compared to the absorbance of standard solutions of sodium nitrite. Sample blanks were prepared for each experiment.

The percentage of the NO-scavenging activity of the extract was calculated with the following equation: % NO-scavenging activity = $100 * ([\text{Nitrites}]_{\text{SNP}} - [\text{Nitrites}]_{\text{sample}} / [\text{Nitrites}]_{\text{SNP}}$). NO-scavenging activity was calculated as the amount of extract that was necessary to reduce 50% of the NO that was generated by SNP (IC_{50}). Samples were analyzed in triplicate.

Inhibition of lipid peroxidation in liposomes

Liposomes were prepared according to Pérez *et al.* (Pérez *et al.*, 2003). Briefly, 25 mg of commercial lecithin was dissolved in 2.15 mL of chloroform, and then 350 μL of methanol was added. This mixture was dried under a nitrogen atmosphere, and, finally, the lecithin was resuspended in 4.5 mL of warm phosphate-buffered saline (PBS 0.01M, pH 7.2) and sonicated for 1 h at 4 $^{\circ}\text{C}$ to form liposomes.

To test the capacity of the polyphenol extracts to protect lipid peroxidation, oxidative stress was induced with AAPH. Fifty microliters of various dilutions of the polyphenol

extracts were mixed with 0.45 mL of liposomes and 0.2 mL of AAPH (final concentration of 10 mM). These solutions were incubated in the dark for 2 h at 37 °C. To determine the malondialdehyde (MDA) concentration, 0.2 mL of 5% TCA (trichloroacetic acid) and 1 mL of 0.75% TBA (thiobarbituric acid) were added, and samples were heated at 96 °C for 30 min. After cooling, 0.25 mL of 3% SDS was added, and the mixture was centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The thiobarbituric acid reactive substances (TBARS) concentration was assessed using the molar absorption coefficient for MDA: $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$. MDA concentrations were plotted against the sample concentration, and the results were expressed as the amount of extract that inhibited 50% of lipid peroxidation (IC₅₀). Samples were tested in triplicate.

Inhibition of lipid peroxidation in liver homogenates

This procedure was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 19-06). Sprague-Dawley rats (220 g ± 20 g), which were obtained from LEBi® (Laboratorio de ensayos biológicos, Universidad de Costa Rica), were anesthetized with CO₂ and sacrificed by decapitation. The liver tissue of each rat was obtained and homogenized in phosphate-buffered saline (PBS 0.01M, pH 7.2) using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 x g for 15 min to reduce the suspended solids. Seventy-five microliters of different concentrations of the polyphenol extracts were added to 0.75 mL of liver supernatant and incubated for 30 min at 37 °C. Subsequently, oxidative stress was induced with TBHP at a final concentration of 1.7 mM and incubated for 1 h at 37 °C. Finally, TBARS were measured as the end products of lipid peroxidation.

TBARS were assayed according to Uchiyama & Mihara (Uchiyama & Mihara, 1978). Briefly, 0.25 mL of liver homogenate, 0.25 mL of 35% TCA and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4) were mixed and incubated for 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added and heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, and the solution was mixed and centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The TBARS concentration was calculated as described previously, and the results were expressed as nmol MDA/g liver tissue. MDA concentrations were plotted against the sample concentration to calculate the IC₅₀.

The assay was performed using liver tissue from 5 rats. To establish the basal level of lipid peroxidation, MDA levels without TBPH were assessed in each liver homogenate. Sample blanks were prepared for each experiment. Samples were tested in triplicate.

Erythrocyte cellular antioxidant activity (ERYCA)

The ERYCA assay was performed according to Gonzalez *et al.* (González *et al.*, 2010). Briefly, human erythrocytes were treated with AAPH (100 μ M) to enhance lipid peroxidation in the presence of the polyphenols extracted from the pasteurized juices. Erythrocyte hemolysis triggered a loss in the light scattering ability (turbidity), which was assessed every 5 min for 6 h. The area under the curve (AUC) for the absorbance decay curve was calculated for each sample and compared to a standard calibration curve of quercetin. ERYCA values were expressed in mmol of quercetin equivalents (QE) per gram of polyphenol extract of the different juices. Samples were analyzed in triplicate.

Inhibition of intracellular ROS

The levels of intracellular ROS were assessed using the DCFDA oxidation-sensitive fluorescent probe. Vero cells, which is a line of African green monkey kidney cells (ATCC, Cell No. CCL-81; Rockville, MD, USA) were grown in EMEM+ L-glutamine medium containing 10% inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (20 μ g/mL). The cells were cultured in a 24-well plate at a density of 5×10^5 cells/well and pre-treated with different concentrations of the polyphenol extracts for 20 h. Later, the cells were washed twice with PBS, and oxidative stress was induced with TBHP (0.7 mM) for 2 h. Thirty minutes before the end of the TBHP incubation, the DCFDA probe (5 μ M) was added to the cells, internalized and oxidized by intracellular ROS to 2',7'-dichlorofluorescein. After washing with PBS twice, the intensity of the intracellular fluorescence was measured using a flow cytometer (FACS-Scalibur, Becton-Dickinson). For each analysis, 10000 events were counted.

3.2.2.f Statistical analysis

The results from each experiment represent the means \pm standard error of at least three independent experiments. An analysis of variance (ANOVA) followed by a Tukey post hoc test was used to compare the differences between the polyphenol extracts: Non-pasteurized (**NPJ**), pasteurized at 75 °C (**JP75**) and pasteurized at 92 °C (**JP92**).

3.2.3 Results and Discussion

The effects of thermal treatments on the phenolic composition of blackberry extracts are shown in Table 3.2. Neither of the two pasteurization conditions that were evaluated in this study significantly altered the concentrations of the total polyphenols or HPLC-ellagitannins compared to those in the non-pasteurized juice.

Previous studies have shown that the effect of thermal treatments on total phenolic concentrations is variable. For example, the polyphenol content of a mango juice treated at 90 °C for 60s was reduced by 38% (Santhirasegaram *et al.*, 2013), but no significant alteration in the total phenolic concentration was evident for peach pieces pasteurized at 90 °C for 5 min (Oliveira *et al.*, 2012). This variability confirms that the effect of thermal treatment depends on the severity of the heat process, the specific factors that are characteristic of the food matrix and the sensitivity of the different phytochemicals to temperature (Rawson *et al.*, 2011).

Ellagitannins are one of the predominant components of the blackberry *R. adenotrichos* (Mertz *et al.*, 2007) and are characterized as heat-stable compounds in the literature (Hager *et al.*, 2010; Mena *et al.*, 2013). A study on pomegranate juice demonstrates that the content of punicalagin and ellagic acid were not affected after different thermal conditions, 65 to 90 °C (Mena *et al.*, 2013). Additionally, Hager *et al.* (Hager *et al.*, 2010) compared pasteurized (90°C) and non-pasteurized blackberry juices and did not find a decrease in the concentrations of six different types of ellagitannins, including sanguinin H6 and lambertanin C, which are present in the blackberry *R. adenotrichos*, which was used in the present study (Mertz *et al.*, 2007).

The anthocyanins of the extracts obtained from the blackberry juices were quantified by HPLC and showed a significant decrease (7%) in the pasteurized juices compared to those in non-pasteurized juice (Table 3.2). Anthocyanins have been described as molecules that are more sensitive to decomposition during thermal processes than other non-anthocyanin, polyphenolic compounds (Piasek *et al.*, 2011; Rawson *et al.*, 2011; Mena *et al.*, 2013). However, there is a variability in the sensibility to thermal decomposition among anthocyanins (Srivastava *et al.*, 2007; Zhang *et al.*, 2012). Previous publications have shown that the type of glycosylation in the cyanidins affects the sensitivity to thermal treatment. Piasek *et al.* (Piasek *et al.*, 2011) compared the influence of pasteurization on the composition of blue-berried honeysuckle juice and chokeberry juice

and concluded that cyanidin-3-glucoside made the blue-berried honeysuckle juice more thermally resistant than chokeberry juice, whose dominant anthocyanin is cyanidin-3-galactoside. Heating treatment of blueberry extracts has shown that cyanidin-3-glucoside is more stable than their galactoside equivalent (Srivastava *et al.*, 2007). In the case of blackberry juice, it was reported previously that cyanidin-3-glucoside is more stable than cyanidin-3-malonyl glucoside during different pasteurization and storage conditions (Zhang *et al.*, 2012, Gancel *et al.*, 2011).

Table 3.2 Chemical characterization of the polyphenol extracts obtained from the pasteurized blackberry juices

	Total polyphenols <i>mg GA /g of extract</i>	HPLC-Anthocyanins <i>mg C3G /g of extract</i>	HPLC-Ellagitannins <i>mg EA /g of extract</i>
NPJ	704 ± 9 ^a	195 ± 1 ^a	328 ± 3 ^a
JP75	679 ± 7 ^a	191 ± 0 ^b	335 ± 15 ^a
JP92	714 ± 6 ^a	181 ± 0 ^c	308 ± 1 ^a

Each value is the mean ± SE of three replicate experiments. Means in columns followed by different letters (a,b,c) differed significantly (p<0.05). C3G: cyanidin-3glucoside, GA: gallic acid, EA: ellagic acid

The free radical-scavenging capacity of the polyphenols extracted from the blackberry juices was compared using different methods, and the results are shown in Table 3.3. The scavenging activity against DPPH and NO was significantly lower for the pasteurized juices compared to the non-pasteurized juice. The reductions in the DPPH-scavenging capacity for the JP75 and JP92 juices were 26% and 27%, respectively, and the reductions of the NO-scavenging capacity for the JP75 and JP92 juices were 15% and 16% of their original activities, respectively. For DPPH- or NO-scavenging capacity, no significant differences were found between the JP75 and JP92 juices. The radical-scavenging capacity, as measured by the ORAC method, was not significantly different between the non-pasteurized and pasteurized juices.

The decrease in the DPPH- and NO-scavenging capacities due to thermal treatments could be explained by changes in the chemical structures of the phenolic compounds. It had been reported that thermal treatments cause cleavage of covalent bonds and modifications in the number and arrangements of hydroxylated groups and glycosylations of anthocyanins (Zhang *et al.*, 2012, Srivastava *et al.*, 2007). The severity and nature of these chemical re-arrangements determine the loss of bioactivity by anthocyanins (Rawson *et al.*, 2011). In the case of blackberry, the main anthocyanins that are detected

are cyanidin-3-glucoside and cyanidin-3-malonyl glucoside (Mertz *et al.*, 2007). These compounds are highly hydroxylated and include a 3-hydroxyl group in the B ring of the flavonoids, which is considered a relevant contributor to its antioxidant activity (Oliveira *et al.*, 2012; Seeram & Nair, 2002). Consequently, arrangement in the hydroxyl group of the B ring, which was caused by thermal treatments, could have an important impact on the antioxidant activity of blackberry juice.

The results listed in Table 3.3 show differences between the free radical-scavenging activities that were determined by each antioxidant assay. Specifically, the ORAC technique did not show significant differences between the non-pasteurized and the pasteurized juices, which contradicted the DPPH- and NO-scavenging techniques. This lack of correlation between the antioxidant assays has been largely discussed and explained in the scientific literature (Niki, 2011; Niki, 2010; Huang *et al.*, 2005). However, in the specific context of this study, it should be emphasized that each phenolic compound that was present in the blackberry juice was differently affected by heat treatments and each phenolic compound contributed in a different way to the antioxidant activity against a specific radical (Oliveira *et al.*, 2012). Moreover, the free radicals that were used in this study, DPPH, NO or peroxy radical (for ORAC), presented specific kinetic and stoichiometric factors that determined the performance of the antioxidant (Niki, 2011). Therefore, the combination and interaction of these aspects could explain the variability between the free radical-scavenging activities of the different assays.

Table 3.3 Free radical-scavenging of the polyphenol extracts that were obtained from the pasteurized blackberry juices

	DPPH <i>IC</i> ₅₀ (µg/mL)	ORAC <i>mmol TE/g of extract</i>	NO Scavenging <i>IC</i> ₅₀ (µg/mL)
NPJ	5.05 ± 0.35 ^a	7.69 ± 0.16 ^a	165 ± 5 ^a
JP75	6.36 ± 0.20 ^b	7.34 ± 0.08 ^a	189 ± 7 ^b
JP92	6.42 ± 0.24 ^b	7.77 ± 0.07 ^a	191 ± 3 ^b

Each value is the mean ± SE of three replicate experiments. Means in columns followed by different letters (a,b) differed significantly ($p < 0.05$).

The assessment of the free radical-scavenging capacity is an indicator of the antioxidant capacity of a compound; however, other assays that show the capacity to inhibit lipid peroxidation provide data with more biological relevance. The assessment of the capacity to inhibit lipid peroxidation involves factors that interact with other biological components and the mobility of the antioxidant in a specific microenvironment (Niki,

2011). For this study, the inhibition of lipid peroxidation was assessed in artificial lecithin liposomes, rat liver homogenates and human erythrocytes (ERYCA). AAPH (in the liposome model and ERYCA) and TBHP (in the liver homogenate model) were used because of their ability to be metabolized into free radical intermediates that crossed cellular membranes, leading to the production of highly reactive hydroxyl radicals that initiated lipid peroxidation. Figure 3.7 shows the lipid peroxidation inhibitory capacity of the polyphenols extracted from the blackberry juices in the three models.

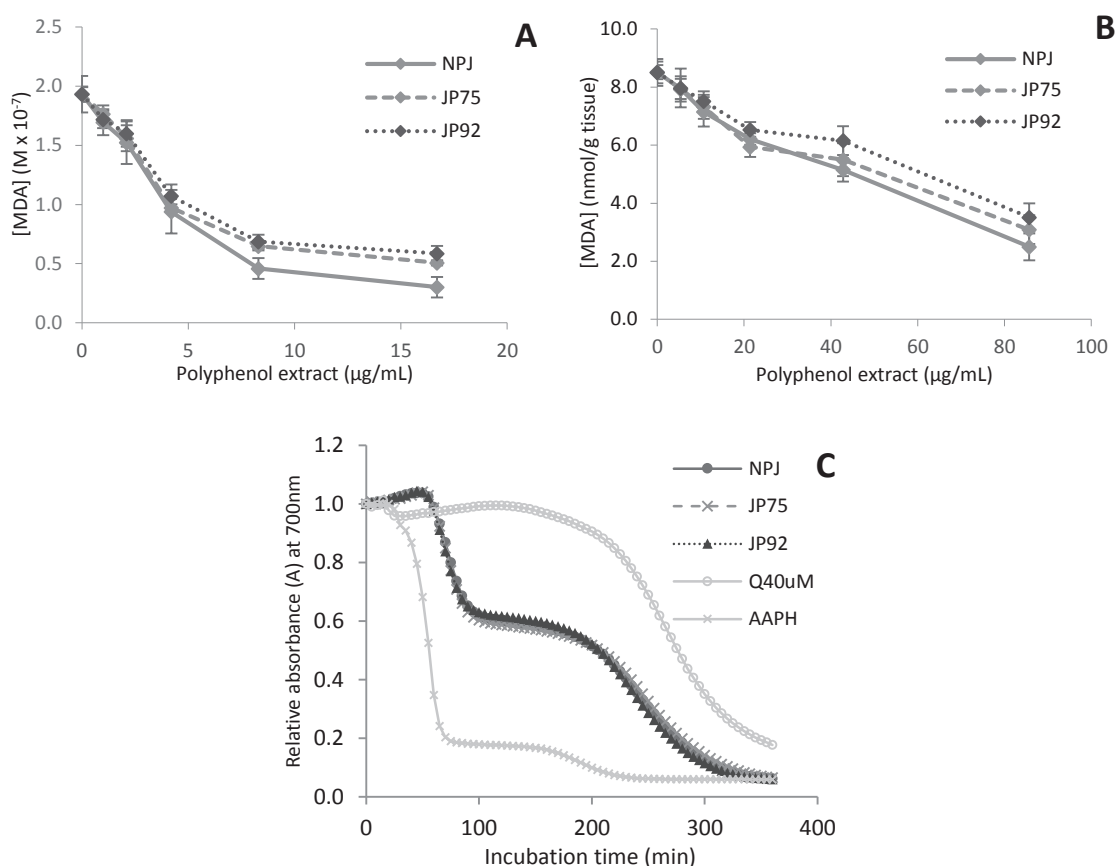


Figure 3.7 Inhibitory capacity of the polyphenol extracts that were obtained from the pasteurized blackberry juices against lipid peroxidation in liposomes (A), liver homogenates (B) and erythrocytes (C). Each value is the mean \pm S.E. (three independent experiments).

Figure 3.7A shows that the polyphenol extracts from all the juices (pasteurized and non-pasteurized) lowered the levels of lipid peroxidation in the liposomes in a dose-dependent manner; the concentration of polyphenols that were necessary to inhibit 50% of the MDA concentration in liposomes (IC_{50}) was $3.9 \pm 0.7 \mu\text{g/mL}$ for NPJ, $3.9 \pm 0.1 \mu\text{g/mL}$ for JP75 and $4.0 \pm 0.5 \mu\text{g/mL}$ for JP92. In Figure 3.7B, a similar inhibitory effect was observed for the rat liver homogenates, with an IC_{50} of $49.4 \pm 2.7 \mu\text{g/mL}$ for NPJ, $49.9 \pm 5.1 \mu\text{g/mL}$ for JP75 and $57.7 \pm 5.3 \mu\text{g/mL}$ for JP92. Figure 3.7C, shows the protective effect of the

polyphenol extract against erythrocyte lysis that was caused by lipid peroxidation and was followed for 360 min. This activity was reported as quercetin equivalents and showed values of 3.65 ± 0.15 mmol QE/g of extract for NPJ, 3.74 ± 0.18 mmol QE/g of extract for JP75 and 3.70 ± 0.18 mmol QE/g of extract for JP92. No significant differences were found for the peroxidation inhibitory capacity of the three juices in all of the assays: liposomes, liver homogenates and ERYCA. These results demonstrates that thermal treatment did not cause a significant decrease in the potential of blackberry polyphenols to protect against lipid peroxidation.

The deleterious effects of ROS are due to lipid peroxidation altering the integrity of the cell membranes and to the modification of intracellular signaling pathways that play crucial roles in the pathophysiology of several diseases (Finkel, 2011; Valko *et al.*, 2007). Because of this, our study evaluates the antioxidant effects of polyphenols inside of cells using the probe DCFDA. Figure 3.8 shows that the blackberry polyphenols reduced the induced intracellular ROS in a dose-dependent manner and that this antioxidant capacity was not statistically decreased because of thermal treatments. The concentration of polyphenol extract necessary to scavenge 50% of the induced ROS production was 204 ± 9 $\mu\text{g/mL}$ for NPJ, 219 ± 10 $\mu\text{g/mL}$ for JP75 and 220 ± 9 $\mu\text{g/mL}$ for JP92.

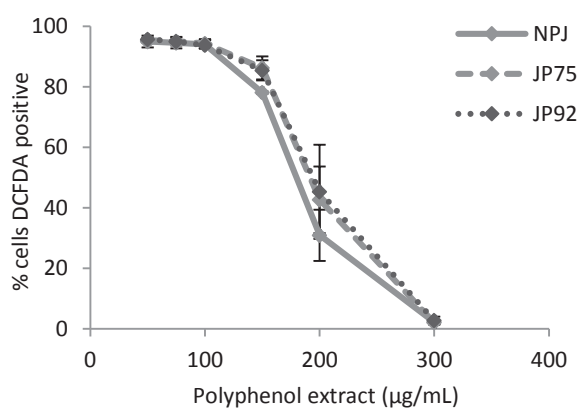


Figure 3.8 Inhibitory capacity of the polyphenol extracts that were obtained from the pasteurized blackberry juices against intracellular ROS.

Each value is the mean \pm S.E. (three independent experiments).

The ability of the blackberry polyphenols to protect against lipid peroxidation and intracellular antioxidant activity was not affected by thermal treatment. Therefore, the decrease in anthocyanin composition that was observed did not significantly affect the biological properties of the blackberry juice in our models. This finding suggests that ellagitannins have an important contribution to the inhibition of lipid peroxidation, as was

reported previously for cloudbberries (*R. chamaemorus*) (Mylnikov *et al.*, 2005). Besides, the lack of changes in naturally occurring antioxidant concentrations may be explained by the loss of these compound being balanced by the simultaneous formation of molecules with novel or improved antioxidant properties (Nicoli *et al.*, 1999).

Regardless of the anthocyanins reduction that was observed in this study, the metabolites that resulted from heat treatment could undergo re-arrangement of hydroxylated groups or glycoside moieties (Rawson *et al.*, 2011; Srivastava *et al.*, 2007); however, this does not necessarily reduce their capacity to inhibit ROS or their ability to protect against lipid peroxidation (Gancel *et al.*, 2011). Hager *et al.* (Hager *et al.*, 2008) suggested that the antioxidant capacity of the polymers formed during heating might compensate for the loss of antioxidant capacity caused by anthocyanin degradation. Seeram and Nair determined that the sugar residues of the anthocyanins (glycosides) decreased their ability to inhibit lipid peroxidation when compared to anthocyanidins (aglycons); therefore, the formation of aglycones during a thermal treatment could be beneficial for the lipid peroxidation inhibitory activity (Seeram & Nair, 2002). Although this is a controversial topic, some authors consider that the appearance of aglycones and simpler flavonoids after pasteurization a positive change because it leads to improved bioavailability (Piasek *et al.*, 2011), and this aspect should be considered when studying the impact of thermal treatments on potential beneficial activity.

Most publications that evaluated the effect of pasteurization in terms of bioactive retention have focused on the changes in the phenolic composition of the fruits and the effects on the antioxidant activity, which are measured as the free radical-scavenging capacity *in vitro*. This study attempted to include results with more relevant, biological impact and revealed that, in spite of the changes in the phenolic composition and radical-scavenging capacity, the pasteurized blackberry juice maintained the biological properties related to the ability to protect against lipid peroxidation and inhibition of intracellular ROS. However, an evaluation of the *in vivo* impact on the health-promoting properties of blackberry-pasteurized juices is still needed.

Acknowledgments

We thank Florida Bebidas S.A. and Cooperativa de Productores de Leche, Dos Pinos R.L. for facilitating the use of the pasteurization equipment. Financial support for this work was funded by CONARE FS project no. 801-B1-655.

3.3 Effect of an *in vitro* digestion on the antioxidant capacity of the tropical highland blackberry polyphenols (*Rubus adenotrichos*)

Effet d'une digestion in vitro sur la capacité antioxydante des polyphénols de la mûre tropicale de montagne (Rubus adenotrichos)

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Article in preparation / Article en cours de préparation

Abstract

Increasing interest for berries polyphenols occurred as *in vitro* and *in vivo* health benefits properties have been demonstrated, however these effects are largely dependent of their bioavailability. This study evaluated the antioxidant activity of different samples of blackberry juice representative of the major stages of the digestion process. The HPLC analysis of the samples obtained after the *in vitro* digestion showed that the gastric digestion had only slight impact on polyphenolic composition. On the contrary the pancreatin/bile digestion had marked effects on polyphenolic composition, particularly in the ratio anthocyanins-ellagitannins. The free radical-scavenging capacity was reduced significantly only for the dialyzed fraction, which showed a 33% and 35% less activity against nitrogen derived radicals DPPH and NO respectively. The capacity to inhibit lipid peroxidation showed no significant differences in the IC₅₀ values in the liposomes model, the liver cells or in the human erythrocytes. The assessment of the inhibitory capacity of intracellular ROS showed significant differences only for the dialyzed fraction with an IC₅₀ of 196 ± 8 µg/mL, that almost duplicate the IC₅₀ of the non-digested sample (106 ± 2 µg/mL). Our results suggest that the blackberry polyphenols could exert their antioxidant capacity after its passage through the GIT. However the dialyzed fraction, which represents the serum available polyphenols, suffer a partial depletion of its antioxidant ability and this could be attributed to the absence of ellagitannins.

KEYWORDS

Blackberry, *Rubus adenotrichos*, antioxidant activity, lipid peroxidation, polyphenols bioavailability

3.3.1 Introduction

The biological activity of berries phenolics have attracted interest of the consumers and different studies had documented *in vitro* and *in vivo* activities that could be related with benefits for human health. Specifically, the berries phenolics have been associated with a strong free radicals scavenging activity, prevention of cellular lipids oxidation, inhibition of oxidant enzymes, modulation of signal transduction, cancer prevention, anti-inflammatory properties and improvement of cardiovascular risk profiles (Paredes-López *et al.*, 2010; Bowen-Forbes *et al.*, 2010; Basu *et al.*, 2010; Basu & Lyons, 2012; Szajdek & Borowska, 2008). However the health effects of polyphenols are largely dependent upon their bioavailability. To validate the beneficial effects of the berries phenolics it is important to consider the levels of absorption and modifications that these compounds suffer during the digestion (D'Archivio *et al.*, 2010).

The bioavailability of each phenolic compounds is affected by several factors as the molecular structure, glycosylation levels, molecular weight, esterification, food matrices or food processing (Paredes-López *et al.*, 2010; D'Archivio *et al.*, 2010). Because of this, it is important to study the bioavailability of each dietary source of polyphenols.

The tropical blackberries, from the *Rubus spp* group, contain anthocyanins, as their main flavonoid (Mertz *et al.*, 2007; Kaume *et al.*, 2012). Unlike other flavonoids, anthocyanins can be absorbed in the intact glycoside form or in several other anthocyanin metabolites, such as methylated glycosides, glucuronides of anthocyanidins, sulfoconjugate of cyanidin, and anthocyanidins. In spite of this, the absorption of blackberry anthocyanins had been reported to be low, often between 0.02 and 1.8% of the ingested amounts (Felgines *et al.*, 2005; Kaume *et al.*, 2012; He & Giusti, 2010).

Another important type of polyphenols reported for blackberries are the ellagitannins (Mertz *et al.*, 2007; Kaume *et al.*, 2012). Some studies had hypothesized that these molecules undergo partial hydrolysis at the pH levels of the small intestine and the remaining ellagitannins are hydrolyzed by colon microbiota to ellagic acid. Later, the ellagic acid followed a gradual metabolism to produce urolithins, which are molecules more lipophilic and easily absorbed (Espín *et al.*, 2007). It has been suggested that differences in the colonic microbiota composition can result in variation in the quantity and types of urolithins absorbed and excreted (Cerdá *et al.*, 2005).

The aim of this study was to evaluate changes in the blackberry juice antioxidant activity of different samples that represent the major stages of the digestion process. These samples were obtained from an *in vitro* digestion technique and the comparison between them was done assessing the anthocyanin and ellagitannins concentrations, the extracellular and intracellular free radical-scavenging capacity and evaluating the ability to inhibit lipid peroxidation.

3.3.2 Materials and Methods

3.3.2.a Chemicals

All the solvents for HPLC and polyphenol purification were obtained from JT Baker (Griesheim, Germany). Amberlite XAD-7, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azobis-2-methyl-propion-amidine-dihydrochloride (AAPH), *tert*-butyl hydroperoxide (TBHP), thiobarbituric acid (TBA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,7-dichlorodihydrofluorescein diacetate (DCFDA), quercetin, fluorescein, pepsin, bile salts and pancreatin were acquired from Sigma Aldrich (St. Louis, MO, USA). The standards ellagic acid was obtained from Fluka (Buchs, Switzerland), and cyanidin-3-glucoside was obtained from Extrasynthese (Lyon, France). For cell culture, EMEM medium, L-glutamine, fetal bovine serum, streptomycin, penicillin and trypsin were purchased from Sigma Aldrich (St. Louis, MO, USA). For the Griess reagent, sodium nitroprusside (SNP), sulfanilamide, naphthylethylenediamine dihydrochloride, and sodium nitrite were acquired from Merck (Darmstadt, Germany).

3.3.2.b Blackberry juice preparation

Full-ripe blackberries (*Rubus adenotrichos* Schltdl. cv. 'vino') were collected from different farms in the province of Cartago, Costa Rica (altitude 1864 – 2517 m, latitude 09° 39' 57.1"N - 09°44'40.3"N, longitude 83°53'32.1"W - 84°00'06.3"W). A microfiltrated juice was prepared according to Vaillant *et al.* (Vaillant *et al.*, 2008) Blackberries were pressed and the juice treated with a commercial enzymatic preparation, Ultrazym (Novozymes, Bagsvaerd, Denmark) for 1 h at 35 °C with constant agitation. The microfiltration was performed in a tubular ceramic membrane (Membralox® 1 P19-40, Pall Exekia, Bazet, France) with an average pore size diameter of 0.2 µm.

3.3.2.c *In vitro* digestion

To simulate the physiochemical changes underwent by polyphenols in the upper GIT, particularly stomach and small intestine, an *in vitro* digestion method previously described (Gil-Izquierdo *et al.*, 2002; Coates *et al.*, 2007) has been adapted. Briefly, 100 ml of a microfiltrated blackberry juice (**Sample 1**) were subjected to a pepsin-HCl digestion, using 31500 units of pepsin and the pH was adjusted to 2 by addition of concentrated HCl. The digestion was conducted at 37 °C with continuous agitation for 2 h (**Sample 2**). Later, 60 ml of the post gastric digestion (sample 2) were transferred to a 400 ml beaker which contained two cellulose dialysis tubes (molecular cut-off 12 kDa) filled with sufficient NaHCO₃ solution to reach the pH of the gastric digestion to 7 (The required amount of NaHCO₃ was previously evaluated). The beaker was sealed with parafilm and incubated at 37 °C with continuous agitation until the pH reached 7 (30 min approximately) and then a mixture of pancreatin (4 g/L)-bile extract (25 g/L) was added and incubated for 2 h more. At the end of the incubation, the juice solution outside the dialysis tubes was recovered (**Sample 3**), representing the compounds that would reach the colon and the solution inside the dialysis tube was recovered (**Sample 4**) and represents the serum available material. After the digestion sample 3 and 4 were acidified to pH 2 and centrifuged before the polyphenol purification.

3.3.2.d Polyphenol purification

The four samples obtained from the *in vitro* digestion were subjected to a polyphenol purification using an Amberlite XAD-7 column (150 mm x 20 mm) packed in water. The different samples were loaded onto the column and then washed with water to remove sugars. Finally, the phenolic compounds were eluted with methanol/water (80:20) and concentrated under vacuum, freeze-dried and stored at -30 °C for further analysis.

3.3.2.e Analytical characterization of the blackberry juices

Phenolic compounds by HPLC

The polyphenol extracts obtained from the digestion samples were analyzed by HPLC for anthocyanins and ellagitannins following the protocol described by Mertz *et al.* (Mertz *et al.*, 2007) and Acosta-Montoya *et al.* (Acosta-Montoya *et al.*, 2010). Briefly, the HPLC quantitative analysis was performed with a Dionex liquid chromatography system equipped with a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) and an endcapped reversed-phase Lichrospher ODS-2 column (250 mm x 4.6

mm i.d., 5 μ m) (Interchim, Montluçon, France). Previously, Mertz *et al.* (Mertz *et al.*, 2007), identified the sanguin H6, lambertianin C and cyanidin-malonyl-glucoside following the same HPLC procedure by an additional hyphenation of the diode array detector (DAD) to an electrospray Ion Trap Mass spectrometry detector (ESI-TRAP-MS/MS). As the same method was followed in this paper, an identification attempt of the phenolic compounds was done based on Mertz *et al.* publication (Mertz *et al.*, 2007). The quantification of polyphenols in the digestions samples was performed using calibration curves established with standards of ellagic acid for ellagitannins and cyanidin-3-glucoside for anthocyanins.

3.3.2.f Antioxidant assays

DPPH radical-scavenging activity

The radical-scavenging activity of the digestion samples was evaluated by assessing the direct DPPH-scavenging activity in the polyphenols extracts (Kim *et al.*, 2004). DPPH (0.25 mM) was prepared in methanol, and 0.5 mL of this solution was incubated with 1 mL of various sample dilutions. Mixtures were incubated at room temperature in the dark for 30 min, and the absorbance of DPPH was measured at 517 nm. Sample blanks were prepared for each dilution. The percentage of the radical scavenging activity of the extract was plotted against the sample concentration to calculate the IC₅₀, which is the amount of extract necessary to reach the 50% radical scavenging activity. The samples were analyzed in triplicate.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Ou *et al.* (Ou *et al.*, 2002). Fluorescein was used as a fluorescent probe, and oxidation was induced with AAPH. Assays were performed using spectrofluorimeter equipment (Biotek Instruments, Winooski, VT, USA). ORAC values were expressed as mmol of trolox equivalents per gram of polyphenol extract (mmol TE/g of extract).

Nitric Oxide scavenging activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and rapidly converted into the stable product nitrite. The nitrite concentration was measured by the Griess reaction (Balakrishnan *et al.*, 2009).

SNP (5 mM) was mixed with different concentrations of the digested polyphenols and incubated for 60 min in direct light to enhance the NO production. Later, the Griess

reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H₃PO₄) was added and the absorbance was read at 540 nm and referred to the absorbance of standard solutions of sodium nitrite. Sample blanks were prepared for each experiment. NO scavenging activity was calculated as the amount of extract necessary to reduce 50% of the NO generated by SNP (IC₅₀). The samples were analyzed in triplicate.

Inhibition of lipid peroxidation in liposomes

Liposomes were prepared according to Pérez *et al.* (Pérez *et al.*, 2003). Briefly, 25 mg of commercial lecithin was dissolved in 2.15 mL of chloroform, and then 350 µL of methanol was added. This mixture was dried under a nitrogen atmosphere, and finally, the lecithin was resuspended in 4.5 mL of warm phosphate-buffered saline (PBS) and sonicated for 1 h at 4 °C to form liposomes.

To test the capacity of the extracts to protect lipid peroxidation, oxidative stress was induced with AAPH. Fifty microliters of various dilutions of the digested polyphenols were mixed with 0.45 mL of liposomes and 0.2 mL of AAPH with a final concentration of 10 mM. These solutions were incubated in the dark for 2 h at 37 °C. To determine the malondialdehyde (MDA) concentration, 0.2 mL of 5% TCA (trichloroacetic acid) and 1 mL of 0.75% TBA (thiobarbituric acid) were added, and samples were heated at 96 °C for 30 min. After cooling, 0.25 mL of 3% SDS was added, and the mixture was centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The thiobarbituric acid reactive substances (TBARS) concentration was assessed using the molar absorption coefficient for MDA: $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$. MDA concentrations were plotted against the sample concentration, and the results were expressed as the amount of extract that inhibits 50% of lipid peroxidation (IC₅₀). The samples were tested in triplicate.

Inhibition of lipid peroxidation in liver homogenates

This procedure was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 19-06). Sprague-Dawley rats (220 g ± 20 g), obtained from LEBi® (Laboratorio de ensayos biológicos, Universidad de Costa Rica), were anesthetized with CO₂ and sacrificed by decapitation. The liver tissue of each rat was obtained and homogenized in phosphate-buffered saline (PBS) using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 x g for 15 min to reduce the suspended solids. Seventy-five microliters of different concentrations of the digested

polyphenols were added to 0.75 mL of liver supernatant and incubated for 30 min at 37 °C. Subsequently, oxidative stress was induced with TBHP at a final concentration of 1.7 mM and incubated for 1 h at 37 °C. Finally, TBARS were measured as the end products of lipid peroxidation.

TBARS were assayed according to Uchiyama & Mihara (Uchiyama & Mihara 1978). Briefly, 0.25 mL of liver homogenate, 0.25 mL of 35% TCA and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4) were mixed and incubated for 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added and heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, mixed and centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated as described previously, and the results were expressed as nmol MDA/g liver tissue. MDA concentrations were plotted against the sample concentration to calculate the IC₅₀.

The assay was performed using liver tissue from 5 rats. To establish the basal level of lipid peroxidation, MDA levels without TBPH were assessed in each liver homogenate. Sample blanks were prepared for each experiment. The samples were tested in triplicate.

Erythrocyte cellular antioxidant activity (ERYCA)

ERYCA assay was performed according to Gonzalez *et al.* (González *et al.*, 2010). Briefly, human erythrocytes were treated with AAPH (100 µM) to enhance lipid peroxidation in the presence of polyphenols extracted from the digested samples. The erythrocytes hemolysis triggered a loss in the light scattering ability (turbidity) which was assessed each 5 min for 6 h. The absorbance decay curve (AUC) was calculated for each sample and compared with a standard calibration curve of quercetin. ERYCA values were expressed in mmol of quercetin equivalents (QE) per gram of polyphenol extract from the different samples.

Inhibition of intracellular ROS

The levels of intracellular ROS were assessed using the DCFDA oxidation-sensitive fluorescent probe. Vero cells, a line of African green monkey kidney (ATCC, Cell No. CCL-81; Rockville, MD, USA) were grown in EMEM+ L-glutamine medium containing 10% inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (20 µg/mL). The cells were cultured in a 24-well plate at a density of 5×10^5 cells/well and were pre-treated with different concentrations of the digested polyphenols for 20 h. Later, the cells

were washed twice with PBS and oxidative stress was induced with TBHP (0.7 mM) for 2 h. Thirty minutes before the end of TBHP incubation, the probe DCFDA (5 μ M) was added to the cells, further internalized and oxidized by the intracellular ROS to 2'7'-dichlorofluorescein. After washing with PBS twice, the intensity of the intracellular fluorescence was measured using flow cytometer (FACS-Scalibur, Becton-Dickinson). For each analysis, 10000 events were counted.

3.3.2.g Statistical analysis

The results from each experiment represent the means \pm standard error of at least three independent experiments. An analysis of variance (ANOVA) followed by a Tukey post hoc test was used to compare the differences between the samples obtained from the *in vitro* digestion.

3.3.3 Results and Discussion

The HPLC analysis of the blackberry digested samples showed in Table 3.4 demonstrated that the gastric digestion (S2) did not provoke a statistically significant change in the concentration of anthocyanins and ellagitannins. However a slight increase in anthocyanins was observed after the pepsin treatment as it have been reported for other similar *in vitro* juices digestions, such as in pomegranate juice (Pérez-Vicente, et al., 2002) and chokeberry juice (Bermudez-Soto *et al.*, 2007). The consensus explanation given for this increase is that the pH used in the pepsin digestion (pH 2.0) lower the initial sample pH causing an increase of the flavylum cation in the solution making the anthocyanins a larger portion of the total (McDougall *et al.*, 2005) (Pérez-Vicente *et al.*, 2002) (Bermudez-Soto *et al.*, 2007).

Contrarily to the gastric digestion, the nondialyzed (S3) and the dialyzed fraction (S4) underwent significant changes in the concentrations of the phenolic compounds (Table 3.4). The nondialyzed fraction (S3) of this study retained 46% of anthocyanins and 66% of the ellagitannins whereas the dialyzed fraction (S4) kept 19% of anthocyanins and 6.8% of the ellagitannins. These recovery percentages evidence a different anthocyanin-ellagitannins proportion in the S3 and S4 fraction compared with the original juice; the S3 become more concentrated in ellagitannins and the sample S4 in anthocyanins. This suggests that *in vivo* the blackberry anthocyanins will be mostly available at the serum and the ellagitannins will be mainly maintained in the colon.

Table 3.4 Anthocyanins and ellagitannins concentrations in the blackberry digested samples

	HPLC-Anthocyanins <i>mg C3G /L digested juice</i>	HPLC-Ellagitannins <i>mg EA /L digested juice</i>
S1	480 ± 37 ^a	353 ± 13 ^a
S2	580 ± 15 ^a	394 ± 24 ^a
S3	220 ± 19 ^b	233 ± 6 ^b
S4	91 ± 4 ^c	24 ± 3 ^c

Each value is the mean ± SE of three replicate experiments. Means in columns followed by different letters differed significantly ($p < 0.05$). C3G: cyanidin-3glucoside, EA: ellagic acid

The anthocyanin recovery rates reported for other fruits treated with similar *in vitro* digestion showed very variable results: 67% in the case of raspberry (McDougall *et al.*, 2005), 57% for a chokeberry juice (Bermudez-Soto *et al.*, 2007) and only 18% for a pomegranate juice (Pérez-Vicente *et al.*, 2002). The variation in the degradation levels of these molecules, depends mainly of the specific chemical composition of the anthocyanins present in each fruit. Different patterns of hydroxylation and mainly the type of sugar moieties determine the sensibility of the anthocyanins to the pancreatic-bile digestion (McGhie *et al.*, 2003). As well, the particular food matrix of each fruit could influence the levels of polyphenol degradation (D'Archivio *et al.*, 2010).

The anthocyanins of the blackberry juice digested in this study showed a higher amount of diffusion through the dialysis tube (19%) compared with similar *in vitro* digestions of raspberry (5%) (McDougall *et al.*, 2005) or pomegranate (2.4%) (Pérez-Vicente *et al.*, 2002). The better recovery of anthocyanins in the dialyzed fraction could be attribute to the fact that the blackberry presents only one major anthocyanin, cyanidin-3-glucoside, (Mertz *et al.*, 2007; Felgines *et al.*, 2009; Del Rio *et al.*, 2010) whereas raspberries and pomegranates presented 2 or 3 main anthocyanins (Pérez-Vicente *et al.*, 2002; McDougall *et al.*, 2005). As well, the simple chemical structure and glycoside moiety of the cyanidin-3-glycoside could favor the rates of bioabsorption through the dialysis tube. It has been reported in rats models fed with blackberry that the cyanidin-3-glycoside is absorbed in the intact glycoside form and the absorption is readily and efficient, suggesting that the small molecular size of this compound simplifies the diffusion (Felgines *et al.*, 2009; Talavera *et al.*, 2004; Talavéra *et al.*, 2003). Previous studies showed that *in vivo*, cyanidin-3-glucoside are absorbed by passive diffusion or by sodium-dependent glucose transporters facilitating the appearing in blood circulation (He & Giusti 2010; McGhie *et al.*, 2003). Furthermore, under *in vivo* conditions, the unabsorbed anthocyanins could be

metabolized via intestinal microflora generating simple compounds, such as phenolic acids that might be absorbed at the colon level and contribute to the serum antioxidant capacity (Coates *et al.*, 2007). Aura *et al.*, demonstrates that the anthocyanins in the colon undergo cleavage of the sugar moiety followed by ring fission of the cyanidin aglycone, producing 3,4-dihydroxybenzoic acid (Aura *et al.*, 2005).

Contrary to the anthocyanins, the ellagitannins had been less studied in similar *in vitro* digestion models as the one performed in this study. To our knowledge the recovery rates of ellagitannins after an *in vitro* digestion had been reported only for raspberry. For this fruit, some ellagitannins were poorly recovered (less than 20%), however, other ellagitannins as Sanguin H-6, increased a 6-fold in their concentration and this is explained due to the breakdown of Sanguin H-10 (Coates *et al.*, 2007). The blackberry juice digested in this study presented an important 73% of ellagitannins recovery (S3+S4); this suggest that ellagitannins are stable to the GIT conditions and only suffer minor re-arranges keeping their ellagitannins identity as it was described for raspberries (Coates *et al.*, 2007).

For the blackberry juice, the absorption of ellagitannins through the dialysis tube was minimum and most of these molecules were maintained in the nondialyzed fraction (S3). Lambertanin C and Sanguinin H6 are the main ellagitannins detected in *Rubus adenotrichos*, and these compounds presented large molecular masses of 2804 and 1869 g/mol respectively (Mertz *et al.*, 2007), that would not permit them to diffuse in the dialysis tube used in this study. The small amount of ellagitannins detected in the dialyzed fraction (S4) correspond probably to ellagic acid molecules that are release from ellagitannins under mild alkaline pH conditions of the small intestine, thus without the need of enzymes leading to an absorption in the GIT (Larrosa *et al.*, 2006; Espín *et al.*, 2007). The ellagitannins maintained in the nondialyzed fraction (S3) under physiological conditions will be transformed by the microbial flora to ellagic acid and subsequently to urolithins and that will facilitated the intestinal uptake (Espín *et al.*, 2007).

Even though the biological activities of anthocyanins and ellagitannins are broadly reported in the scientific literature (He & Giusti, 2010; Bowen-Forbes *et al.*, 2010; Larrosa *et al.*, 2006), it is important to evaluate this polyphenols in a similar composition to that found in colon or in plasma rather than the crude homogenates. Because of this, the samples obtained from the blackberry digestion were lyophilized and then their biological activities evaluated in this study. The main objective was to determine if the

changes in structures or the proportions of the different polyphenols cause an effect in their antioxidant properties.

The free radical-scavenging activity is one of the important functions of antioxidants, because of this, the digested samples were evaluated against different free radicals and the results are shown in Table 3.5. Compared to the other samples, the dialyzed fraction (S4) showed a significant decrease of the scavenging activity against nitrogen derived radicals (DPPH and NO scavenging techniques) but the same fraction presented no significant differences when is evaluated against peroxy radicals (ORAC method). The inconsistency of the results regarding different techniques that evaluate antioxidant capacity is well documented in several publications. (Huang *et al.*, 2005; Niki, 2011; Niki, 2010). Factors as the chemical structure of the radical, the kinetic and stoichiometric conditions of the radical-antioxidant interaction and the use of competition systems provoke variations between the methods. However, according to Niki, it is more appropriate to measure the antioxidant activity against peroxy radicals, as in ORAC technique, because this radical plays an important role as a chain-carrying radical in lipid peroxidation (Niki, 2011).

The fact that in this study the dialyzed fraction (S4) kept part of the antioxidant capacity is in agreement with previous *in vivo* results in human volunteers that reported a 5 to 30% increase in plasma antioxidant capacity after the ingestion of different berries products. (Paredes-López *et al.*, 2010; Basu *et al.*, 2010). These complementary studies demonstrated that the berries polyphenols could be absorbed and could exert their antioxidant capacity after its passage through the GIT.

Table 3.5 Free radical-scavenging of the digested blackberry polyphenols

	DPPH <i>IC</i> ₅₀ (µg/mL)	ORAC <i>mmol TE/g of extract</i>	NO Scavenging <i>IC</i> ₅₀ (µg/mL)
S1	5.10 ± 0.20 ^a	5.94 ± 0.41 ^a	197 ± 19 ^a
S2	4.75 ± 0.03 ^a	6.30 ± 0.42 ^a	183 ± 14 ^a
S3	5.30 ± 0.15 ^a	5.86 ± 0.27 ^a	217 ± 13 ^{ab}
S4	6.79 ± 0.06 ^b	6.56 ± 0.18 ^a	267 ± 21 ^b

Each value is the mean ± SE of three replicate experiments. Means in columns followed by different letters differed significantly ($p < 0.05$).

A possible benefit of the antioxidant capacity of the polyphenols could be the inhibition of lipid peroxidation. Antioxidants could scavenge free radicals and impede the oxidation of lipids, preventing disturbances on the structure, integrity and fluidity of the biomembranes, modifications of low and high density lipoproteins and generation of pro-atherogenic and pro-inflammatory forms (Niki, 2012). In this study, the digested polyphenols inhibited the lipid peroxidation in the three substrates evaluated: artificial lecithin liposomes, rat liver homogenates and human erythrocytes (ERYCA). Figure 3.9 (A, B, C) shows that for the three models tested, the blackberry phenols of each digestion samples inhibited the levels of malondialdehyde in a dose-dependent manner. Table 3.6 showed that there are no significant differences in the IC₅₀ values of the digested samples, however there was a tendency to reduce the inhibitory capacity in the nondialyzed (S3) and dialyzed (S4) fractions, reaching a maximum loss of 20%.

The results showed in Table 3.4 and Table 3.6 demonstrate that even though the nondialyzed (S3) and the dialyzed fraction (S4) changed the ratio of anthocyanins and ellagitannins, compared to the nondigested sample (S1), the potential to protect against lipid peroxidation suffer a minor decrease. This suggested that both anthocyanins and ellagitannins contribute to the protection against the lipid peroxidation as it has been reported in previous studies. Seeram & Nair, explained that the presence of hydroxyl groups on the B-ring of anthocyanins confers stability to the flavonoid phenoxyl radicals, limiting the lipid peroxidation reactions in which the radicals can participate (Seeram & Nair, 2002). Reddy *et al.* and Bialonska *et al.* showed that different tannin components of pomegranate could exert a strong antioxidant activity even after they are metabolized (Reddy *et al.*, 2007; Bialonska *et al.*, 2009).

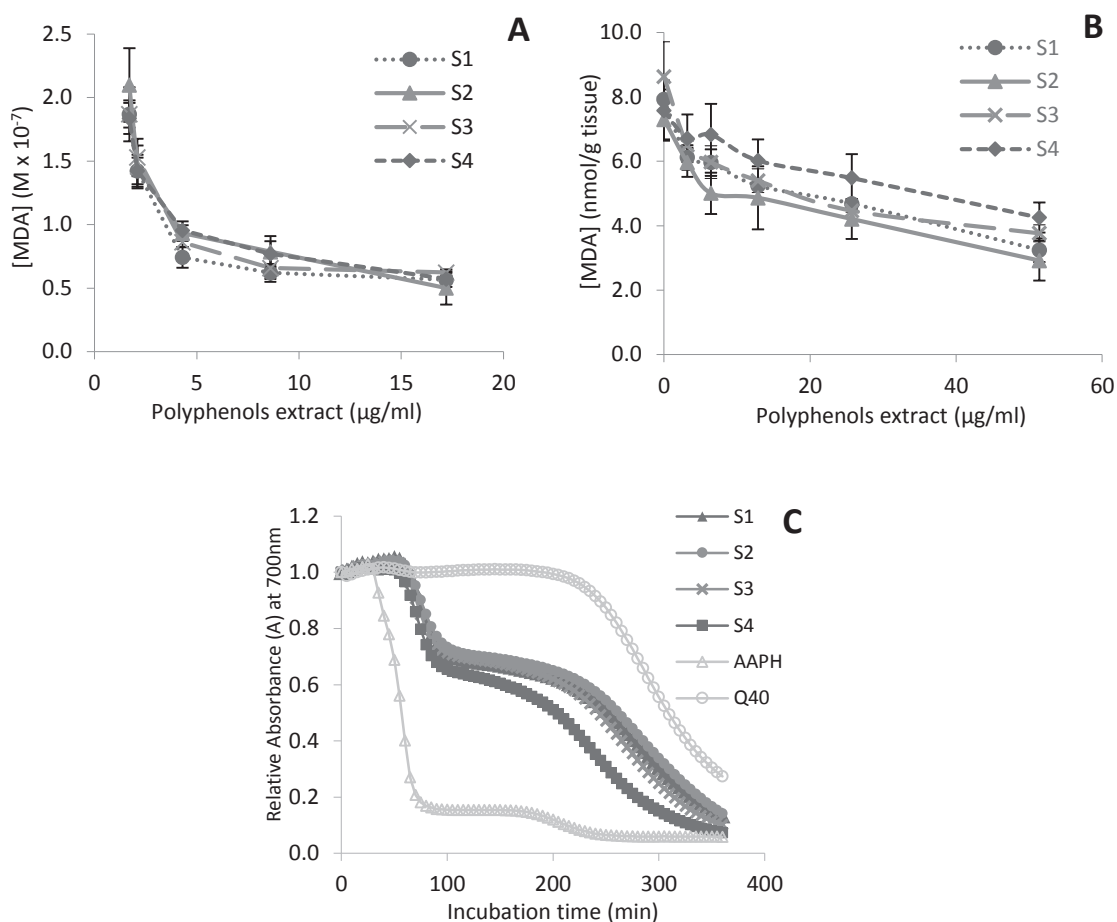


Figure 3.9 Inhibitory capacity of the digested blackberry juice polyphenols against lipid peroxidation in liposomes (A), liver homogenates (B) and erythrocytes (C). Each value is the mean \pm S.E. (three independent experiments).

Table 3.6 Lipid peroxidation inhibitory capacity of the digested blackberry polyphenols

	Liposomes IC ₅₀ ($\mu\text{g/ml}$)	Rat liver homogenate IC ₅₀ ($\mu\text{g/ml}$)	ERYCA mmol QE/g of extract
S1	5.03 \pm 0.49 ^a	32.7 \pm 4.8 ^a	3.87 \pm 0.19 ^a
S2	4.27 \pm 0.27 ^a	29.2 \pm 5.0 ^a	3.88 \pm 0.41 ^a
S3	5.36 \pm 0.62 ^a	37.0 \pm 7.1 ^a	3.80 \pm 0.25 ^a
S4	5.84 \pm 0.41 ^a	39.1 \pm 6.3 ^a	3.54 \pm 0.25 ^a

Each value is the mean \pm SE of three replicate experiments. Means in columns followed by different letters differed significantly ($p < 0.05$).

The lipid peroxidation techniques discussed above involve a direct extracellular interaction radical-antioxidant which is particularly important to avoid damage of the cell membranes. Intracellular ROS are also harmful when the levels are increased and that has been implicated in the modification of biological essential molecules and consequently in

the progress of various diseases (Reuter *et al.*, 2010). The assessment of the inhibitory capacity of intracellular ROS involves a more complex scenario than the lipid peroxidation techniques. The incubation of the polyphenols in a cellular environment caused additional chemical changes of the bioactive components. Additionally, the cellular transporters and the interaction with internal metabolites could modify the antioxidant capacity of the polyphenols. Figure 3.10 show that the digested polyphenols reduce the intracellular ROS in a dose-dependent manner. The concentration of polyphenols necessary to scavenge the 50% of the ROS (IC_{50}) was $106 \pm 2 \mu\text{g/mL}$ for sample 1, $124 \pm 2 \mu\text{g/mL}$ for sample 2, $132 \pm 9 \mu\text{g/mL}$ for sample 3 and $196 \pm 8 \mu\text{g/mL}$ for sample 4. The IC_{50} of the dialyzed sample (S4) showed a statistical significant decrease of 85% in the inhibitory activity compared with the nondigested sample.

The important decrease in the inhibitory capacity of intracellular ROS of the dialyzed fraction (S4) could be explained by the reduced amounts of ellagitannins. Ellagitannins, have been reported to be hydrolyzed to ellagic acid under neutral pH environment and this metabolite is more easily transport across the membrane (Larrosa *et al.*, 2006; Espín *et al.*, 2007). Also, intermediates of the ellagitannins metabolism, such as ellagic acid, have been described to have higher antioxidant activity than their precursors (Reddy *et al.*, 2007; Bialonska *et al.*, 2009). So, it could be suggested that the 20 h of incubation of cells with the extract, favor hydrolysis of ellagitannins and enhances the antioxidant capacity of samples 1, 2 and 3 but in a minor level for the dialyzed fraction (S4) which is less concentrated in ellagitannins.

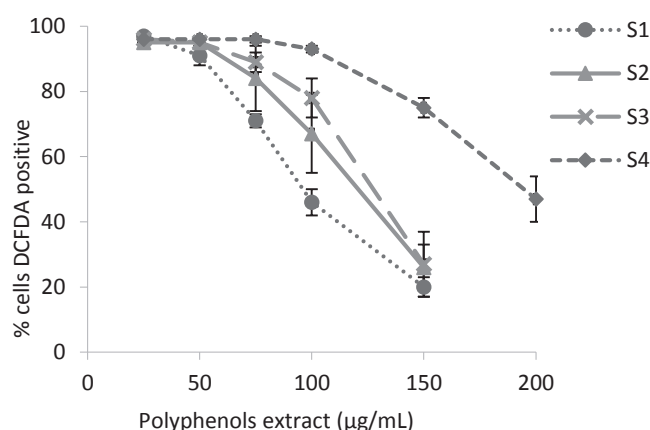


Figure 3.10 Inhibitory capacity of the digested blackberry juice polyphenols against intracellular ROS. Each value is the mean \pm S.E. (three independent experiments).

The results obtained with the different methods used in this study indicate that the blackberry polyphenols subjected to the GIT conditions retain largely their antioxidant capacity. Only the fraction of polyphenols dialyzed (S4), which represents the serum available polyphenols, suffer a partial depletion of its antioxidant ability in the different techniques and this could be attributed to the absence of ellagitannins. It is necessary to perform *in vivo* experiments to confirm the results of our study, mainly because the simulated digestion used did not take in account the effect of microflora of the GIT that degraded polyphenols into smaller phenolic compounds that could be absorbed and contribute to the serum antioxidant capacity. This phenomenon had been demonstrated to play an important role in the case of the ellagitannins (Espín *et al.*, 2007). Nevertheless, the *in vitro* procedure performed in this study mimics the physiochemical changes during the digestion and provide a similar composition to that found *in vivo*, this provide more realistic extracts and reduce the overestimation of biological activities that occur when crude extracts are tested.

3.4 Hypoglycemic, hypolipemic and antioxidant effects of blackberry beverage consumption in streptozotocin-induced diabetic rats

Effets hypoglycémiants, hypolipémiants et antioxydants de la consommation d'une boisson de mûre chez des rats rendus diabétiques par la streptozotocine (STZ)

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Article in preparation / Article en cours de préparation

Abstract

Abnormal glucose metabolism, hyperlipidemia profiles and high levels of radical oxygen species (ROS) are classic features of the development and progression of diabetes. This study evaluates the effect of the consumption of two different blackberry beverages at 25% and 12.5% given orally for 40 days to rats with STZ induced diabetes. The lower dose of blackberry (12.5%) provoked a decrease in glucose (-10.4%), triglycerides (-4.6%) and cholesterol (-21.0%) compared to the control diabetic group. These differences were not statistically significant however, the highest dose of blackberry (25%) showed a significant decrease in glucose (-48.6%), triglycerides (-43.5%) and cholesterol (-28.6%). The plasma antioxidant capacity (PAC) values were also improved because of the blackberry consumption. Whereas the diabetic control group lost 12% of their PAC compare to the control healthy rats, the rats that drank the blackberry beverage lost around 4-5% of their PAC. The higher dose of blackberry (25%) reduced the levels of lipid peroxidation in plasma (-19%) and in kidney tissue (-23%). Regarding the catalase activity, the blackberry intake did not present any effect, suggesting that this berry attenuated the oxidative stress via scavenging activities rather than improving the activities of antioxidant enzymes. The ability to increase the serum antioxidant capacity and decrease the lipid peroxides together with the hypoglycemic and hypolipidemic effect caused by blackberry consumption provides promising data for the selecting blackberry as a dietary complement or adjuvant to the pharmacological management of diabetes.

KEYWORDS

Blackberry juice, *Rubus adenotrichos*, diabetes mellitus, dyslipidemia, plasma antioxidant capacity, lipid peroxidation

3.4.1 Introduction

Abnormal glucose homeostasis and hyperlipidemia profiles are some of the major public health problems in most developed and developing countries. Both disorders play essential roles in the course of chronic illnesses, such as obesity, diabetes and cardiovascular diseases (WHO, 2011). It is well known that diet plays a major role in the development of these metabolic abnormalities, but this factor could also participate as a non-pharmacological complement to classical chronic diseases therapy (Sears & Ricordi, 2012).

Blackberries have been described as fruits with high content of phenolic compounds, especially anthocyanins and ellagitannins (Kaume *et al.*, 2012). These compounds were associated with different health benefits on cardiovascular diseases, cancer, diabetes, inflammatory processes and age-related neurodegenerative diseases (He & Giusti, 2010; Kaume *et al.*, 2012). The mechanisms by which the phenolic compounds confer these health benefits are mainly related to relief of oxidative stress ability. Indeed oxidative status unbalance could be the primary cause of the disease, or a secondary damage linked to the illness (Paredes-López *et al.*, 2010; Finkel, 2011).

High levels of radical oxygen species (ROS) have been linked to the development and progression of diabetes with insulin resistance and hyperglycemia. These free radicals can alter the intracellular signal pathways (Finkel, 2011) and lead to cellular organelles damages, causing lipid peroxidation, protein carbonylation and DNA guanylation (Maritim *et al.*, 2003). Moreover, oxidative stress is described to play an important role in the cardiovascular and renal complications in diabetes as nephropathies, arteriosclerosis and retinopathies (Sedeek *et al.*, 2012). In animal models, pharmacological strategies have been applied to increase antioxidant tissues levels and partially reverse the oxidative stress associated to diabetes and insulin resistance (Anderson *et al.*, 2009). Berries, could be studied as a source of exogenous antioxidants obtained from diet that could play an important role in managing hyperglycemic and hyperlipidemic profiles and homeostasis. For this reason, the present study aims to evaluate the effect of the consumption of a blackberry beverage on selected biochemical parameters and the antioxidant status of streptozotocin induced diabetic rats.

3.4.2 Materials and Methods

3.4.2.a Blackberry juice preparation

Full-ripe blackberries (*Rubus adenotrichos* Schltdl. cv. 'vino') were collected from different farms in the province of Cartago, Costa Rica (altitude 1864 – 2517 m, latitude 09° 39' 57.1"N - 09°44'40.3"N, longitude 83°53'32.1"W - 84°00'06.3"W). A microfiltrated juice was prepared according to Vaillant *et al.* (Vaillant *et al.*, 2008). Blackberries were pressed and the juice treated with a commercial enzymatic preparation, Ultrazym (Novozymes, Bagsvaerd, Denmark) for 1 h at 35 °C with constant agitation. The microfiltration was performed in a tubular ceramic membrane (Membralox® 1 P19-40, Pall Exekia, Bazet, France) with an average pore size diameter of 0.2µm.

Two different blackberry beverages were prepared at 25% and 12.5% from the microfiltrated juice. Both beverages were sweetened with a sucralose artificial sweetener (Splenda®, Mc Neil Nutritionals, USA).

3.4.2.b Analytical characterization of the blackberry juice

Phenolic compounds by HPLC

A 50% blackberry beverage was analyzed by HPLC for anthocyanins and ellagitannins following the protocol described by Mertz *et al.* (Mertz *et al.*, 2007) and Acosta-Montoya *et al.* (Acosta-Montoya *et al.*, 2010). Briefly, the HPLC quantitative analysis was performed with a Dionex liquid chromatograph system equipped with a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) and an endcapped reversed-phase Lichrospher ODS-2 column (250 mm x 4.6 mm i.d., 5 µm) (Interchim, Montluçon, France). The quantification of polyphenols in the juice was performed using calibration curves established with standards of ellagic acid for ellagitannins and cyanidin-3-glucoside for anthocyanins.

Total polyphenol content

Total phenolic content was determined with Folin-Ciocalteu assay modified by George *et al.* (George *et al.*, 2005). Results were expressed as milligrams of gallic acid equivalent (GAE) per liter. Each sample was analyzed in four replicates.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Ou *et al.* (Ou *et al.*, 2002). Fluorescein was used as a fluorescent probe, and oxidation was induced with AAPH. Assays were performed using spectrofluorimeter equipment (Biotek Instruments). ORAC values were expressed as mmol of trolox equivalents (mmol TE/L).

Vitamin C and ascorbic acid content

Total vitamin C content from the blackberry beverage was determined by the quantification of ascorbic acid (AA) and dehydroascorbic (DHA) (Hernández *et al.*, 2006; Wechtersbach & Cigic, 2007). Briefly, AA was extracted using a metaphosphoric acid solution (4%) and DHA was reduced to AA with a tris-[2-carboxyethyl]-phosphine solution (40 mM). Both products were analyzed by HPLC with a C18 column, 5 μ m, 250 x 4.60 mm (Phenomenex Luna, Torrance, CA), and the AA peak was identified and quantified using commercial standards of AA (Sigma, St. Louis, USA). DHA content was calculated by subtracting the initial AA content from the total AA content after the conversion.

Moisture content and soluble solids (%Brix)

The blackberry beverage was analyzed for Moisture Content and Soluble solids (%Brix) using standard AOAC methods (AOAC, 2010).

3.4.2.c Animals

The protocol described in this manuscript was approved by the Institutional Committee for Care and Handling of Experimental Animals of the Universidad de Costa Rica (CICUA # 35-11).

Male Sprague-Dawley rats weighing 200 g \pm 20 g were used in this study. The rats were acclimatized to laboratory conditions for 1 week before the diabetes induction. They were fed ad libitum with regular laboratory pellet diet and water.

Induction of experimental diabetes

Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, Saint-Louis, MO, US) in fasted rats at a dose of 60 mg/kg body weight. STZ was dissolved in cold citrate buffer (0.1 M, pH=4.0) immediately before use. After 72 h, rats with fasting blood

glucose of 250 mg/dl or more were used for the study. Non-diabetic controls received a cold citrate buffer injection. Treatment with the blackberry juice was started on the third day after STZ injection and continued until day 40.

Experimental design

The rats were divided into 5 groups, with 6 to 8 rats on each group. All the groups received regular laboratory pellet diet *ad libitum*. Some groups were given water to drink and others a blackberry beverage, in both cases *ad libitum*. The distribution of the groups was as follows:

Group 1-STZ-H₂O (n=8): STZ-induced diabetic control rats which were given water to drink.

Group 2-STZ-BB25% (n=8): STZ-induced diabetic rats which were given a blackberry beverage diluted to 25% with water.

Group 3-STZ-BB12.5% (n=8): STZ-induced diabetic rats which were given a blackberry beverage diluted to 12.5% with water.

Group 4-Control-Water (n=6): normal control rats which were given water to drink.

Group 5-Control-BB 25% (n=6): normal rats which were given a blackberry beverage diluted to 25% with water.

All groups were maintained for 40 days with the same treatment. Liquid and food intake was monitored daily and body weight was monitored weekly. The fasting glucose level was measured weekly by using glucose reagent strips (Accu chek®, Roche diagnostics, Indianapolis, USA) on blood samples obtained from tail. On day 41, after 12 h of fasting, all rats were sacrificed by decapitation. Blood was collected for biochemical analysis and some portions of liver and kidney were taken out, washed in ice-cold PBS, homogenized and frozen for further analysis.

3.4.2.d Biochemical analysis

Blood chemistry

The estimation of serum glucose, triglycerides, cholesterol, urea, creatinine, uric acid, AST and ALT was done in ROCHE Cobas c501 chemistry analyzer, by using ROCHE kits.

Plasma antioxidant capacity (PAC)

Antioxidant capacity of plasma samples was assessed on the basis of the DPPH free radical-scavenging activity by a modified method of Janaszewska (Janaszewska & Bartosz, 2002). Plasma samples were treated with perchloric acid (3% final concentration) to precipitate the proteins. The samples were centrifuged at 13000 x g for 15 min at 4 °C. The supernatant was recovered, diluted 1:10 in PBS (80 µl) and incubated with 500 µl of DPPH 0.1 mM for 20 min. Finally the absorbance was measured at 517 nm and the results expressed % of scavenging activity with respect to the control without plasma.

Lipid peroxidation assay

Lipid peroxidation in liver, kidney and plasma was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS), which were expressed in terms of malondialdehyde (MDA) content.

The liver or kidney tissue of each rat was homogenized in phosphate-buffered saline (PBS) using Ultraturrax T-25 equipment (Ika-Labortechnik, Staufen, Germany) to obtain a 20% tissue suspension. The suspension was centrifuged at 9000 x g for 15 min to reduce the suspended solids. 0.25 mL of the supernatant was mixed with 0.25 mL of 35% TCA and 0.25 mL of Tris-HCl buffer (50 mM, pH 7.4), and incubated for 10 min at room temperature. Then, 0.5 mL of 0.75% TBA was added and heated at 100 °C for 45 min. After cooling, 0.5 mL of 70% TCA was added, mixed and centrifuged at 2500 x g for 15 min. The absorbance of the supernatant was measured at 532 nm. The TBARS concentration was assessed using the molar absorption coefficient for MDA: $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$, and the results were expressed as µmol MDA/g tissue. For plasma, the homogenization was omitted and the results are expressed as µmol/ml of plasma.

Catalase activity

The catalase activity was determined in liver and kidney homogenates following the Aebi method (Aebi, 1984). Briefly, 995 µl of H₂O₂ (10 mM) was mixed with 5 µl of tissue homogenate (20%) and the decomposition of H₂O₂ was followed by monitoring the absorption at 240 nm. The activity was calculated by using a molar absorption coefficient and the enzyme activity was defined as nmol of dissipating H₂O₂ in 30s per mg of protein.

3.4.2.e Statistical analysis

Data are expressed in mean \pm SE for each group. The comparison between the groups was done using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test. Comparisons with p values < 0.05 were considered to be statistically significant.

3.4.3 Results and Discussion

The main characteristics of a blackberry beverage 50% are shown in Table 3.7. The low pH of the beverage was counteracted with an artificial sucralose to obtain taste acceptance by rats. The composition of the blackberry beverage revealed a low concentration of vitamin C and ascorbic acid, but a high concentration of total polyphenols and a high ORAC value with respect to other berries (Guerrero *et al.*, 2010). These patterns suggest that the antioxidant capacity is mainly caused by the polyphenols present in the juice, as suggested in the literature for other berries (Battino *et al.*, 2009).

The HPLC chromatogram of the blackberry beverage reveals similar retention times for the polyphenols previously identified by Mertz *et al.* using HPLC-DAD/ESI-TRAP-MS (Mertz *et al.*, 2007). Since this study followed the same HPLC method and the same blackberry species as Mertz *et al.*, this suggests that the blackberry beverage, contains four major polyphenols from two groups: the anthocyanins (cyanidin 3-glucoside and cyanidin-3-malonyl glucoside) and the ellagitannins (lambertianin C and sanguin H-6).

Table 3.7 Main characteristics of blackberry beverage 50% (*Rubus adenotrichos*)

Ph	2.75
Soluble solids (°Brix)	7.53
Moisture content (%)	92.8 \pm 0.1
ORAC (mmol of trolox equivalent/L)	14.6 \pm 0.3
Total polyphenols (mg gallic acid/L)	1245 \pm 16
Anthocyanins by HPLC (mg cyanidin-3glucoside/L)	260 \pm 2
Ellagitannis by HPLC (mg ellagic acid/L)	96 \pm 1
Ascorbic acid (mg/1000g)	< 1mg/1000g
Vitamin C (mg/1000g)	< 2mg/1000g

Table 3.8 summarizes the initial and final body weight as well as the liquid and food consumption of all the groups of rats. All diabetic groups presented a significant loss of body weight at the end of the treatment as compared to control non-diabetic water and

blackberry control groups, whose weight gain was 80% and 78%, respectively. Other authors reported similar patterns for plant extracts tested in STZ models. In these studies the extract treatment did not demonstrate an improvement in body weight, despite that they modulated the oxidative stress and presented a hypoglycemic and hypolipidemic activity (Tan *et al.*, 2005; Sefi *et al.*, 2011; Pushparaj *et al.*, 2007). The higher loss of weight in the diabetic rats that drank blackberry beverage 25% was developed the first week because the lack of acceptance of the beverage by the animals. The next 5 weeks the loss of weight was similar to the other diabetic groups (data not shown).

Food and liquid intake were significantly higher in STZ-H₂O and STZ-BB12.5% groups compared to the non-diabetic controls. However, the highest dose of blackberry tested in this experiment (STZ-BB25%) demonstrated a significant decrease in the food and liquid intake compared to the STZ-H₂O group, getting closer to the intake levels of non-diabetic control groups (Table 3.8). This reduction could be the result of better glycemic control produced by the blackberry intake.

No significant differences in body weight, food intake or liquid consumption were evident between the control group that drank water and the control group that drank blackberry beverage, showing that the blackberry does not affect any of these parameters in normal rats.

Table 3.8 Effect of blackberry consumption on body weight, food and liquid intake for streptozotocin induced diabetic rats

Treatment group	Body weight (g)		Water intake (ml/rat/day)	Food intake (g/rat/day)
	<i>initial</i>	<i>Final</i>		
STZ -H ₂ O	203 ± 3 ^a	153 ± 5 ^a	215 ± 7 ^a	34 ± 1 ^a
STZ-BB12.5%	204 ± 7 ^a	170 ± 12 ^a	229 ± 10 ^a	30 ± 1 ^a
STZ-BB25%	208 ± 3 ^a	120 ± 4 ^b	113 ± 8 ^b	18 ± 1 ^b
C-H ₂ O	208 ± 2 ^a	376 ± 8 ^c	34 ± 1 ^c	21 ± 1 ^b
C-BB 25%	201 ± 4 ^a	358 ± 6 ^c	35 ± 2 ^c	19 ± 1 ^b

Values are expressed as mean ± SE. On the same column, values assigned with the same letter are not significantly different at $p < 0.05$.

Table 3.9 shows the effects of blackberry consumption, for 40 days, on blood glucose levels and lipid profiles of the rats. The diabetic rats showed an increase in glucose, triglycerides and cholesterol compared to the non-diabetic groups. The hyperglycemia and dyslipidemia are commonly linked in diabetic states because of an unbalance in some

metabolic pathways. The high levels of lipids in the diabetic rats could be explained because insulin deficiency leads to an increase in lipogenesis and a decrease in the lipoprotein lipase activity, involved triglycerides hydrolysis. Moreover, the insulin deficit does not permit their normal inhibitory action on the HMG-CoA reductase, causing an increase in the cholesterol biosynthesis (Henderson, et al., 2013).

Despite the higher glycemic and lipidemic profiles induced by the STZ, the oral administration of the lower dose of blackberry beverage (STZ-12.5%) provoked a decrease in glucose (-10.4%), triglycerides (-4.6%) and cholesterol (-21.0%) compared to the STZ-H₂O group. These differences were not statistically significant mainly because of the high variability of the values between rats. However, the highest dose of blackberry beverage (STZ-BB25%) showed a significant decrease in glucose (-48.6%), triglycerides (-43.5%) and cholesterol (-28.6%) compare to the STZ-H₂O group. In the case of triglycerides and cholesterol parameters, the values of the STZ-BB25% group returned to the levels of the control non-diabetic groups.

Considering the low bioavailability of blackberry polyphenols (Felgines *et al.*, 2005; Felgines *et al.*, 2009; Kaume *et al.*, 2012), the most reliable mechanism for the hypoglycemic and hypolipemic effect of the blackberry is associated to the inhibition of the digestive enzymes by polyphenols and a consequently reduction in the concentration of free glucose and lipids that are able to be absorbed in gastrointestinal tract and reach the blood. It have been reported for some berries an inhibitory activity against α -amilase, α -glucosidase and pancreatic lipase activities (Basu & Lyons, 2012; Johnson *et al.*, 2011, McDougall *et al.*, 2005). Furthermore, a competitive binding of polyphenols with sodium-glucose co-transporter 1 (SGLT-1) has been reported, (Wootton-Beard & Ryan, 2011; He & Giusti, 2010) which permits to suggest a decrease in the glucose uptake in the intestine enhancing in this way the hypoglycemic effect. Other possible mechanisms suggested in the literature for the hypoglycemic and hypolipidemic effects of different plants include the stimulation of peripheral glucose utilization and an increase of glucose removal from blood. Also, a progressive regeneration of the damaged β cells was proposed in some STZ models (Sefi *et al.*, 2011; Wacho *et al.*, 2012). Nevertheless, the specific mechanism for the hypoglycemic and hypolipidemic effect of the blackberry components needs further investigation.

Table 3.9 shows that blackberry consumption does not have an effect in biochemical parameters of kidney function, such as urea, creatinine and uric acid. For these factors the

diabetic rats showed increased values regarding to the non-diabetic rats, but the treatment with blackberry did not improve significantly these parameters. A similar situation was displayed in Table 3.9, for the evaluation of the hepatocellular injury measured by ALT and AST activity. The activity of ALT was increased significantly in the diabetic rats evidencing a liver damage. However, these higher values were not reduced significantly in rats that consumed blackberry. The AST activity did not increase in the diabetic rats, probably because AST is a mitochondrial isoenzyme release only after a sever destruction of hepatic cells.

Table 3.9 Effect of blackberry consumption on blood chemistry of streptozotocin induced diabetic rats

	STZ-H ₂ O	STZ-BB12.5%	STZ-BB25%	C-H ₂ O	C-BB25%
Glucose*	701±26 ^a	628±58 ^a	360±53 ^b	137±11 ^c	143±10 ^c
Triglycerides*	131±9 ^a	125±20 ^{ab}	74±8 ^{cd}	74±6 ^{bd}	56±3 ^d
Cholesterol*	133±6 ^a	105±7 ^{ab}	95±13 ^b	95±4 ^b	89±3 ^b
Urea*	69±4 ^a	68±4 ^a	60±4 ^a	19±1 ^b	21±1 ^b
Creatinine*	0.2±0.0 ^a	0.1±0.0 ^a	0.2±0.0 ^a	0.3±0.0 ^b	0.3±0.0 ^b
Uric Acid*	1.2±0.1 ^a	1.2±0.1 ^a	1.1±0.1 ^a	2.1±0.2 ^b	1.8±0.2 ^b
ALT [#]	133±10 ^a	113±17 ^a	116±13 ^a	59±5 ^b	48±2 ^b
AST [#]	170±13 ^a	183±44 ^a	211±21 ^a	201±28 ^a	149±8 ^a

Data are expressed as mean ± SE. On the same raw, values assigned with the same letter are not significantly different at $p < 0.05$. * mg/dl, # U/L

Table 3.10 describes some indicators of the antioxidant status as plasma antioxidant capacity (PAC) and the catalase activity in liver and kidney. Also, Table 3.10, reported the lipid peroxidation levels as an indirect evidence of free radical damage.

The PAC values demonstrates that the blackberry consumption improves the antioxidant capacity to intermediate levels between the non-diabetic groups and the STZ-H₂O group. Whereas the STZ-H₂O group lost 12% of their PAC compare to the control non-diabetic groups, the groups that drunk the blackberry beverage only lost around 4-5% of their PAC. Improvements of PAC values because of berries consumption have been documented mainly in human trials (Paredes-López, et al., 2010) (Basu, et al., 2010).

Table 3.10 Effect of blackberry consumption on antioxidant parameters of streptozotocin induced diabetic rats

		STZ-H ₂ O	STZ-BB12.5%	STZ-BB25%	C-H ₂ O	C-BB25%
PAC*	Plasma	36.0±1.7 ^a	38.8±0.7 ^{ab}	39.1±0.7 ^{ab}	40.8±0.8 ^b	40.9±1.0 ^b
[MDA] [#]	Plasma	5.3±0.2 ^a	4.9±0.1 ^{ab}	4.3±0.2 ^b	4.5±0.1 ^b	4.6±0.1 ^b
[MDA] ^{##}	Liver	15.3±1.2 ^a	13.8±0.4 ^a	14.3±1.2 ^a	13.4±1.1 ^a	13.3±0.6 ^a
[MDA] ^{##}	Kidney	8.7±0.4 ^a	7.1±0.8 ^{ab}	6.7±0.5 ^{ab}	5.7±0.3 ^b	6.1±0.2 ^b
Catalase ⁺	Liver	182±10 ^a	210±9 ^a	209±7 ^a	197±9 ^a	204±11 ^a
Catalase ⁺	Kidney	125±8 ^a	133±9 ^a	94±4 ^a	186±10 ^b	183±10 ^b

Data are expressed as mean ± SE. On the same raw, values assigned with the same letter are not significantly different between them at $p < 0.05$. * Plasma antioxidant capacity (% plasma scavenging capacity against DPPH), # $\mu\text{mol/ml}$, ## nmol/g tissue , + U/mg protein

The levels of lipid peroxidation were measured by MDA quantification and the increased MDA concentrations used as an indication of the damage caused by oxidative stress. The lower dose of blackberry beverage (STZ-12.5%) manages to decrease the levels of lipid peroxidation in kidney (-19%) and plasma (-7.5%) to intermediate levels between the non-diabetic groups and the STZ-H₂O group, however the values are no significantly different from the STZ-H₂O group. Additionally, the higher dose of blackberry beverage (STZ-BB25%) reduced the levels of lipid peroxidation to values statistically different ($p < 0.05$) than the STZ-H₂O diabetic group in plasma (-19%) reaching values similar to the non-diabetic rats. Some previous *in vitro* assays on human LDL and lecithin liposomes also demonstrated a protective effect of blackberries to lipid peroxidation (Kaume *et al.*, 2012) however our study confirmed an *in vivo* effect. The confirmation of this *in vivo* activity is important because the literature demonstrates that even though some antioxidants are good free radicals scavengers they do not necessarily succeed protecting the lipid damage *in vivo* (Maritim *et al.*, 2003).

The blackberry beverage tested in this experiment achieved protection from lipid peroxidation, and according to the scientific literature, this attribute has important consequences in preventing the alteration of the transbilayer fluidity gradient and the activities of membrane-bound enzymes and receptors. This lipid integrity in the membrane is an important feature to avoid structural or functional abnormalities that are associated with the progression of diabetes and its complications, for example atherosclerosis (Ma, 2012).

In diabetic conditions, there is a decay in the catalase activity, because of an uncontrolled production of hydrogen peroxide caused by the autoxidation of glucose, protein glycation and lipid peroxidation (Sefi *et al.*, 2011; Aizzat *et al.*, 2010). In our STZ model, the reduction of the catalase activity was evident in the kidney tissue of the STZ-H₂O group compared with the non-diabetic groups. The blackberry intake did not increase the activity to reach the control groups, suggesting that even though the blackberry relief oxidative stress in the animals (as it was demonstrated in the PAC and MDA values), is not by improving the activity of antioxidant enzymes.

The MDA levels and catalase activity in liver tissue were similar between the diabetic and the non-diabetic groups, contrary to what was observed in kidney tissue or plasma. Other studies involving STZ models, coincided that MDA and catalase alterations were not evident in the liver tissue of diabetic animals (Tan *et al.*, 2005; Pushparaj *et al.*, 2000). Tan *et al.* suggested that the liver has a higher capacity to cope with oxidative stress, compared to other organs, and that is the reason why some oxidative markers are not affected in diabetic conditions (Tan *et al.*, 2005).

No significant differences in glucose, lipid profile, renal and liver function parameters, PAC, lipid peroxidation and catalase parameters were evident between non-diabetic group that drank water and the non-diabetic group that drank blackberry beverage. These results demonstrated that the blackberry components do not affect any of these parameters in normal rats.

The improvement of the oxidative profile (PAC, MDA) in the diabetic rats that consumed blackberry beverage, demonstrated that the intake of this fruit modulates the antioxidant status in different tissues, and this may play a role in the metabolic complications related to diabetes mellitus disorder, such as hyperglycemias and hyperlipidemias. Many authors suggest that plant extracts with hypoglycemic effects accomplish their effect in part because of their antioxidant capacities (Wacho *et al.*, 2012; Aizzat *et al.*, 2010; Pushparaj *et al.*, 2000; Sefi *et al.*, 2011; Mukai *et al.*, 2011).

The ability to increase the serum antioxidant capacity and decrease the lipid peroxides together with the hypoglycemic and hypolipidemic effect caused by blackberry consumption suggested a potential to prevent some of the secondary complications of severe diabetes. However, the specific mechanism to explain these possible health beneficial properties need further investigation.

Publications Summary

- The first publication showed that the tropical highland blackberry polyphenols presented an important free radical-scavenging activity, a capacity to inhibit lipid peroxidation and a capacity to inhibitory the production of superoxide, nitrites and IL-6. Some of these abilities are probably related to the modification of redox sensible signaling molecules in the cells, such as the transcription factor NF- κ B. However, the specific mechanisms of action still need to be elucidated and the biological activities need to be verified using *in vivo* models. In spite of that, the biological activities demonstrate for the blackberry could have a positive role in different pathological process.
- The pasteurization conditions tested in the second publication of this study did not impact importantly the polyphenol concentrations or the biological activities. Total polyphenols and ellagitannins were not affected and anthocyanins and free radical-scavenging were slightly reduced. However, the lipid peroxidation and intracellular ROS were not affected because of thermal treatment. These minor impact of pasteurization process in the antioxidant activities may be explained because the loss of some compound due to temperature is balanced by the simultaneous formation of molecules with novel or improved antioxidant properties.
- The results showed after the *in vitro* digestion demonstrated that the gastric digested sample (S2) and the pancreatin/bile non dialyzed fraction (S3) retained similar levels of antioxidant activities than the non-digested blackberry juice (S1). Contrarily the dialyzed fraction (S4) showed significant reduced activities in the free radical assays and the intracellular ROS inhibition. These results evidence that the factor with main impact on the biological activities of the blackberry polyphenols after the GI conditions is the retention of ellagitannins in the colon rather than chemical changes in the phenolic compounds. Nevertheless, under *in vivo* conditions: the unabsorbed ellagitannins could be metabolized by the intestinal microflora generating simple compounds such as ellagic acid or urolithins that might be absorbed at the colon and contribute to the serum antioxidant capacity.

- The rats-STZ model used in the fourth publication showed a decrease in glucose and lipid serum concentrations because of a blackberry beverage consumption. As well, the blackberry consumption modulate the antioxidant status of the animals, particularly in the levels of lipid peroxidation and the plasma antioxidant capacity. This *in vivo* model suggests that the blackberry intake could have a possible beneficial properties, however the mechanisms of action need to be clarify to confirm this claim.

Chapter 4: Complementary Results

4.1 Inhibition of lipid peroxidation by ellagic acid

Despite ellagic acid is not one of the main components of the blackberry polyphenols, ellagitannins are abundant in this species. Studies in pigs have demonstrated that ellagitannins are hydrolyzed to ellagic acid due to the intestinal pH, and then the gut microbiota in the small intestine metabolized the ellagic acid to urolithins D, C, A and B (Espín *et al.*, 2007). For this reason, ellagic acid became an important intermediate in the ellagitannins metabolism and it is important to evaluate its biological activities.

This section showed the results of the inhibitory capacity against lipid peroxidation evaluated for a commercial ellagic acid (Fluka, Buch, Switzerland). For this purpose, we followed the techniques described in the sections 2.3.4 and 2.3.5 of the materials and methods chapter.

The results showed that the concentration of ellagic acid that was necessary to decrease 50% of the MDA concentration in control liposomes (IC_{50}) was $3.01 \pm 0.37 \mu\text{g/ml}$ for the liposomes and $4.6 \pm 0.2 \mu\text{g/ml}$ for the liver tissue (Figure 4.1). These results are significantly better ($p < 0.05$) than the values obtained for the blackberry extract (chapter 3, publication 1), $7.0 \pm 0.5 \mu\text{g/ml}$ for the liposomes and $20.3 \pm 4.2 \mu\text{g/ml}$ for the liver tissue.

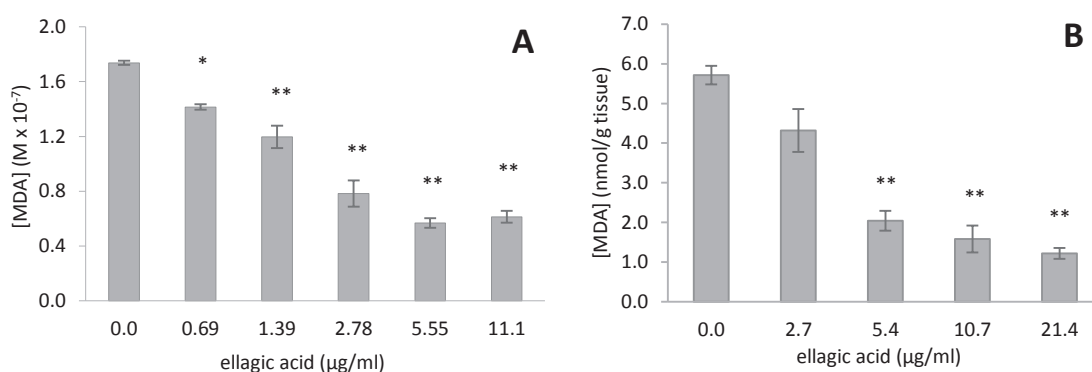


Figure 4.1 Inhibitory capacity of ellagic acid against lipid peroxidation in liposomes (A) and liver homogenates (B). Each value is the mean \pm S.E. (three independent experiments). * $p < 0.05$, ** $p < 0.01$ compared to controls without blackberry treatment.

The high inhibitory activity of lipid peroxidation showed by the ellagic acid could contribute *in vivo* to the antioxidant capacity of the blackberry, because the release of this compound from the ellagitannins during digestion could balance the loss of naturally antioxidants compounds of the blackberry (Nicoli *et al.*, 1999).

4.2 Inhibition of superoxide anion production by the digested blackberry samples

The samples S1, S3 and S4 obtained after the *in vitro* digestion methodology described in the section 2.1.3 were evaluated for their capacity to inhibit superoxide anion production following the protocol described in the section 2.3.8. Sample 2 (S2) was not analyzed since it has a similar chemical composition to Sample 1 (S1).

The results of the superoxide inhibitory capacity of digested polyphenol extracts S1, S3 and S4 are showed in Figure 4.2, and they demonstrated that there are no significant differences ($p < 0.05$) between the non-digested polyphenol extract (S1) and the pancreatic/bile digested polyphenol extracts, non-dialyzed (S3) or dialyzed (S4). The concentration of phenolic extract that was necessary to decrease 50% of the superoxide production (IC_{50}) was $155 \pm 6 \mu\text{g/ml}$ for S1, $145 \pm 11 \mu\text{g/ml}$ for S2 and $147 \pm 14 \mu\text{g/ml}$ for S4.

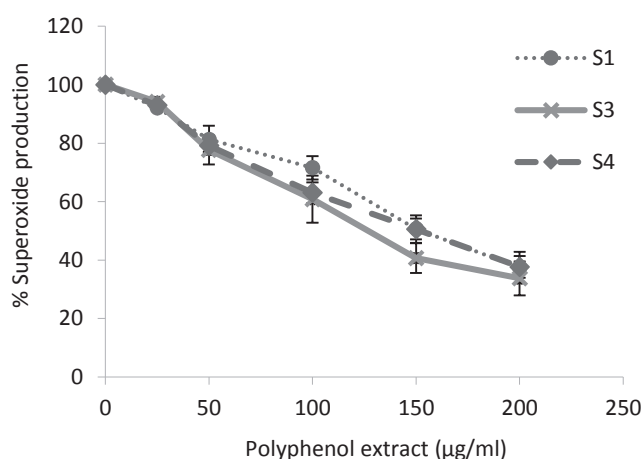


Figure 4.2 Inhibitory capacity of the digested blackberry polyphenols extracts against superoxide anion production.

Each value is the mean \pm S.E. (three independent experiments).

The similar IC_{50} values of the non-digested polyphenol extract (S1) and the pancreatic/bile digested polyphenol extracts, non-dialyzed (S3) or dialyzed (S4), suggests that the compound involved in these biological activities are preserved after the digestion enzyme treatments. Contrarily, industrial production of the blackberry juice greatly affected the capacity to inhibit superoxide production. Whereas the IC_{50} of the polyphenol extract prepared from the blackberry fruit showed a value of $80 \pm 4 \mu\text{g/ml}$ (extract of publication 1), the polyphenols extract prepared from the juice (S1, publication 2) showed a value of $155 \pm 6 \mu\text{g/ml}$. This reduction could be explained by the elimination of bioactive compounds in the presscake during the clarification steps or by the hydrolysis of antioxidant molecules induced by the pectinolytic enzymes (Manach *et al.*, 2004; Hager *et al.*, 2008; Hager *et al.*, 2010).

Chapter 5: General Discussion

The first publication of this thesis demonstrates that blackberry phenolic compounds have an important antioxidant capacity, not only reflected in high DPPH and ORAC values but also showed by an *in vitro* capacity to inhibit, superoxide production, lipid peroxidation and inflammation. All these conditions are implicated in common pathologies, such as cardiovascular disease, cancer, metabolic syndrome, neurological decline and diabetes (Wootton-Beard & Ryan, 2011). These biological activities are evidence of the protective effects of berry consumption as it has been reviewed for other berries (Paredes-López *et al.*, 2010; Del Rio *et al.*, 2010; Basu & Lyons, 2012; Basu *et al.*, 2010). However, it is recognized that extrinsic conditions based on geographical origin, as well as different climatic and soil growing conditions, could be recognized to cause infra-specific differences between the same species (Díaz *et al.*, 2008).

The characteristic phenolic profile of the *Rubus adenothichos* species consisting of the anthocyanins cyanidin-3-glucoside and cyanidin-3-malonyl-glucoside and the ellagitannins lambertianin C and sanguin H-6, changed in different magnitudes in blackberry fruit extract used in publication 1 and blackberry juice extracts pasteurized and digested of publications 2 and 3. The first difference is the reduction in ellagitannins concentration from blackberry fruit extract used in the publication 1 (409 µg/mg of lyophilizate) and blackberry microfiltrated juice extracts used in publications 2 and 3 (328-353 µg/mg of lyophilizate). Gancel *et al.* documented the effect of industrial processing of blackberries and suggested that the removal of cell-wall fragments, seeds and the thin peel of the drupelets from the fruit during the microfiltration reduce the polyphenol concentrations available in the final product (Gancel *et al.*, 2011). Particularly seeds are documented in some studies as rich sources of ellagitannins (Larrosa *et al.*, 2006).

The second modification in polyphenol concentrations of blackberry samples used in these studies, was the changes induced by the pasteurization process. However, this thermal treatment did not cause relevant modifications in the polyphenol profile; only the anthocyanins showed a significant reduction of a 7% in the higher temperature tested (92°C). Ellagitannins, which are the predominant components of the blackberry *R. adenotrichos*, are characterized as heat-stable compounds in the literature (Hager *et al.*,

2010) (Mena *et al.*, 2013) and this feature was congruent with the results obtained in this study.

The third important difference in the polyphenol profile of blackberry samples analyzed in these studies was the effect induced by the dialysis process in the *in vitro* digestion. The dialyzed fraction practically did not contain ellagitannins and mostly contained anthocyanins. These demonstrated that under physiological conditions, the polyphenol blackberry profile could change drastically, and hence, the importance to study the bioavailability of the molecules with potential benefit effects. In particular, ellagitannins are phenolic compounds with complex aspects involved in their bioavailability, especially because of the participation of colonic flora (Espín *et al.*, 2007; Larrosa *et al.*, 2006). Ellagitannins fermentation by colonic flora releases ellagic acid and the complementary results of this study showed that this metabolic intermediate have high inhibitory activities against lipid peroxidation, even better than the natural antioxidants compounds of blackberry extract. Thereby ellagic acid release could have an important contribution to the total antioxidant capacity of blackberry.

Analyzing together the phenolic changes in all the samples analyzed in these studies, it is evident that blackberry anthocyanins and ellagitannins are strongly resistant to industrial processes, such as microfiltration and pasteurization. This robustness is mainly caused by the kind of saturated structures and glycosylations of the anthocyanins and ellagitannins present in the blackberry, which provide more stability (Rawson *et al.*, 2011; Srivastava *et al.*, 2007). However, as it was showed in the *in vitro* digestion model, these same robust chemical structures do not permit an easy absorption in the gastrointestinal tract, especially in the case of the large complexes that form the ellagitannins.

Most of the studies in scientific literature that evaluated the effects of digestion or thermal treatments focused on the changes in the phenolic composition. However, these phenolic changes do not necessarily correspond to deterioration of the health promoting activities of polyphenols. Because of this reason, the studies performed in this thesis tried to use other more relevant antioxidants assays which are closer to physiological scenarios, such as the evaluation of the inhibitory capacity against lipid peroxidation or the evaluation of intracellular radical scavenging activity. However, the antioxidant assays used demonstrated variability and lack of correspondence between them. This phenomenon is well documented in scientific literature, which explained that each assay evaluates the biological activity against diverse radicals and by different mechanisms (Huang *et al.*,

2005; Niki, 2010; Niki, 2011). In particular, are important the differences in the chemical structure and the kinetic or stoichiometric factors of the free radicals used in each assay. These differences could cause variations in the performance of the same antioxidant in different assays (Niki, 2010; Niki, 2011).

The DPPH technique and the NO-scavenging assay, which measured the activity against nitrogen derived radicals, showed better correlation between the antioxidant activity and changes in the phenolic composition caused by the pasteurization process or the *in vitro* digestion. ORAC assay and the assays measuring the inhibition of lipid peroxidation (liposomes, liver homogenates and ERYCA), all evaluate the activity against peroxy radicals. These assays showed less correspondence between the biological activity and the changes in the phenolic content of the samples. This suggests that the most stable components of blackberry extracts, probably ellagitannins, are the ones responsible of the activity against the peroxy radical (Battino *et al.*, 2009; Lee *et al.*, 2012). According to Niki, these kind of assays which evaluate the antioxidant activity against peroxy radicals have better biological significance, because this radical is the one with most toxic effects physiologically and it is involved in chain-carrying radical reactions (Niki, 2011).

The intracellular inhibitory activity of ROS tested for the different samples in this thesis demonstrated that blackberry polyphenols could reach the cytosol of the mammal cells and exert their antioxidant capacity inside the cell. This is an important feature of blackberry polyphenols, because part of the oxidative stress associated to several pathologies occur inside of cells, and the signaling pathways affected by deleterious ROS mostly happened in the cytosol or mitochondria environment (Reuter *et al.*, 2010; Wootton-Beard & Ryan, 2011). For this reason, it is crucial for natural antioxidants to be able to reach the intracellular environment, as it was evidenced for the blackberry polyphenols.

The *in vitro* studies usually are criticized because they used polyphenol aglycones or sugar conjugates rather than their active metabolites. However, the *in vivo* model used in this study (Publication 4) demonstrated that blackberry is capable to modulate the oxidative stress and produce a hypoglycemic and hypolipidemic activity in a mammal organism (rats). This *in vivo* evidence together with the *in vitro* assays performed in the digested samples (Publication 3) provides promising data that target the blackberry as a source of biologically available polyphenols that exert health benefits. The kind of assays performed in this thesis, particularly *in vivo* studies and digestion models, helps to

document the bioavailability of the active compounds of the fruits. This is particularly important because “in the field of functional foods, it is not important to know how much of a nutrient is present in specific food or dietary supplement, but it is even more important to know how much of it is bioavailable” (D’Archivio *et al.*, 2010).

The mechanisms of action linked to biological activities reported in diabetic rat models or in other antioxidant assays performed in this thesis still need to be elucidated. However it is probable that the general reduction of oxidative stress in the cells may play a role in preventing the metabolic unbalance related to the different pathologies (Wacho *et al.*, 2012; Aizzat *et al.*, 2010; Pushparaj *et al.*, 2000; Sefi *et al.*, 2011; Mukai *et al.*, 2011).

Chapter 6: Conclusion and Perspectives

The blackberry species evaluated in this study exhibits promising biological activities that need to be complemented with more studies to determine the inclusion of this fruit in the group of functional foods. A strong antioxidant activity was demonstrated *in vitro*: a scavenging activity against free radicals, an inhibitory effect on superoxide production, a protective effect against lipid peroxidation and also an intracellular ROS scavenging activity. Besides, blackberry polyphenols demonstrated an *in vitro* capacity to modulate some molecules involved in the inflammatory pathways (NO, iNOS, IL-6). However, more research is needed to fully understand which molecules are effective and what mechanisms are involved in the biological activities documented. Specifically in the case of lipid peroxidation it is necessary to identify if the lower levels of MDA are only a result of a radical scavenging activity or if other mechanisms are involved. For the ability to inhibit superoxide production it is required to elucidate which protein of the NADPH pathway is modulated by the blackberry polyphenols. In the case of intracellular ROS scavenging activity, it is essential to clarify the mechanism of internalization of the phenolic compounds and the possible interactions of these compounds with normal signal transduction pathways. For the anti-inflammatory activity it is necessary to evaluate the effects of polyphenols on other inflammatory molecules, such as cytokines or secondary messengers. Besides for all these biological activities it is required to confirm if they occur during *in vivo* scenarios.

The pasteurization conditions evaluated in this study did not show significant decreases in the antioxidant capacity in most of the assays used. This suggested that the blackberry phytochemicals, mainly the ellagitannins, are stable and not easily affected or degraded by thermal treatments. However other pasteurization conditions could be tested, particularly longer periods of thermal treatment and the effect of dissolved oxygen during pasteurization. Also, to have a better approach to the impact of industrial production of blackberry juice, it is necessary to evaluate the effect of other industrial processes, such as enzyme treatments, homogenization, sieving, cooling-freezing, storage, etc. As well, the impact of novel non-thermal processing technologies used to inactivate microorganisms, such as ozone processing, ultrasound, radiation and pulse electric fields, may be studied.

The *in vitro* digestion performed in this study demonstrated that biological activities were maintained largely in the fractions kept in the gastrointestinal tract. This suggests that blackberry polyphenols could exert their antioxidant capacity in GIT tissues and produce benefits in some stomach or colon pathologies. The dialyzed fraction representing the serum available fraction, even if it was partially diminished in their phenolic content, showed that the polyphenols absorbed retained an important part of their biological activities, suggesting that systemic effects of blackberry consumption are possible. However, to complete the study of blackberry polyphenols bioavailability it is still necessary to evaluate the interactions between berry phenolics and colon microflora. It is known that these bacteria cause substantial structural modifications in the polyphenols, producing metabolites that could be absorbed by the colon and contribute to the bioactivity of ingested polyphenols (Espín *et al.*, 2007), however this has to be studied particularly for the blackberry polyphenols. To achieve these additional objectives, some *in vitro* anaerobic fecal fermentation models could be applied. As well, *in vivo* studies with animals may be helpful to evaluate the phenolic biodistribution.

Finally, the *in vivo* assay performed in this study with diabetic rats showed promising results concerning the lipidic and glycemic profile and also the serum and tissue antioxidant status. However, it is necessary to clarify the mechanisms of action of blackberry polyphenols in their hypoglycemic and hypolipidemic activities. It is also important to elucidate the bioactive components of the blackberry beverage. The diabetic rats model could be repeated with different doses of blackberry juice and longer periods of intake. However, to evaluate if the results obtained in rats are transposable to humans, human trials are required.

Despite that fact that the specific mechanism to explain the biological activities of blackberry polyphenols need further investigation, this work provides promising data for selecting blackberry as a candidate to be include in the group of functional foods.

Chapter 7: References

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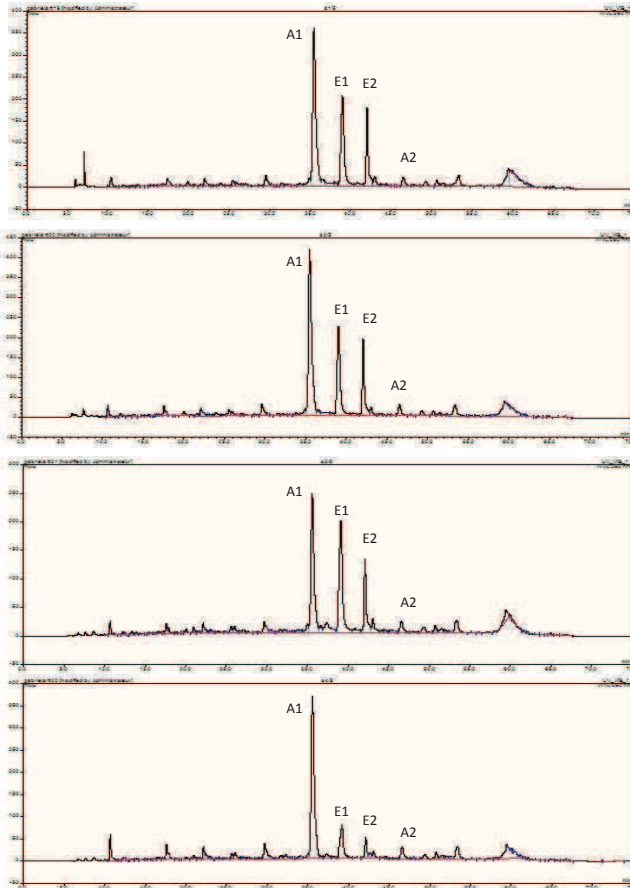
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Chapter 8: Annex

Comparative cromatograms of the digested samples cited in the section 3.3



S1
BB Juice (non digested)

S2
BB Juice + Pepsin
(gastric conditions)

S3
BB Juice + Pepsin + Pancreatin
(non dialyzed)

S4
BB Juice + Pepsin + Pancreatin
(Dialyzed)

Corresponding tentative identification of peaks according to Mertz *et al.* 2007

A1 Cyanidin-3-glucoside

A2 Cyanidin-3-malonyl glucoside

E1 Lambertianin C

E2 Sanguin H-6

Study of biological activities of the tropical highland blackberry polyphenols and the impact of digestion and pasteurization processes.

Abstract: This study evaluated biological activities of blackberries (*Rubus adenotrichos* Schlttdl.) and the effects of digestion or pasteurization processes on these activities. The blackberries phenolic extract showed an ORAC value of 4.34 ± 0.14 mmol TE/g, higher than those of quercetin and ellagic acid. It also protected liposomes and liver homogenates against lipid peroxidation; with IC_{50} of 7.0 ± 0.5 and 20.3 ± 4.2 $\mu\text{g/mL}$, respectively. The blackberry polyphenols inhibited nitrite production in J774A.1 cells stimulated with LPS+IFN γ due to down-regulation of iNOS protein expression, suggesting an anti-inflammatory potential.

The biological activities of the fruits could be affected by the industrial processes. The pasteurization of a blackberry juice caused a 7% decrease in the anthocyanins concentration of the highest temperature tested, 92 °C. For the same sample, a significant reduction of 27% and 15% were showed in the DPPH and NO scavenging capacity, respectively. However, no significant differences were evident neither for the peroxidation inhibitory capacity nor for the intracellular antioxidant activity after the pasteurization step.

The antioxidant capacity is largely dependent of their bioavailability and because of this a blackberry juice was digested *in vitro* to obtain samples that represent the major stages of the digestion process. The gastric digestion had slight impact on polyphenolic composition while the pancreatin/bile digestion had marked effects on polyphenolic composition, particularly in the ratio anthocyanins-ellagitannins. These changes in phenolic composition caused in the dialyzed fraction a reduction of 33% and 35% in the activity against nitrogen derived radicals DPPH and NO respectively. As well the capacity to inhibit intracellular ROS was decrease by an 85% in the dialyzed fraction. Nevertheless, no significant differences were evident in the capacity to inhibit lipid peroxidation.

Finally, this study used an *in vivo* model with diabetic rats to evaluate the effect of the consumption of a blackberry beverage. The blackberry intake provoked a decrease in plasmatic glucose (-48.6%), triglycerides (-43.5%) and cholesterol (-28.6%) levels in the diabetic rats with respect to the diabetic controls animals. The diabetic rats which consumed blackberry, improved (+7%) the plasma antioxidant capacity (PAC) values and reduced the levels of lipid peroxidation in plasma (-19%) and in kidney tissue (-23%). This study suggests that blackberry could be considered as a candidate for the group of functional foods even when considering the bioavailability aspects of the phenolic compounds and the pasteurization effects.

Key words: Blackberry, *Rubus adenotrichos*, antioxidant activity, anti-inflammatory activity, diabetes mellitus, pasteurization, bioavailability.

Étude des activités biologiques des polyphénols de la mûre tropicale de montagne et détermination de l'impact des processus de digestion et de pasteurisation.

Résumé: Dans cette étude nous avons évalué les activités biologiques de la mûre tropicale de montagne (*Rubus adenotrichos* Schltdl.) et les effets de la digestion ou des procédés de pasteurisation sur ces activités. L'extrait polyphénolique de mûre présente un pouvoir antioxydant déterminé par la méthode ORAC de 4.34 ± 0.14 mmol TE/g, plus élevé que celui de la quercétine et de l'acide ellagique. L'extrait de mûre présente des propriétés protectrices des liposomes et des homogénats de foie contre la peroxydation lipidique avec des IC_{50} de 7.0 ± 0.5 et 20.3 ± 4.2 mg/ml, respectivement. Les polyphénols de mûre inhibent la production de nitrite dans par les macrophages murins J774A.1 stimulées par le mélange LPS, IFN. Cette activité est confirmée par la diminution du taux de NO synthase inductible dans ces mêmes cellules.

Les activités biologiques des fruits pourraient être affectés par les procédés industriels. Le processus de pasteurisation du jus de mûre a provoqué une diminution d'environ 7% de la concentration en anthocyanes pour la température la plus élevée évalué, 92°C. Pour le même échantillon, une réduction significative de 27% de l'activité anti-oxydante mesurée par la méthode DPPH et de 16% de la capacité de complexations des radicaux NO. Toutefois, aucune différence significative n'a été mise en évidence autant pour la capacité d'inhibition de la peroxydation que pour l'activité anti-oxydante intracellulaire.

Les effets *in vivo* sont largement dépendants de la biodisponibilité des composés phénoliques et de ce fait un jus de mûre a été digéré *in vitro* pour obtenir des échantillons qui représentent les grandes étapes du processus de digestion. La digestion gastrique a peu d'influence au contraire de la digestion intestinale sur la composition polyphénolique de l'extrait de mûre en particulier au niveau du rapport anthocyanines/elagitannins. La fraction dialysable présente une réduction de 33% et de 35% de l'activité contre les radicaux dérivés de l'azote DPPH et NO, respectivement. De même, la capacité d'inhiber au niveau intracellulaire les radicaux libres (ROS) a diminué d'environ 85%. Toutefois, aucune différence significative n'a été mise en évidence au niveau de la capacité d'inhiber la peroxydation lipidique.

Finalement, cette étude a utilisé un modèle *in vivo* pour évaluer l'effet de la consommation d'une boisson de mûre chez des rats diabétiques. L'apport de mûre a provoqué une diminution du glucose (-48.6%), des triglycérides (-43.5%) et du taux de cholestérol (-28.6%) plasmatique des rats diabétiques par rapport aux témoins diabétiques qui n'ont pas consommé la boisson de ce fruit. De plus, les rats diabétiques qui consomment de la mûre ont vus améliorées les valeurs de la capacité antioxydante du plasma (+7%), tandis que les niveaux de peroxydation lipidique dans le plasma (-19%) et dans les tissus rénaux (-23%) ont été réduits. Cette étude suggère que la mûre pourrait être considérée comme un candidat pour le groupe des aliments fonctionnels, même si l'on considère les aspects de biodisponibilité et les effets du procédé de pasteurisation.

Mots clés: Mûre, *Rubus adenotrichos*, activité anti-oxydante, activité anti-inflammatoire, diabète sucré, pasteurisation, biodisponibilité.