UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Facultad de Ciencias de la Actividad Física
y del Deporte

Development of weight-loss products based on natural polyphenols: Diet, exercise and microbiota interactions

INTERNATIONAL PHD TESIS

PhD Program in Biomedicine Research Human performance,
Physical Activity and Health Research Group

MIRIAM GELABERT REBATO

Las Palmas de Gran Canaria, 2022

Dedicada a la meva família;

als meus pares, al meu germà i al Sergi.

"La meva vida té llum gràcies a vosaltres.
La vostra presència és l'arbre que em sustenta,
les arrels del qual sempre troben l'aigua,
l'aigua que donarà el fruit del futur.
I jo, agraïda
celebro al que neix i al que mor
sense oracions i sense Déus
així com el poble camina sobre la pols.
Cansats del dia tornem ansiosos
a l'ambrosia nit de la llar,
sentint l'amor i l'harmonia,
ens omplim de gratitud.
La gratitud dona sentit al passat,
porta pau al present
i crea una visió pel demà.
Així que no deixem d'aprendre
perquè la vida no deixa mai d'ensenyar-nos.
Caminem junts el viatge de la vida,
no acceptem el seu destí,
ja que només així
seguirem omplint
els anys de vida."

MGR

ULPGC Universidad de Las Palmas de Gran Canaria | Escuela de Doctorado

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

ESCUELA DE DOCTORADO

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Tesis Doctoral presentada por Dª **Miriam Gelabert Rebato.**

Dirigida por el Dr. D **José Antonio López Calbet.**

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Las Palmas de Gran Canaria, a 13 de febrero de 2022

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Study 1 – Journal Article

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Study 2 – Journal Article

Gelabert-Rebato M, Wiebe JC, Martin-Rincon M, Galvan-Alvarez V, Curtelin D, Perez-Valera M, Habib JJ, Pérez-López A, Vega T, Morales-Alamo D, Calbet JAL. Enhancement of Exercise Performance by 48 Hours, and 15-Day Supplementation with Mangiferin and Luteolin in Men. *Nutrients.* 2019;11(2):344.

Study 3 – Journal Article

Gelabert-Rebato M, Martin-Rincon M, Galvan-Alvarez V, Gallego-Selles A, Martinez-Canton M, Vega-Morales T, Wiebe JC, Fernandez-Del Castillo C, Castilla-Hernandez E, Diaz-Tiberio O, Calbet JAL. A Single Dose of The Mango Leaf Extract Zynamite® in Combination with Quercetin Enhances Peak Power Output During Repeated Sprint Exercise in Men and Women. *Nutrients*. 2019;11(11):2592.

Study 4 – Journal Article

Martin-Rincon M, Gelabert-Rebato M, Galvan-Alvarez V, Gallego-Selles A, Martinez-Canton M, Lopez-Rios L, Wiebe JC, Martin-Rodriguez S, Arteaga-Ortiz R, Dorado C, Perez-Regalado S, Santana A, Morales-Alamo D,, Calbet JAL. Supplementation with a Mango Leaf Extract (Zynamite®) in Combination with Quercetin Attenuates Muscle Damage and Pain and Accelerates Recovery after Strenuous Damaging Exercise. *Nutrients*. 2020; 12(3):614.

Other contributions

Study 1 – Oral Presentation

Gelabert-Rebato M, Wiebe JC, Martin-Rincon M, Gericke N, Perez-Valera M, Curtelin D, Galvan-Alvarez V, Lopez-Rios L, Morales-Alamo D, Calbet JAL. *Mangifera indica* L. Leaf extract in combination with luteolin or quercetin enhances VO_2peak and peak power output, and preserves skeletal muscle function during ischemia-reperfusion in humans. *23th Annual congress of the European College of Sports Science*. 4-7th July 2018, Dublin, Ireland.

Study 2 – Oral Presentation

Gelabert-Rebato M, Wiebe JC, Martin-Rincon M, Galvan-Alvarez V, Curtelin D, Perez-Valera M, Habib JJ, Perez-Lopez A, Vega T, Morales-Alamo D, Calbet JAL. Enhancement of exercise performance by 48 hours, and 15-day supplementation with mangiferin and luteolin in men. *24th Annual congress of the European College of Sport Science*. 3-6th July 2019, Prague, Czech Republic.

Study 3 – Oral Presentation

Gelabert-Rebato M, Galvan-Alvarez V, Martin-Rincon M, Gallego-Selles A, Martinez-Canton M, Morales-Alamo D, Calbet JAL. A single dose of the mango

leaf extract Zynamite® in combination with quercetin enhances peak power output during repeated sprint exercise in men and women. *International Sport Forum Madrid.* 15-16th November 2019, Madrid, Spain.

Study 4 – Poster Presentation

Gelabert-Rebato M, Martin-Rincon M, Galvan-Alvarez V, Gallego-Selles A, Martinez-Canton M, Lopez-Rios,L, Wiebe JC, Martin-Rodriguez S, Arteaga R, Dorado C, Perez-Regalado S, Santana A, Morales-Alamo D, Calbet JAL. Supplementation with a mango leaf extract (Zynamite®) in combination with quercetin attenuates muscle damage and pain and accelerates recovery after strenuous damaging exercise. *25th Annual congress of the European College of Sports Science.* 28-30th October 2020, VIRTUAL Congress.

Study 5 - Poster Presentation and YIA award

Gelabert-Rebato M, Martin-Rincon M, Pérez-Valera M, Galvan-Alvarez V, Morales-Alamo D , Dorado C, Boushel R, Jostein H, Calbet JAL. Sex differences in metaboreflex activation and functional reserve during exercise to exhaustion revealed by post-exercise ischaemia and repeated supramaximal exercise. *26th Annual congress of the European College of Sports Science.* 8-10th September 2021, VIRTUAL Congress.

Certificate in Appendix 1

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INTRODUCTION

RESEARCH STAY ABROAD

The PhD candidate completed an International research stay abroad in the following destination:

- Institute of Sports Medicine, Bispebjerg Hospital.

Supervisor: Michael Kjær, MD DMSci; Head of Department, Chief Physician at the Institute of Sports Medicine, Bispebjerg Hospital and Professor at the Universitiy of Copenhagen.

Mentor: Abigail Louise Mackey, PhD; Associate professor and head of research group at the Center of Healthy aging, Department of Biomedical Sciences, University of Copenhagen and Institute of Sports Medicine, Bispebjerg Hospital.

Date: from 12th May 2021 to 12th September 2021.

Duration: 4 months.

Certificate in Appendix 2.

I

ABSTRACT

ABSTRACT

Background

The benefits of fruits, vegetables and herbs on human health has been well known for centuries. Plant polyphenols originating from these foods share a powerful antioxidant effect, as well as anti-inflammatory properties decreasing the level of reactive oxygen species in the human body. Adequate intake of polyphenols could confer health benefits, especially regarding obesity and chronic diseases. Obesity and sedentary lifestyle are among the most prevalent health problems worldwide, and the treatment against obesity through exercise and a proper diet could be improved by adding supplements of natural polyphenol extracts. For these reasons, the aim of this thesis was to find a specific polyphenolic combination, based on natural polyphenols, to develop new nutritional supplement formulations with positive effects specifically on physical performance, body composition and general health for human subjects. Therefore, the main goals of the present PhD thesis were: 1) to determine the acute and prolonged effects of oral supplementation with a combination of luteolin (peanut husk extract (PHE) containing 95% luteolin) and mangiferin (mango leave extract (MLE) containing 60% mangiferin, Zynamite®) at low and high doses may enhance exercise performance, muscle metabolism, and brain and muscle oxygenation (*Study 1*); 2) to determine whether MLE administered in two different formulations: one with quercetin (Q) and tiger nut extract (TNE), and another with luteolin, has an enhancing effect on sprint performance and if protects skeletal muscle from the negative effects of ischemia-reperfusion (IR) applied immediately at the end of sprint exercise (*Study 2*); 3) to determine whether a single dose of Zynamite® in combination with a small amount of quercetin, or with quercetin combined with sunflower lecithin (SFL) administered 1 h before exercise, increases exercise performance during repeated sprint exercise, and if these effects can be improved by adding sunflower lecithin

(*Study 3*); 4) to determine whether Zynamite® administered in combination with a small amount of quercetin, facilitates recovery after repeated damaging exercise and if attenuates exercise induced muscle damage (EIMD) and pain.

Material and Methods

The present PhD thesis is composed by 4 studies in which young men and women volunteered for participation (12 men aged 21.3 ± 2.1 years (*Study 1*), 17 men aged 22.7 ± 2.1 and 13 women aged 27.0 ± 2.2 years (*Study 2*), 26 men aged 23.1 ± 2.2 and 24 women aged 23.5 ± 2.9 years (*Study 3*), 30 men aged 23.1 ± 2.5 and 18 women aged 23.3 ± 2.4 years (*Study 4*). The protocol of the main experiment slightly changed between the 3 first studies, but in all of them sprints and IR were performed, as well as testing different polyphenolic treatments (mainly containing mangiferin, luteolin and quercetin) compared to placebo (maltodextrin) on sports performance. The treatment time, the polyphenolic combination and the doses changed according to the study. Briefly, anthropometrics (body mass and height) and body composition (by dual-energy x-ray absorptiometry) were evaluated. All sprints were performed with the same cycle ergometers that measure the instantaneous peak power output (Wpeak) and mean power output (Wmean). The maximal fat oxidation rate (MFO), maximal oxygen uptake (VO_2max) and anaerobic capacity (maximal accumulated oxygen deficit), were measured with a calibrated metabolic cart and analyzed the respiratory variables breath by breath and averaged every 20 s during the tests. Cerebral and muscle oxygenation was measured using near-infrared spectroscopy employing spatially resolved spectroscopy to obtain the tissue oxygenation index (TOI). Moreover, muscle pain and effectiveness of concealment were collected. Moreover, in the fourth article, EIMD was induced by a ten-kilometer race followed by 100 drop jumps.

Results

During sprint exercise, the polyphenolic combinations compared to placebo improved exercise performance, facilitated muscle oxygen extraction, and improved brain oxygenation. Polyphenolic treatments also improved oxygen extraction during IR and sport performance just after ischemia-reperfusion (*Studies 1-3*). However, there were no differences between low and high dose of MLE+Lut combination on exercise performance (*Study 1*). MLE supplementation increased Wpeak and improved Wmean by 5.0–7.0 % (*Study 2*), as well as Wpeak during the first three Wingate tests of the third study (by 2.8 and 3.8 % in MLE+Q+SFL and MLE+Q trials, respectively). After ischemia, MLE+Q+TNE and MLE+Lut treatments significantly increased Wpeak (by 19.4 % and 10.2 %, respectively) and Wmean (by 11.2 % and 6.7 %, respectively) compared to placebo. Although VO_2 was not increased in the first study, in the second study women showed a greater peak VO_2 (5.8 % more) during repeated sprints after MLE administration, coinciding with better brain oxygenation. Mean VO_2 during sprints was unchanged in all studies, suggesting increased efficiency or recruitment of the anaerobic capacity after MLE ingestion. In *Study 3*, MLE attenuated the metaboreflex hyperpneic response after ischemia, which may have improved *Vastus Lateralis* O_2 extraction ($P = 0.056$), and reduced pain during ischemia ($P = 0.068$). Moreover, *Study 4* showed that polyphenol supplementation attenuated the muscle pain felt after the 10 km race ($P = 0.035$) and the loss of jumping performance and mechanical impulse ($P < 0.04$) 24 h later. In men, the polyphenols attenuated the increase of serum myoglobin and alanine aminotransferase ($P < 0.05$) (*Study 4*), besides blood lactate concentration was 5.9 % lower after the ingestion of Zynamite®+Q ($P = 0.049$) (*Study 3*). However, blood lactate, acid-base balance, and plasma electrolytes responses were not altered by the supplements (*Study 2*).

Conclusions

Acute and prolonged supplementation with MLE+Lut enhances performance, muscle O_2 extraction and brain oxygenation during sprint exercise, at high and low doses. In addition, these effects were observed following 48 h and 15 days of supplementation without significant differences between the two doses tested (*Study 1*). MLE extract combined with either quercetin and tiger nut extract or luteolin exerts a remarkable ergogenic effect, increasing muscle power in fatigued subjects and enhancing peak VO_2 (especially with MLE+Q+TNE) and brain oxygenation in women, during prolonged sprinting. Importantly, the combination of MLE+Q+TNE improves skeletal muscle contractile function during ischemia-reperfusion. In addition, both polyphenolic combinations tended to reduce the pain evoked by the occlusions (*Study 2*). Furthermore, a single dose of Zynamite® combined with quercetin one hour before exercise improves repeated-sprint performance and muscle O_2 extraction and mitochondrial O_2 consumption during ischemia. No advantage was obtained from the addition of phospholipids (sunflower lecithin) (*Study 3*). Finally, a single dose of Zynamite® combined with quercetin, administered one hour before competition, followed by three additional doses every eight hours, attenuates muscle pain and damage, and accelerates the recovery of muscle performance (*Study 4*).

II

RESUMEN GENERAL

RESUMEN GENERAL

Introducción

El impacto beneficioso de las frutas, hierbas y verduras en la salud humana es bien conocido desde hace siglos. Los polifenoles vegetales originarios de estos alimentos poseen un poderoso efecto antioxidante, así como propiedades antiinflamatorias que disminuyen el nivel de especies reactivas de oxígeno en el cuerpo humano. La ingesta adecuada de polifenoles podría conferir beneficios para la salud, especialmente en relación con la obesidad y las enfermedades crónicas. La obesidad y el sedentarismo se encuentran entre los problemas de salud más prevalentes a nivel mundial, por esta razón, el tratamiento contra la obesidad a través del ejercicio y una dieta adecuada podría mejorarse mediante la adición de suplementos de extractos de polifenoles naturales. Por consiguiente, los principales objetivos de la presente tesis doctoral fueron: 1) determinar los efectos agudos y prolongados, en dosis altas y bajas, de la suplementación oral con una combinación polifenólica de luteolina (extracto de cáscara de cacahuete (PHE) que contiene un 95 % de luteolina (Lut)) y mangiferina (extracto de hoja de mango (MLE) que contiene 60 % de mangiferina, Zynamite®) en la mejora del rendimiento del ejercicio, la oxigenación muscular y cerebral y, el metabolismo muscular (*Estudio 1*); 2) si MLE administrado en dos formulaciones diferentes: una con quercetina (Q) y extracto de chufa (TNE), y la otra con luteolina, tienen un efecto potenciador sobre el rendimiento de esprint y si protegen el músculo esquelético de los efectos negativos de la isquemia-reperfusión (IR) aplicada inmediatamente al final del ejercicio de esprint (*Estudio 2*); 3) determinar si una dosis única de Zynamite® en combinación con una pequeña cantidad de quercetina, o con quercetina combinada con lecitina de girasol (SFL) (administrada 1 h antes del ejercicio), aumenta el rendimiento deportivo durante el ejercicio de esprint repetido y, si estos efectos pueden mejorarse agregando lecitina de girasol (*Estudio 3*); 4) para determinar si Zynamite® administrado en

combinación con una pequeña cantidad de quercetina, facilita la recuperación después del ejercicio dañino repetido y, si atenúa el dolor y el daño muscular inducido por el ejercicio (EIMD) *(Estudio 4).*

Material y métodos

La presente tesis doctoral está compuesta por 4 estudios en los que hombres y mujeres jóvenes se ofrecieron como voluntarios para participar (12 hombres de 21.3 ± 2.1 años (*Estudio 1*), 17 hombres de 22.7 ± 2.1 años y 13 mujeres de 27.0 ± 2.2 años (*Estudio 2*), 26 hombres de 23.1 ± 2.2 años y 24 mujeres de 23.5 ± 2.9 años (*3*), 30 hombres de 23.1 ± 2.5 años y 18 mujeres de 23.3 ± 2.4 años (*Estudio 4*). El protocolo del experimento principal cambió ligeramente entre los 3 primeros estudios, pero en todos ellos se realizaron esprints e IR, además de probar diferentes tratamientos polifenólicos (principalmente a base de mangiferina, luteolina y quercetina) frente a placebo (maltodextrina) sobre el rendimiento deportivo. El tiempo de tratamiento, la combinación polifenólica y las dosis cambiaron según el estudio. Brevemente, se evaluaron la antropometría (masa corporal y altura) y la composición corporal (mediante absorciometría de rayos X de energía dual). Todos los esprints se realizaron con los mismos cicloergómetros que miden la producción de potencia pico instantánea (Wpeak) y potencia media (Wmedia). La oxidación máxima de grasas (MFO), el consumo máximo de oxígeno (VO_2max) y la capacidad anaeróbica (déficit máximo de oxígeno acumulado), se midieron con un carro metabólico calibrado y, se analizaron las variables respiratorias respiración a respiración y se promediaron cada 20 s durante los test. La oxigenación cerebral y muscular se midió mediante espectroscopia de infrarrojo cercano empleando espectroscopia de resolución espacial para obtener el índice de oxigenación tisular (TOI). Además, se registró el dolor muscular y la eficacia de la ocultación del tratamiento. Además, en el

cuarto artículo, el EIMD fue inducido por una carrera de diez kilómetros seguida de 100 saltos con caída.

Resultados

Durante el ejercicio de esprint, las combinaciones polifenólicas en comparación con el placebo mejoraron el rendimiento del ejercicio, facilitaron la extracción de oxígeno muscular y mejoraron la oxigenación del cerebro. Los tratamientos polifenólicos también mejoraron la extracción de oxígeno durante la IR y el rendimiento deportivo justo después de la isquemia-reperfusión (*Estudios 1-3*). Sin embargo, no hubo diferencias entre la combinación de dosis baja y alta de MLE+Lut en el rendimiento del ejercicio (*Estudio 1*). La suplementación con MLE aumentó la Wpeak y mejoró la Wmedia en un 5.0–7.0 % (*Estudio 2*), así como la Wpeak durante las tres primeras etapas del tercer estudio (2.8 y 3.8 % de aumento en MLE+Q+SFL y MLE+Q respectivamente). Después de la isquemia, los tratamientos con MLE+Q+TNE y MLE+Lut aumentaron significativamente la Wpeak (en un 19.4 % y un 10.2 %, respectivamente) y la Wmedia (en un 11.2 % y un 6.7 %, respectivamente) en comparación con el placebo. Aunque el VO_2 no aumentó en el primer estudio, en el segundo estudio las mujeres mostraron un pico de VO_2 mayor (5.8 % más) durante los esprints repetidos después de la administración de MLE, coincidiendo con una mejor oxigenación cerebral. El VO_2 medio durante los esprints se mantuvo sin cambios en todos los estudios, lo que sugiere una mayor eficiencia o reclutamiento de la capacidad anaeróbica después de la ingesta de MLE. En el *Estudio 3*, el MLE atenuó la respuesta hiperpneica metaborrefleja después de la isquemia, lo que pudo haber mejorado la extracción de O_2 del vasto lateral ($P = 0.056$) y reducido el dolor durante la isquemia ($P = 0.068$). Además, el *Estudio 4* mostró que la suplementación con polifenoles atenuó el dolor muscular después de la carrera de 10 km ($P = 0.035$) y la pérdida de rendimiento de salto e impulso mecánico ($P < 0.04$) 24 h después. En hombres,

los polifenoles atenuaron el aumento de la mioglobina sérica y la alanina aminotransferasa ($P < 0.05$) (*Estudio 4*), además la concentración de lactato en sangre fue 5.9 % menor después de la ingesta de Zynamite®+Q ($P = 0.049$) (*Estudio 3*). Sin embargo, los complementos no alteraron las respuestas del lactato sanguíneo, el equilibrio ácido-base y los electrolitos plasmáticos (*Estudio 2*).

Conclusiones

La suplementación aguda y prolongada con MLE+Lut mejora el rendimiento, la extracción de O_2 muscular y la oxigenación cerebral durante el ejercicio de esprint, a dosis altas y bajas. Además, estos efectos se observaron tras 48 h y 15 días de suplementación, sin diferencias significativas entre las dos dosis ensayadas (*Estudio 1*). El extracto de MLE combinado con quercetina y extracto de chufa o luteolina ejerce un notable efecto ergogénico, aumentando la potencia muscular en sujetos fatigados, mejorando el VO_2 máximo (especialmente con MLE+Q+TNE) y la oxigenación cerebral en mujeres, durante carreras de velocidad prolongadas. Es importante destacar que la combinación de MLE+Q+TNE mejora la función contráctil del músculo esquelético durante la isquemia-reperfusión. Además, ambas combinaciones polifenólicas tendían a reducir el dolor provocado por las oclusiones (*Estudio 2*). Además, una dosis única de Zynamite® combinada con quercetina, una hora antes del ejercicio, mejora el rendimiento de los esprints repetidos, la extracción de oxígeno muscular y el consumo de O_2 mitocondrial durante la isquemia. No se obtuvo ninguna ventaja de la adición de fosfolípidos (lecitina de girasol) (*Estudio 3*). Finalmente, una dosis única de Zynamite® combinada con quercetina, administrada una hora antes de la competición, seguida de tres dosis adicionales cada ocho horas, atenúa el dolor y el daño muscular y acelera la recuperación del rendimiento muscular (*Estudio 4*).

III

LIST OF ABBREVIATIONS

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Abbreviations are defined at first mention and used consistently thereafter:

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ALT	Alanine aminotransferase
BMI	Body mass index
Ca^{2+}	Calcium
CNS	Central nervous system
CK	Creatine kinase
DM2	Type 2 diabetes mellitus
EEG	Electroencephalogram
EIMD	Exercise-induced muscle damage
eNOS	Endothelial nitric oxide synthase
Fe^{2+}	Ferrous ion or iron oxidation state +2
Fe^{3+}	Ferric ion or iron oxidation state +3
FiO_2	Fraction of inspired oxygen
GDP	Gross Domestic Product
GM	Gut microbiota
H^+	Hydrogen ions
HR	Heart rate
hs-CRP	High-sensitivity C-reactive protein
IBD	Inflammatory bowel disease

IR	Ischemia reperfusion
K^+	Potassium
MLE	Mango leaf extract
MFO	Maximal fat oxidation
NO	Nitric oxide
NOX	NADPH oxidase or Nicotinamide adenine dinucleotide phosphate-oxidase
NRF2	Nuclear factor erythroid 2-like 2
O_2	Oxygen
OH	Hydroxyl
$PaCO_2$	Partial pressure of carbon dioxide
PEIC	Post-exercise ischemic conditioning
PHE	Peanut shell extract
Pi	Inorganic phosphate
PO_2	Partial pressure of oxygen
Q	Quercetin
RONS	Reactive oxygen and nitrogen species
ROS	Reactive oxygen species
SFL	Sunflower lecitin
TNE	Tigernut extract
TOI	Tissue oxygenation index
VE	Pulmonary ventilation

VO_2	Oxygen uptake
VO_2max	Maximal oxygen uptake
WHO	World Health Organization
Wmean	Mean power output
Wpeak	Peak power output
XO	Xanthine oxidase or xanthine oxidoreductase

*Additional abbreviations are defined at first mention and used consistently thereafter within a given manuscript in the present study.

IV

INTRODUCTION

INTRODUCTION

The beneficial impact of vegetables, fruits, and herbs on human health has been well known for centuries. Today, we understand the reasons for that as many plant-derived products are rich in nutrients, vitamins, minerals, and very importantly, bioactive polyphenols. Briefly, polyphenols are compounds with multiple phenolic groups in their structure, which means that they contain one or more aromatic rings with hydroxyl groups attached to them (explained in depth later). Their chemical structure explains their broad spectrum of biological activities. The health-promoting properties of plant polyphenols comprise anti-inflammatory, anti-allergic, anti-thrombotic, anti-ageing, anti-obesogenic and anti-cancer or anti-mutagenic effects [1-5]. These compounds present powerful antioxidant and anti-inflammatory properties decreasing the level of reactive oxygen species in the human body. Polyphenols can impact the composition of the gut microbiota (which are independently associated with health benefits), and gut bacteria metabolize polyphenols into bioactive compounds that produce clinical benefits [6]. Although deficiencies in polyphenol intake do not result in specific deficiency diseases, adequate intake of polyphenols could confer health benefits, especially with regard to obesity and chronic diseases [6], such as type 2 diabetes mellitus (DM2).

Current social components of obesity and physical inactivity

Obesity and a sedentary lifestyle represent one of the top ten causes of death in developed countries, according to the World Health Organization (WHO). Since the 1980s obesity has nearly tripled worldwide. In 2016, 39 % of adults (18 years old or over) were overweight, and 13 % were obese. In the records of that same year, more than 340 million children and adolescents (ages 5 to 19) were overweight or obese. In addition, this problem also affects children under 5 years

of age, noting that 41 million of them were overweight or obese. Specifically in Spain, The Spanish Society of Cardiology (Sociedad Española de Cardiología (SEC)) indicates that 53.6 % of Spaniards are overweight, according to data from the Nutritional Study of the Spanish Population (Estudio Nutricional de la Población Española (ENPE)) which details that 22 % of Spaniards have obesity and 31.6 %, are overweight. In Canary Islands, the prevalence of obesity is one of the highest in Spain [7, 8].

Obesity contributes to more than 13 types of cancer [9, 10] and a significant fraction of highly prevalent chronic diseases, especially type 2 diabetes mellitus, osteoarthritis, chronic kidney failure, gallstones, hypertension and ischemic coronary artery disease [11-14]. According to a study coordinated by the World Obesity Federation, the high Body Mass Index (BMI) of Spaniards in 2019 cost the country 2.1 % of the Spanish Gross Domestic Product (GDP). Moreover, it has been estimated that in 2060, global costs will increase to 2.4 % of GDP. Obesity has a negative impact on the quality of life of patients [15] and shortens life expectancy [16]. These effects are even more serious when obesity is associated with physical inactivity [17]. The most effective treatment to combat obesity and physical inactivity is exercise combined with a hypocaloric diet [18]. In fact, to tackle the health implications of obesity, becoming more serious during child hood, the European Union (EU) activated the Action Plan 2014–2020 program (EUActionPlan 2014) with the aim to halt the rise of overweight and obesity, through the promotion of a healthy lifestyle characterized by dietary regimen enriched in vitamins, minerals and other healthy micronutrients with a low content of fat and sugars, besides to realise an increased physical activity [19].

On the other hand, the treatment against obesity through exercise and a proper diet could be improved by adding supplements of natural polyphenol extracts. Several studies on cell culture and in animal and human models provide strong evidence for polyphenols' anti-oxidant and anti-inflammatory positive

effect, also on thermogenesis and energy expenditure facilitating weight loss, by acting at molecular level [19].

The Impactur Canarias 2018 report, carried out by the Government of the Canary Islands and Exceltur, reveals that tourism is responsible for 40.4 % of employment and 35 % of GDP. However, the Canary Islands continues to be a region with high unemployment, so it is necessary to promote new forms of employment and economic activity. Weight loss tourism has been experiencing rapid growth in recent years, particularly in Europe, which is the main source market for tourism to the Canary Islands.

Consequently, obesity and sedentary lifestyle are among the most prevalent health problems worldwide. For these reasons, this thesis was aimed originally to design an exercise program to lose weight, to find a specific polyphenolic combination, as well as to elucidate the molecular mechanisms by which exercise acts. At the same time, in collaboration with the local industry (Nektium Pharma S.L) we aimed at developing new nutritional supplement formulations based on natural polyphenols with positive effects on body composition, physical performance and mental health for human subjects. Given the limitations generated by the COVID-19 pandemic it was neither possible to develop experiments with obese patients and polyphenolic treatment nor to investigate possible interactions of these polyphenols with the microbiota.

Therefore, the thesis was focussed on the development of polyphenol-based supplements with potential positive effects on exercise performance and recovery.

Exercise, fatigue, metabolites, and reactive molecules

Fatigue is a complex process which may originate in any structure intervening in the production and control of muscle contractions. Performance-enhancing compounds may exert their effects by facilitating energy supply and utilization, easing central command and motor control, and reducing the negative effects caused by energy depletion, shortage of oxygen (O_2), metabolite accumulation and reactive oxygen and nitrogen species (RONS) on force generation, muscle contraction activation and afferent feedback. Excessive production of RONS during exercise may cause damage to the cellular structures resulting in maladaptation to exercise [20, 21], inflammation [22], muscle [23-25] and cardiac fatigue [26], and impairment of executive and cognitive functions [27]. During exercise, reactive oxygen and nitrogen species are continuously produced by mitochondrial respiration, but xanthine oxidase (also called xanthine oxidoreductase; XO) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase, also named NOX) are also important sources of RONS during exercise [28-30].

Fatigue may be caused, during intense exercise, by a mismatch between oxygen delivery and utilization leading to faster consumption of glycogen stores and activation of the anaerobic metabolism, resulting in hydrogen ions (H^+) and inorganic phosphate (Pi) accumulation, and increased generation of RONS [31-33]. Increased levels of H^+, Pi, and RONS may reduce calcium (Ca^{2+}) release from the sarcoendoplasmic reticulum [6] and diminish troponin calcium sensitivity, diminishing peak power [34, 35]. Exercise performance also depends on the capacity of the nervous system to provide an adequate activation signal for the prescribed task [36]. The central nervous drive is, in turn, modulated by sensory feedback from group III/IV afferents acting at spinal and supraspinal levels [37]. Input from type III and IV muscle afferents may also influence the rate of perceived exertion and contribute to task failure by a central mechanism [38]

although their exact role during whole-body exercise is poorly understood (Fig. 1). This is further complicated by the fact that perceived fatigability depends on many factors including core temperature, hydration, brain oxygenation, blood glucose, and several psychological factors (arousal, executive function, expectations, mood, motivation, pain, and performance feedback) [36].

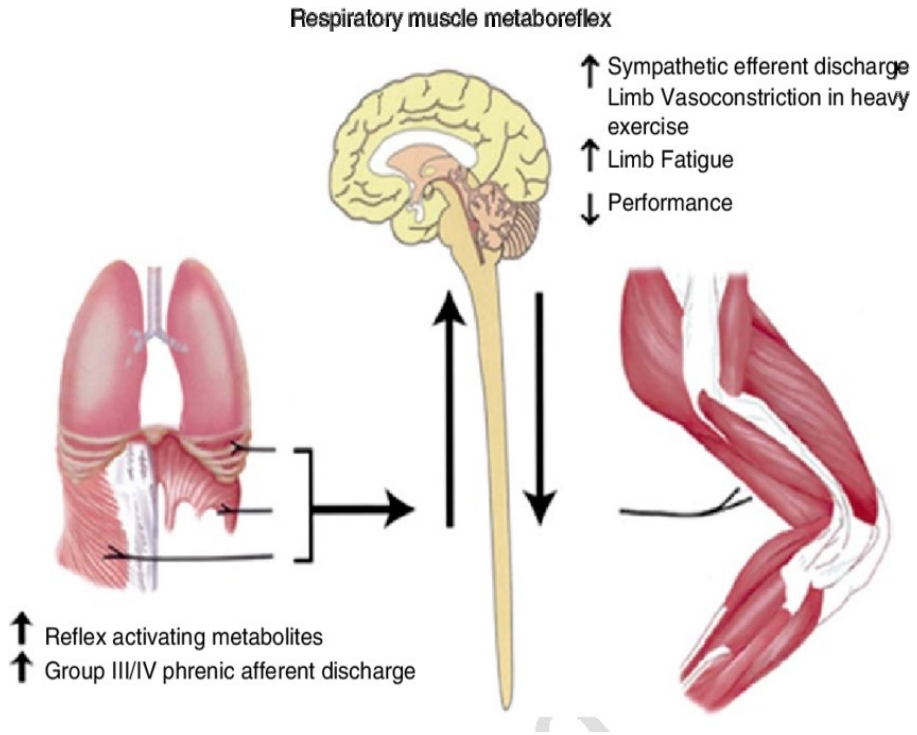

Figure 1. Diagram of the metaboreflex and regulatory mechanisms.

However, it has been shown by the activation of the metaboreflex (pulmonary ventilation (V_E) and heart rate (HR) responses) and O_2 debt per kg of active lean mass, that there are no sex differences in muscle metabolism during recovery after repeated supramaximal whole-body exercise [39].

On the other hand, prolonged or unusual exercise, particularly when involving eccentric muscle contractions, may cause muscle damage [40]. Exercise-induced muscle damage (EIMD) is characterized by muscle soreness, structural disruption, and local inflammation, and it is accompanied by a

temporary reduction of muscle force and exercise performance [41]. In the following hours and days after EIMD, the range of motion is reduced, and some swelling appears in the affected limbs [42]. The risk and magnitude of EIMD is exacerbated when muscle contractions are performed at longer muscle length, faster angular velocity, and with higher forces [41]. Although a mechanical disruption of muscle fibers is thought to be the main mechanism starting EIMD, this is followed by a mild inflammatory response, in which reactive oxygen species (ROS) are involved [43, 44].

Ischemia-reperfusion (IR) may well occur during isometric contractions [45]. IR injury is the damage that occurs when blood supply returns to the tissue after a period of prolonged ischemia, causing metabolic and contractile damage [46]. In skeletal muscle, IR may be produced by occluding limbs with cuffs for short periods of time (seconds to minutes), consequently generating an intermittent vascular occlusion followed by reperfusion. In medicine, ischemia-reperfusion has been used to induce ischemic pre- or post-conditioning to tissue protection, particularly in the myocardium. In sports, IR has been applied immediately after exercise (post-exercise ischemic conditioning (PEIC)) to enhance training adaptation or induce acute improvements in exercise performance [47, 48]. Ischemia-reperfusion may cause a tisular damage which has some resemblances with EIMD, namely an increase in appearance of muscle proteins in the blood as well as increased intracellular calcium concentrations [46, 48, 49]. In our experiments short occlusions were applied immediately at the end of sprint exercise.

To mitigate the potential damage caused by exercise or ischemia-reperfusion due to reactive oxygen and nitrogen species, the role of some natural polyphenols, was investigated.

Polyphenols: classes, composition, food sources and properties

Polyphenols are molecules present naturally in plants but that can also be chemically synthesized. Natural polyphenols are characterized by the presence of more than one phenol group per molecule which provide antioxidant properties, allowing to prevent or delay RONS-elicited cell damage. It is important to note that not all antioxidant molecules are polyphenols, but all polyphenols have antioxidant properties since the presence of phenolic groups in their structure is what gives them these properties. Mostly, antioxidants are molecules capable of retarding or preventing the oxidation of other molecules, but without a specific structural pattern, unlike the polyphenolic structure that content the phenolic rings. Antioxidants are mainly divided into two extensive groups: hydrophilic (water soluble) or hydrophobic (fat soluble). Biologically, the former react with oxidants in the cell cytoplasm and blood plasma, while fat-soluble antioxidants protect cell membranes against lipid peroxidation [50]. Among the antioxidants, there are nutrients, peptides, vitamins and provitamins, thiol donors, antioxidant enzymes, polyphenols, etc. [51]. We will focus on polyphenolic compounds, which are the most extensive group of non-energy substances present in plant origin food, providing beneficial properties for health.

Polyphenols originate mainly in plants, as a product of their secondary metabolism, with defensive functions against environmental stress (humidity, temperature, light, nutrients, etc.) and pathogens (bacteria, fungi, insects, etc.). Plant polyphenols can range from simple molecules (phenolic acids) to highly polymerized compounds (tannins). The number of phenolic rings and the structural elements that polyphenols present will determine the different classes and subclasses of these. Since the late eighties, according to Harborne et al. [52] polyphenols have been divided into, at least, ten different classes depending on their basic chemical structure and taking into account the simple phenols. The

polyphenols (compounds with two or more phenolic rings) can be divided into six different groups: hydroxybenzoic acids, hydroxycinnamic acids / coumarins, chlorogenic acids, xanthones, stilbenes, anthraquinones, flavonoids and lignans) [53]. Flavonoids are quantitatively the predominant group, to the extent that in recent publications natural polyphenols have been classify into flavonoids and non-flavonoids [54].

Flavonoid can be subdivided into 13 classes, highlighting six groups that form the major dietary components: the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins (Fig. 2). The non-flavonoids, which are less abundant in the diet, are classified into coumarins, xanthones, stilbenes and lignans. The general figures and chemical compositions of the main polyphenolic compounds are well described in these reviews [52-54].

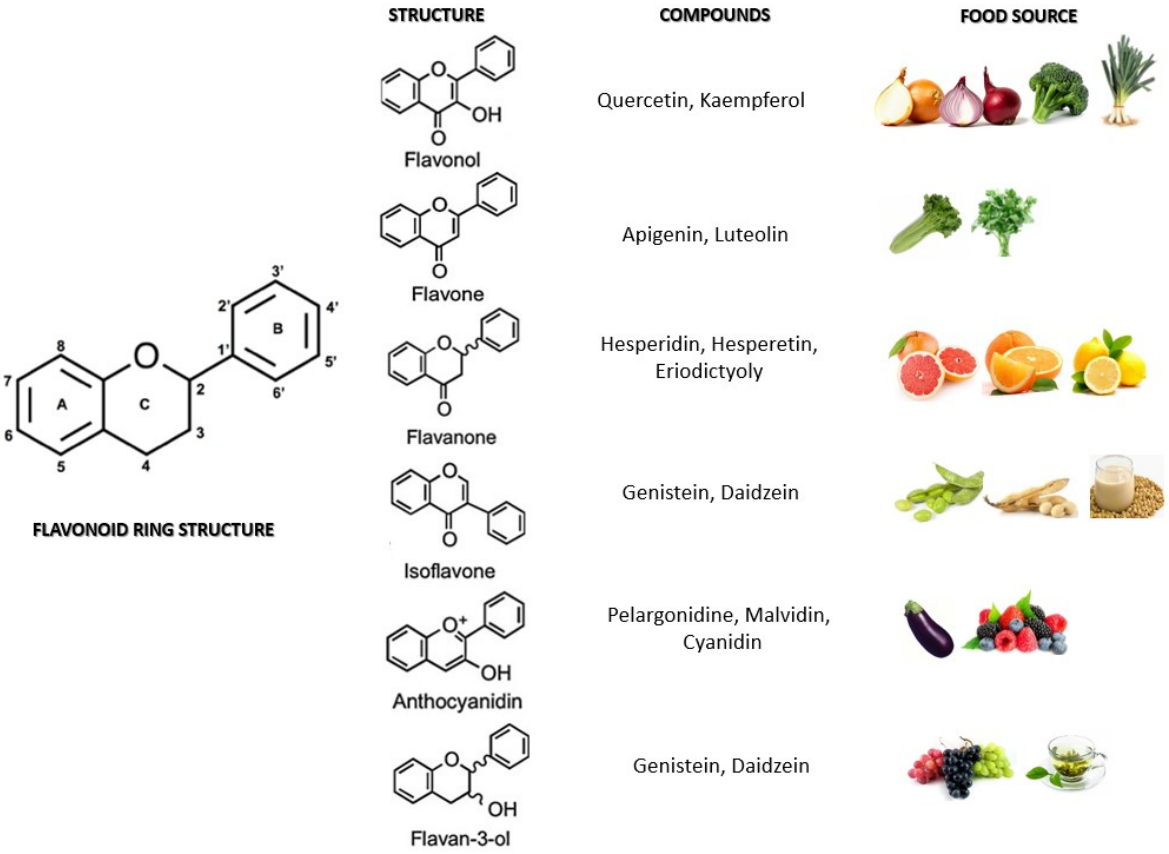

Figure 2. Summary image of the structure of flavonoids, their respective groups, some of their compounds and the food sources where they are found.

Flavonoids

All flavonoids share a common structure consisting of two aromatic rings (A and B) that are bound together by three carbon atoms that form an oxygenated heterocycle (ring C) (Fig. 2). Below are the 6 most representative classes.

Flavonols are a class of flavonoids that have the 3-hydroxyflavone backbone. Their diversity come from the different positions of the phenolic hydroxil (-OH) groups (Fig. 3). Flavonols are one of the most common and widespread flavonoids in food. The most representative compounds in this group are quercetin and kaempferol, which can be found in onions, kale, leek, broccoli, in drinks such as red wine and tea, or in fruits such as blueberries or apricot [53].

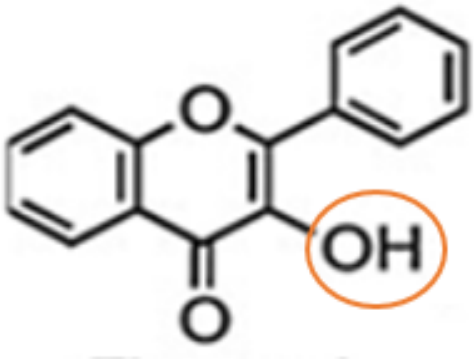

Figure 3. Flavonol structure.

Flavones are much less common than flavonols, although share a similar structure, the former lack oxygenation in C-3, and their structure can vary with hydroxylation, methylation, oxygen and carbonic glycosylation and alkylation (Fig. 4). Common flavones include apigenin, luteolin, tangeritin and chrysin, glycosides, provided in the human diet mostly by tea leaves and herbs (generally celery and parsley), and from some spices [55].

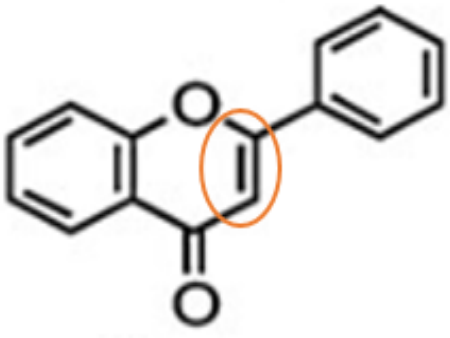

Figure 4. Flavone structure.

Flavanones are characterized by the absence of a D2,3 double bond and the presence of a chiral center at C-2, and are produced as hydroxyl, glycosylated and O-methylated derivatives (Fig. 5). This group is generally glycosylated by a disaccharide at position 7: a neohesperidose, which imparts a bitter taste (like naringin in grapefruit), or a rutinose, which is tasteless.

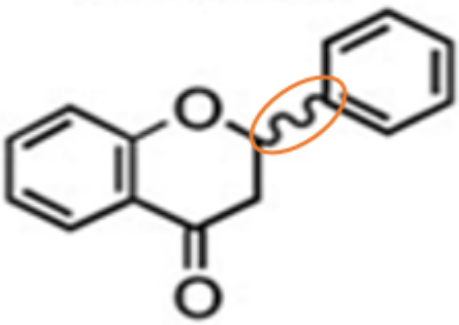

Figure 5. Flavanone structure.

Flavanones are present in high amounts in the citrus flavedo (outermost part of the peel) and the albedo (the fluffy white portion), as well as in the membranes that separate the segments. The most common compounds are hesperidin in grapefruit, hesperetin in oranges, and eriodictyoly in lemons [56].

The isoflavones have ring B attached at C-3 instead of at position C-2 (Fig. 6). Structurally, this group shows similarities with oestrogens and are considered phytoestrogens. Although they are not steroids, the isoflavones have hydroxyls in analogous positions (7' and 4') with the oestradiol molecule. This structure provides some affinity for oestrogen receptors, and capacity to cross the blood-brain barrier [57, 58]. Isoflavones are found almost exclusively in leguminous plants, the main food source being soy and its processed products. This group presents three main molecules: genistein, daidzein and glycitein aglycone [59].

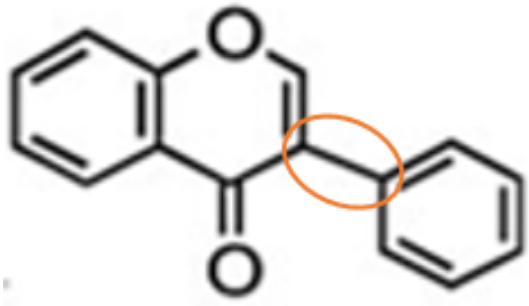

Figure 6. Isoflavone structure.

Anthocyanins are water soluble vacuolar pigments that, depending on their pH, are responsible for the colour of the flowers and fruits of higher plants: from orange and red, to purple and blue or black. The hydroxylation pattern of the B ring of anthocyanins is a major determinant of the colour of these pigments [60] (Fig. 7). The term anthocyanin refers to anthocyanidin glycosides, the most common being pelargonidine, malvidin, and cyanidin. Food plants rich in anthocyanins include vegetables such as aubergine, berries (blackberry, blackcurrant, blueberry, raspberry, etc.) and cereals or legumes such as black rice and black soy [53]. Anthocyanins and polymeric pigments formed from anthocyanins by condensation with other flavonoids are responsible for the red colour of wine [61], which change and transform into various complex structures as the wine ages [53, 62].

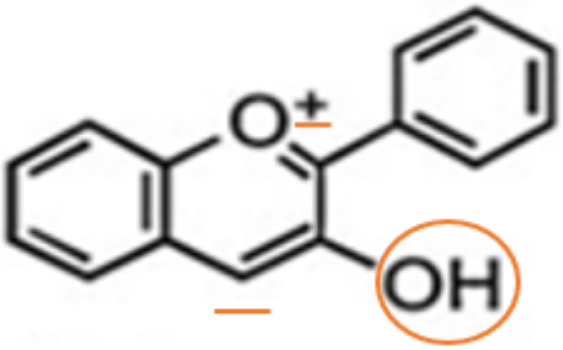

Figure 7. Anthocyanidin structure.

The flavan-3-ols group is also known as flavanols and is one of the most complex subclass of flavonoids, ranging from simple monomers (catechins) to oligomeric and polymeric forms (proanthocyanidins). The two chiral centers at C2 and C3 of the monomeric flavan-3-ol produce four isomers for each level of B-ring hydroxylation (Fig. 8), two of which, (+)-catechin and (-)-epicatechin, are generalized in nature, whereas others like (-)-epiafzelechin have a more limited distribution [63, 64]. Catechin and epicatechin are the main flavanols in fruits, found for example in apricots, as well as in red wine and chocolate, while gallatocatechin, epigallocatechin, and

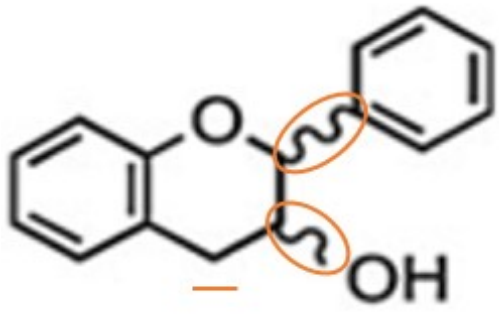

Figure 8. Flavanol structure.

epigallocatechin gallate are found in certain leguminous plant seeds, grapes and tea [65, 66].

Proanthocyanidins, also known as condensed tannins, have an additional chiral center at C4 in the upper and lower units, are catechin dimers, oligomers, and polymers that are linked by C4 to C8 or C6 linkages and are named as type B proanthocyanidins (Fig. 9). Type B proanthocyanidins are formed from (+)-catechin and (-)-epicatechin to create oligomers or polymers.

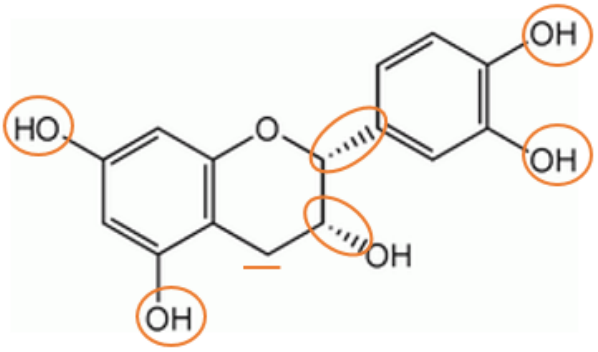

Figure 9. Tannin structure.

Type A proanthocyanidins have an additional ether bond between C2 in ring B of one monomer and C7 in ring A of the other monomer [67]. Proanthocyanidins can appear as polymers of up to 50 units, although there are few determinations of their distinction and average degree of polymerization in food. This is due to the complexity and structural diversity of proanthocyanidins, in addition to the difficulty of separating them from plants. Jean Marc et al 1996, showed that the average degree of polymerization of the grape skin varied from 3 to 80 depending on the fraction analysed [68]. The complexity of this group is so high that researchers are looking for new methods to better determine proanthocyanidin polymers [69-71]. Furthermore, the formation of complexes with salivary proteins varies the composition of these condensed tannins, in the same way that it occurs in the maturation of the fruits that contain them (for example, grapes, kakis, apples, etc.), in the fermentation of beverages (wine, cider, tea, beer, etc.) or in the bitterness of chocolate [72]. Proanthocyanidins that consist exclusively

of (epi) catechin units are called procyanidins, and are the most abundant type of proanthocyanidins in plants.

Non-flavonoids

Coumarins belong to benzopyrone chemical class, commonly found in many medicinal plants and are considered as a lactone. Their structure consists of two six-membered rings with lactone carbonyl groups (Fig. 10). Natural coumarins are subdivided in different classes based on their chemical diversity and complexity: simple coumarins, isocoumarins, furanocoumarins and pyranocoumarins (both angular and linear), biscoumarins and other coumarins such as phenylcoumarins [73]. Coumarins are present in high concentrations in tonka bean (*Coumarouna odorata*, where the class name come from), vanilla grass, sweet clover, cassia cinamon and a large number of cherry blossom trees [74]. This molecule is a colourless crystalline solid with a sweet odour resembling the scent of vanilla and a bitter taste [75]. Coumarins have antioxidant, antibacterial, antifungal, antiviral, anti-proliferative, anti-inflammatory, antidiabetics, anticoagulant and antineurodegenerative properties [73] .

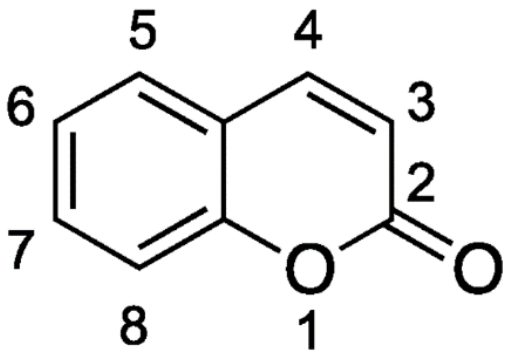

Figure 10. Coumarin structure.

The chemical formula of xanthone is $C_{13}H_8O_2$ (Fig. 11). Its main structure is 9H-xanthen-9-one with a dibenzo-γ-pirone scaffold [76]. In general, xanthones are categorized into six classes based on substitutions on the basic structure of xanthones: simple xanthones, xanthone glucosides (or glycosylated xanthones), prenylated xanthones, xanthonolignoids, bis-xanthones, and miscellaneous xanthones [77]. Natural xanthones occurring in higher plant families (Clusiaceae and Gentianaceae families), fungi, and lichen being the higher plants the main source of this polyphenol [78]. The chemical profile of xanthone nucleus can be highly variable (from R1 to R8, Fig. 11). The interest of this structure comes from the wide range of different substitutions that can generate many diverse compounds with interest for drug development [77].

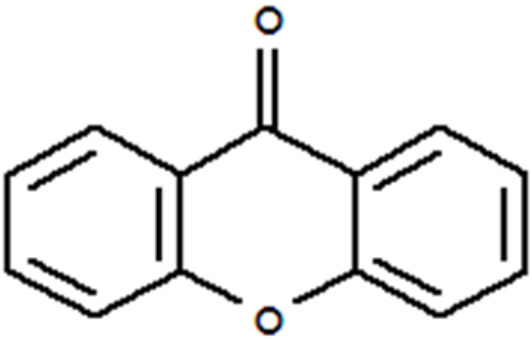

Figure 11. Xanthone structure.

Stilbenes present a C6 – C2 – C6 structure (Fig. 12) and were denominated like phytoalexins produced by plants in response to disease, injury and stress [79] but it also has beneficial health properties as generally all polyphenols, anti-inflammatory, anti-aging and anti-proliferative effects. Although only minor dietary components, these phenolic metabolites can be found in various edible plants, such as grapevines, berries, and peanuts [80]. The most studied stilbene is resveratrol (3,5,4'-

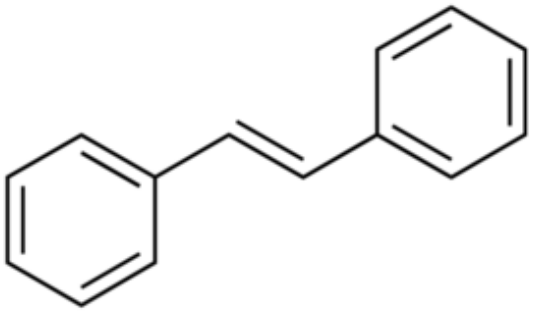

Figure 12. Stilbene structure.

trihdroxystilbene), which occurs as cis and trans isomers, as well as conjugated derivatives [81].

The basic chemical structure of lignans consists of two phenylpropane units linked by a C-C bond between the central atoms of the respective side chains (position 8). The 3-3′, or 8-3′ bonds are observed less frequently; and when present, the dimers are called neolignans [82] (Fig. 13).

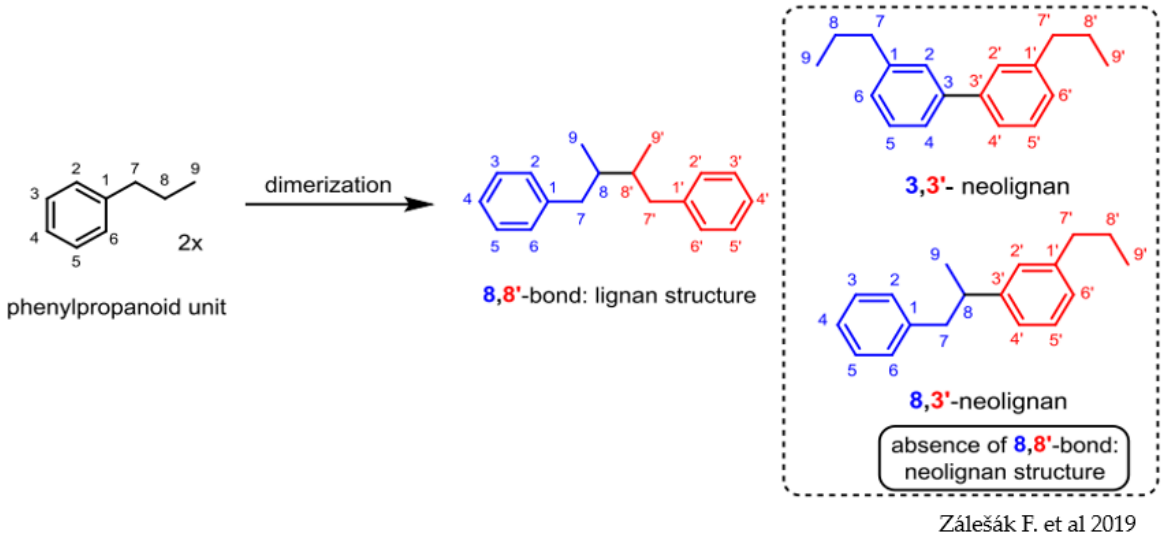

Figure 13. Lignan structure and the conversion to neolignans structure.

The richest dietary source of lignans is flaxseed, although leguminous plants (lentils), cereals, vegetables (garlic, asparagus, carrots) and fruits (pears, prunes) also present them, the concentrations in flaxseeds are almost 1000 times higher than the concentrations in these other food sources [53, 83]. There is a wide range of plant lignans, such as secoisolariciresinol, matairesinol, medioresinol, pinoresinol and lariciresinol, which after ingestion are converted to enterodiol and enterolactone by the human gut microbiota [84-86]. Enterolignan producing bacteria are common in the human population, but interindividual differences are reflected in the concentrations of these enterolignans in the blood, depending

on the concentration of bacteria in each person. Due to their structural similarity to oestrogen, lignans are classified as phytoestrogens, as are the isoflavones mentioned above. Furthermore, it has been shown that physiologically relevant concentrations of enterolignan lead to activation in *vitro* and in *vivo* of estrogen receptors [87].

Polyphenols tested to improve exercise performance: Mangiferin, Quercetin and Luteolin

The original idea for studying these polyphenols came from an etnopharmacologist from Nektium Pharma S.L looking for plants with energising and stimulating effect used in different cultures around the world. A selection of over 100 plants, traditionally used as tonics, hunting aids and to improve physical, psychological and sexual performance, was chemically and pharmacologically studied, and narrowed down to the ten most promising candidates. Even though of different plant families, several of these plants turned out to contain mangiferin as common denominator. A selection of six was studied *in vivo* and eventually the leaves of *Mangifera indica* were chosen due to their *in vivo* effect similar to caffeine and a sustainable supply for industrial purposes. Mango leaves are used in Asia as food and has also been combined with tea due to the similar stimulating effect. For the experiments performed in this thesis, an extract of *Mangifera indica* leaves standardized to mangiferin was used.

Studies performed in humans have reported divergent results which have been attributed to differences in the exercise model, fitness level of the study population and the type of polyphenol tested [88]. The ergogenic potential of some polyphenols such as luteolin, mangiferin quercetin on performance during repeated all-out prolonged sprints had not yet been studied in humans.

Mangiferin (2-b-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone (Fig. 14)) is a xanthone (non-flavonoid polyphenol) originally isolated from *Mangifera indica* L. (*Anacardiaceae*), present in abundance in mango leaves and other plants [89]. Mangiferin is considered a "super antioxidant" capable of specifically protecting against free radical production by the Fenton reaction due to its iron-chelating properties. The Fenton reaction is thought to play an important role as a source of RONS during sprint exercise [28] (Fig. 15), due to the marked acidification elicited by the high glycolytic rates attained during this type of exercise.

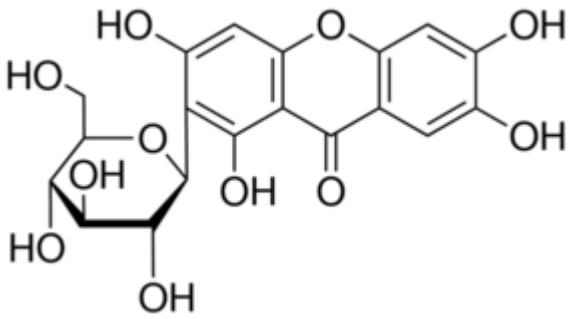

Figure 14. Mangiferin structure.

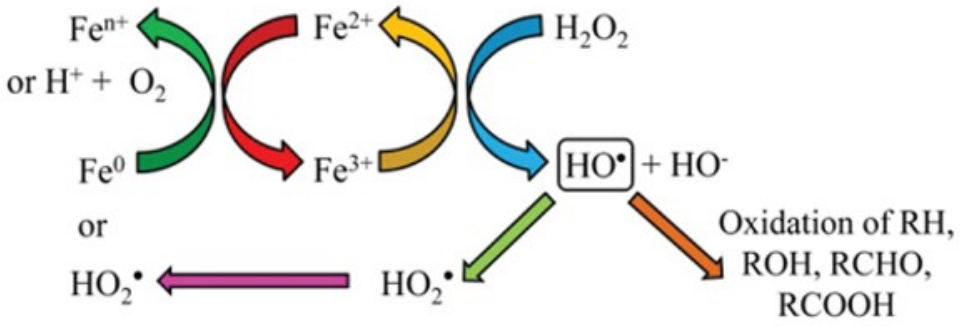

Figure 15. Fenton reaction.

Acidosis accelerates hydroxyl radical production by the Fenton reaction and reduces the activities of the antioxidant enzymes glutathione peroxidase, glutathione S-transferase, and glutathione reductase [90]. Mangiferin has powerful free radical scavenging properties and has been shown to attenuate ischemia-reperfusion injuries in diabetic rats [91], but it remains unknown whether mangiferin attenuates the effects of IR in humans. Mangiferin can

traverse the blood-brain barrier and modulate neurotransmission, potassium (K^+) channels and nociception [92]. Strong stimulation of type III and IV afferents by metabolite accumulation during sprint exercise [93, 94], particularly H^+ and lactate [95] are likely involved in the perception of effort and exercise-induced pain. III/IV muscle afferents discharge inhibits corticospinal drive and could contribute to limit exercise capacity or enhance fatigue sensation [96-99]. Mangiferin has properties which may attenuate III/IV muscle afferent discharge during exercise, either by reducing RONS-mediated stimulation of III/IV muscle afferents or by downregulating the glycolytic rate and interstitial potassium (K^+) accumulation.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone (Fig. 16)) is a well-studied flavonoid polyphenol which is found in several fruits, vegetables, leaves, seeds, and grains. Although quercetin has a low bioavailability due to its poor intestinal absorption [100], this may be improved by an oleaginous vehicle [101] such as tigernut (*Cyperus esculentus*) extract or sunflower lecithin. Quercetin may improve performance during prolonged exercise [102, 103] although its effects in athletes are unclear [104]. In animal models, quercetin attenuates ischemia-reperfusion injuries in several tissues [105-107] including skeletal muscle [108]. A potential sex dimorphism in the responses to polyphenol supplementation have not been specifically tested, although quercetin like mangiferin is a phytoestrogen, capable of binding to and activating oestrogen receptors [109].

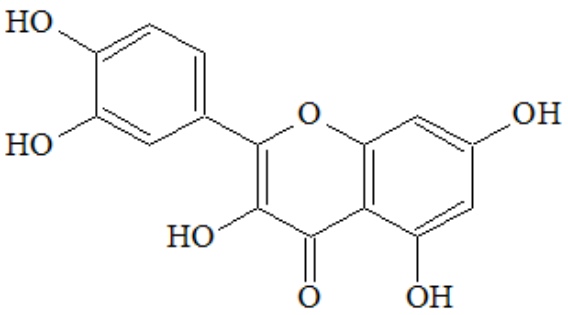

Figure 16. Quercetin structure.

Luteolin (30, 40, 50, 70-tetrahydroxyflavone (Fig. 17)) is one of the most abundant flavones and, like mangiferin and quercetin, is a potent antioxidant and inhibitor of xanthine oxidase [110-113]. Luteolin is also a NADPH oxidase inhibitor [114, 115]. Both enzyme activities, XO and NOX, play a critical role in RONS generation during intense exercise [28] and IR events [116]. Luteolin mitigates ischemia-reperfusion damage in cell cultures [117] and animals [118-122]. Moreover, animal experiments indicate that luteolin may down-regulate the expression of the genes (Cyba, Cybb, Ncf1, Ncf4, and Rac2) encoding the enzymatic subunits of NADPH oxidase [114, 115]. The selective action of these two polyphenols on XO and NOX is particularly interesting since other sources of free radicals would not be inhibited, permitting the signalling events necessary for the normal adaptation to exercise [123].

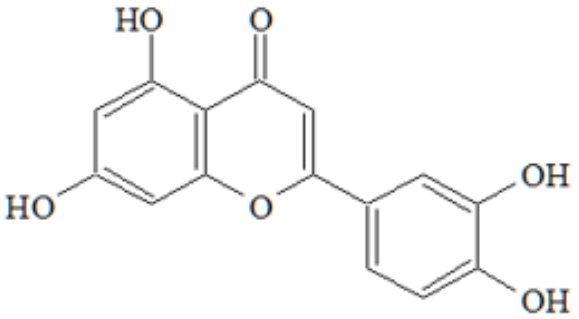

Figure 17. Luteolin structure.

Pilot and first studies with mangiferin, quercetin and luteolin

Initially, once the polyphenols with commercial interest had been chosen, an exercise protocol was developed to see if these substances had a performance-enhancing effect, and if they were able to protect the skeletal muscle from the negative effects of ischemia-reperfusion applied immediately at the end of sprint exercise. Two studies were necessary to establish an adequate dose and type of exercise. Mangiferin dosage was based on the pharmacokinetic study by Hou et al. 2012 [124] which showed a mean residence time close to 7 h, after the ingestion of 0.1 g of pure mangiferin in humans. Luteolin dosage was based on human pharmacokinetic data obtained following the ingestion of an artichoke leaf

extract rich in luteolin [125], and 100 mg of encapsulated luteolin [126]. One of our studies revealed no differences between low (50 mg) and high (100 mg) dose, so the lower dose was administered in the following experiment. The quercetin dose was set to 600 mg, i.e., below the doses previously used in studies reporting positive ergogenic effects [102].

As previously commented, hundreds of natural polyphenols present in edible plants and plant products contribute to the health effects attributed to the consumption of certain foods [127-129]. Most polyphenols have free radical-scavenging capacity [130], while others act as signalling molecules, or have interesting properties as anti-ageing [2, 131], anti-mutagenic [3, 127, 132] and anti-obesogenic [4, 111, 128] compounds. After ingestion, some polyphenols can cross the blood-brain barrier and exert specific effects on the central nervous system (CNS) acting on brain metabolism, neurotransmission, and oxygenation with positive effects on neurogenesis, neurocognitive functions, and mood state [133-135]. Besides, some polyphenols may enhance sports performance [104] and facilitate the adaptation to regular exercise by reducing exercise-induced muscle damage [103]. Although some antioxidants may enhance mechanical efficiency (e.g., acetylcysteine) and improve performance [51], they may also counteract some of the signalling processes necessary for the adaptive responses to exercise [28, 136, 137]. This has prompted the search for products alternative to classical antioxidants capable of modulating redox responses without blunting some beneficial exercise adaptations [138]. The classical approach to counteract RONS during exercise has been the administration of antioxidants compounds with free radical-quenching properties. This approach has been criticized due to the potential interference with some critical signalling events that depend exclusively on free radicals [123, 139, 140]. Nevertheless, it has been reported that supplementation with some polyphenolic compounds could avoid some of the adverse effects on performance observed with the intake of antioxidant vitamins,

like vitamin C during training [123, 140] . Besides, pharmacological inhibition of XO seems to reduce exercise-induced muscle damage both in animals [141] and athletes [142, 143]. Generally, flavonoids might facilitate an increase in mitochondrial Ca^{2+} concentration by acting on the mitochondrial Ca^{2+} uniporter [144]. This may up-regulate the respiratory rate and adenosine triphosphate (ATP) production and stimulate endothelial nitric oxide synthase (eNOS), increasing nitric oxide (NO) production. Interestingly, the cytoprotective effects of flavonoids against ischemia-reperfusion may be enhanced by exercise [145]. Prior to this investigation, no data was available regarding the effects of prolonged mangiferin or luteolin supplementation on exercise performance.

Therefore, we carried out studies to determine the luteolin (PHE) and mangiferin (MLE) dose suitable for making the polyphenolic combination, and their influence on exercise performance after 2 and 15 days of supplementation. Since these two polyphenols can have ergogenic effects through various mechanisms, a specific exercise protocol was designed, which included low intensity, high intensity exercise phases and repeated sprints combined with ischemia-reperfusion episodes. Twelve physical active men were recruited and randomly assigned to two different treatments following a double-blind cross-over counterbalanced design. The placebo group received maltodextrin, while the treatment group (divided in two) received identical looking capsules containing luteolin and mangiferin: some in low doses and the others in high doses (Fig. 18).

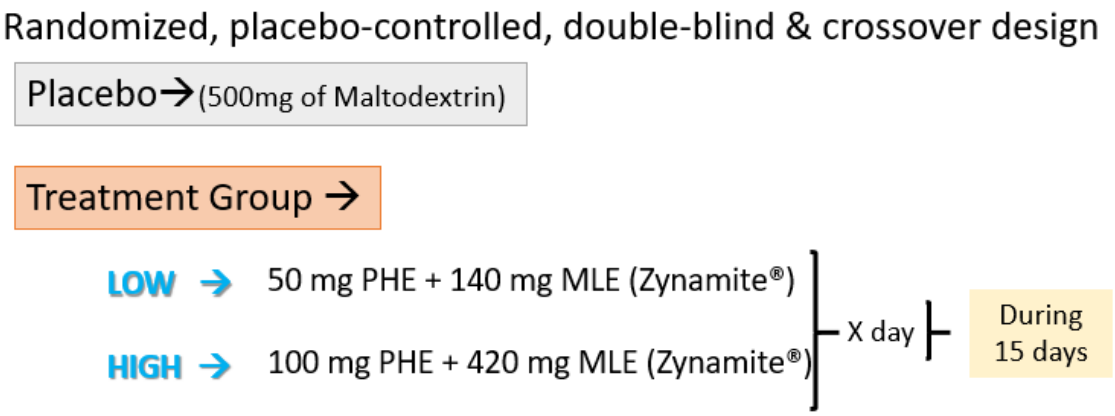

Figure 18. Treatments, doses and protocol of Study 1.

This exercise protocol was long and hard for the participants, but it allowed us to observe how these polyphenolic formulations acted in different types of exercise. The subjects performed an incremental exercise test to determine the maximal fat oxidation capacity (MFO), an incremental test until exhaustion, followed by repeated sprints and a submaximal constant-intensity time trial to exhaustion. These exercise periods were followed by active recovery (unloaded pedalling) or short periods of ischemia followed by reperfusion and sprint exercise. While MFO and incremental test are more robust measures, sprints of different times were used to assess the effects of the supplements on anaerobic capacity, and the time trial was used to evaluate the endurance capacity (since the test likely started with very low glycogen levels, replicating the conditions of the final stages of most endurance competitions).

In a second study two different polyphenol supplementations regimes were tested and compared to placebo. The purpose of this study was to test whether a mango leaf extract (MLE) (60 % by weight mangiferin) administered in two different formulations (one with quercetin and tigernut extract, and another with luteolin) has a performance-enhancing effect in young population

and if protect skeletal muscle from the negative effects of ischemia-reperfusion. (Fig. 19).

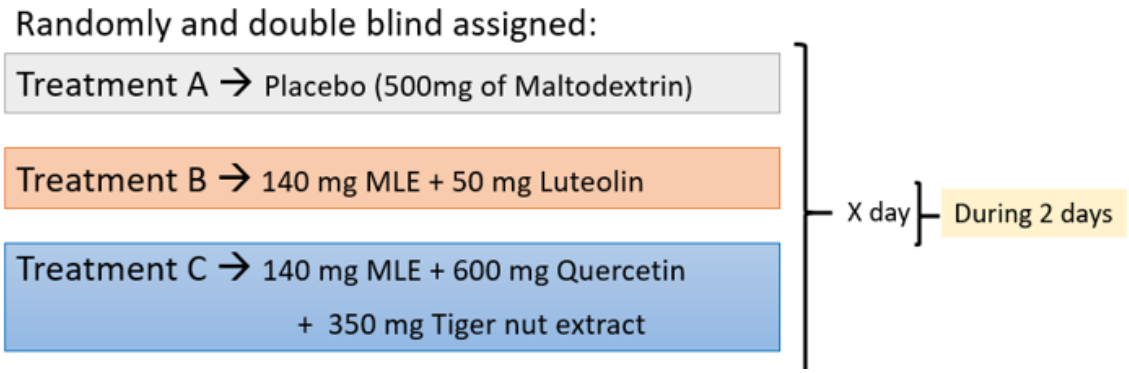

Figure 19. Treatments, doses and protocol of Study 2.

After the first study, it was observed that effect of these polyphenols was most visible in sprint exercise. These positive effects were present after 48 hours, but without increasing after a prolonged dose for 15 days. This means that no tolerance was build up, which would have let to a reduction of the effect, like happens with caffeine after a chronic ingestion. For this reason, the subjects performed a repeated sprint protocol, with different sprints' time (8 s, 15 s, 30 s and 60 s) after taking each of the treatments shown in figure 19.

These studies showed that the polyphenols mixtures tested containing mangiferin or luteolin enhanced sprint performance by improving brain oxygenation and muscle extraction of oxygen. Moreover, these combinations increase peak VO_2 during high-intensity exercise and tended to reduce the pain evoked by the occlusions.

Zynamite® product

Concomitantly, Nektium Pharma S.L carried out additional experiments to determine beneficial effects of the mango leave extract (Zynamite® (patented EP3487491B1, ES2681996AB)) on cognitive function. Zynamite® is incredibly versatile and can be included in different types of supplements, pre-workout powders and energy drinks (Fig. 20).

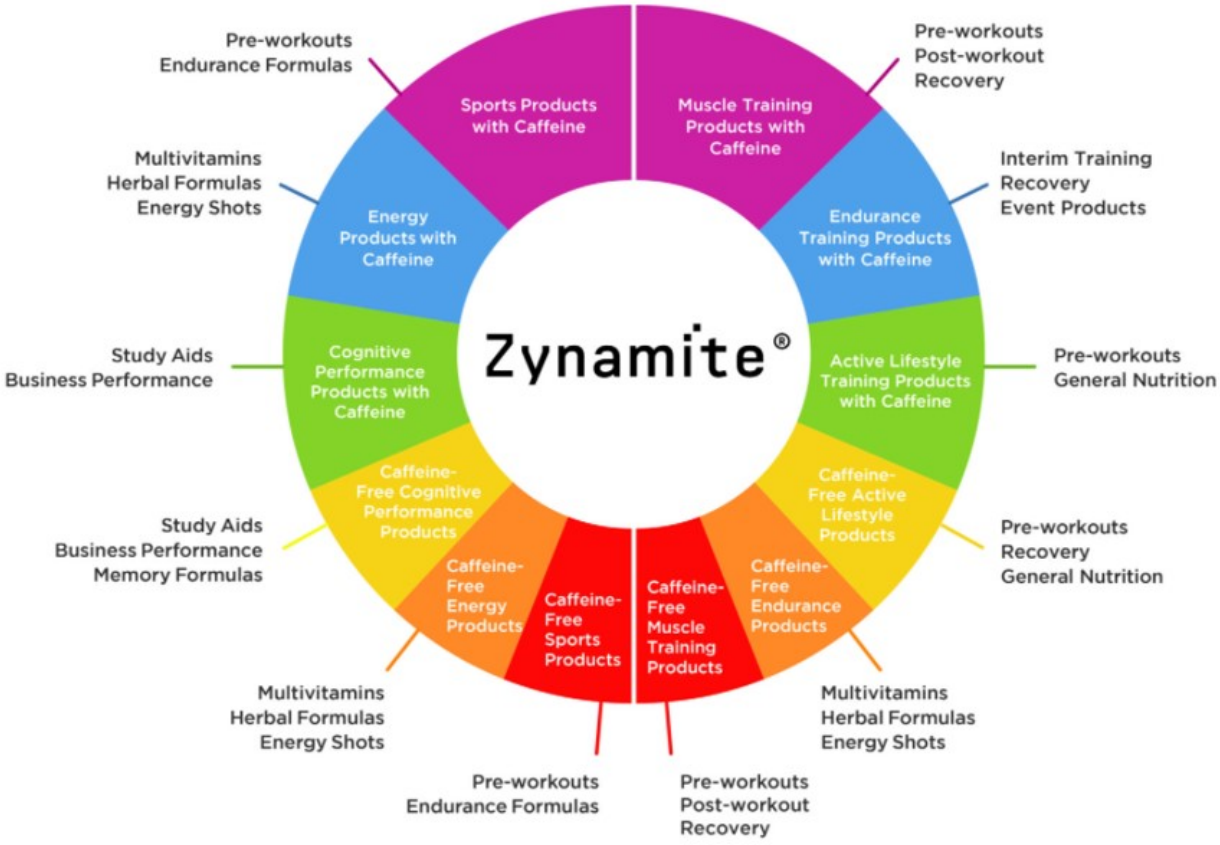

Figure 20. Potential applications of Zynamite® supplementation in different fields.

Regarding the effect of Zynamite® on the brain, it was observed in in-vivo studies that Zynamite® had stimulating effects on the CNS similar to that of caffeine, although the mechanism of action is completely different and without any of the side-effects related to caffeine intake (insomnia, jitters, nervousness,

increased heart rate, etc). The field potential in free-moving rats and the induction of long-term potentiation in the hippocampus (brain section mainly involved in long-term memory storage) were analysed [146] The researchers concluded that Zynamite® and caffeine both stimulate the CNS and increases cognitive function. Besides the combination of a low dose caffeine and Zynamite® had a synergistic effect in attenuating nearly all frequencies in all areas of the brain, which can be explained by the different mechanism of action of Zynamite® and caffeine [146]. The following study was conducted to determine if mangiferin (being the primary bioactive component of MLE), is responsible for the neurocognitive activity of Zynamite®, and if the effects of MLE in the central nervous system have translational potential. It was conducted through an in *vitro* and in *vivo* study and two double-blind, randomized, placebo-controlled crossover clinical trials [147]. The investigators concluded that mangiferin is the main bioactive constituent of MLE due to both isolated mangiferin and MLE produced similar effects in long-term potentiation within the hippocampus and in electroencephalogram (EEG) in rats (unpublished data). Besides, according to the results of the two clinical trials, it was concluded that mango leaf extract can modulate brain electrical activity during challenging cognitive tasks, significantly increase reaction time, and decrease self-reported mental fatigue compared to a placebo. Moreover, in both human clinical trials, MLE was well tolerated and there were no reported cardiovascular side effects. Although sample size was relatively small with only 16 individuals, these positive effects were noticed after just a single dose of mango leaf extract at 500 mg [147]. Finally, in the third cognitive study the researchers tested the effects of a single dose of Zynamite® (300 mg MLE) on cognitive function and mood. It was reported, in a sample of 70 healthy young adults, a significantly improved performance on "Accuracy of Attention", "Episodic Memory" and "Cognitive Demand Battery sub-section of the assessment" factors [148].

Single dose of Zynamite® improves performance, attenuates muscle damage and pain and accelerates recovery after strenuous damaging exercise

Two additional studies were carried out taking into account the results obtained previously. Since it was shown that the mango leaf extract rich in mangiferin Zynamite® improved exercise performance when combined with luteolin or quercetin ingested at least 48 h prior to exercise, the next study aimed at determining whether a single dose of Zynamite® administered in combination with a small amount of quercetin (140 mg), or with quercetin combined with sunflower lecithin (lipidic vehicle), increases exercise performance during repeated-sprint exercise and if improve muscle contractile capacity and muscle oxygenation during this process to exhaustion.

To determine whether a single dose of Zynamite® + quercetin administered one hour before exercise increases repeated-sprint performance, twenty men and twenty women who were physically active were randomly assigned to three treatments following a double-blind cross-over counterbalanced design (Fig. 21).

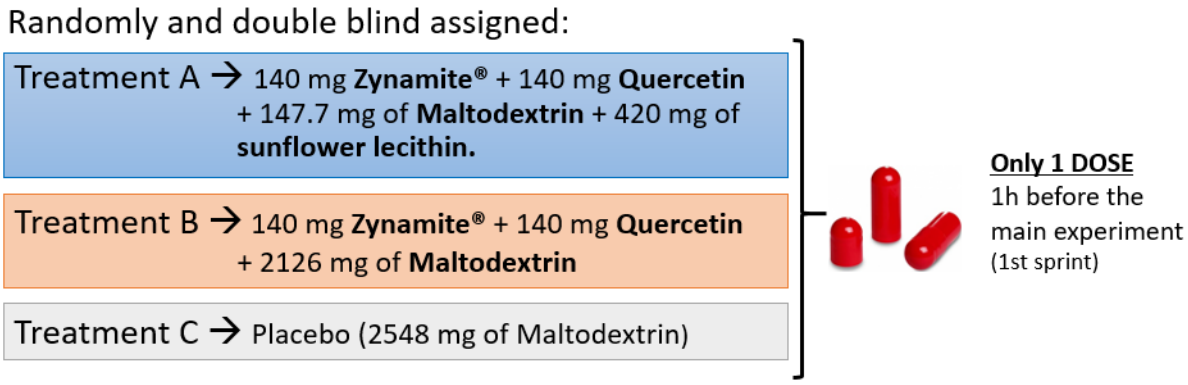

Figure 21. Treatments, doses and protocol of the Study 3.

The specific protocol consisted of three Wingate tests spaced by 4 minutes of unloaded pedalling plus a 1-minute occlusion and immediately at the end of the occlusion a 15-second sprint.

As a result, a single dose of Zynamite® combined with quercetin administered one hour before exercise improved muscular performance and O_2 extraction. Besides, the study confirmed that Zynamite® combined with quercetin facilitates mitochondrial O_2 consumption during ischemia, a situation which is observed during prolonged isometric contractions in many sports disciplines. Finally, adding sunflower phospholipids to the Zynamite®-quercetin mixture had no additional beneficial effects.

In the last study included in this thesis, the main purpose was to determine whether Zynamite® administered in combination with quercetin, facilitates recovery after repeated damaging exercise and attenuates EIMD and pain. With this purpose twenty-four women and thirty-three men were randomly assigned to two treatment groups matched by sex and pre-tests performance, and then completed the main experiment: running a 10 km race followed by 100 drop jumps to elicit EIMD. One hour prior to competition, and every eight hours after for 24 h, they ingested the treatment (Fig. 22).

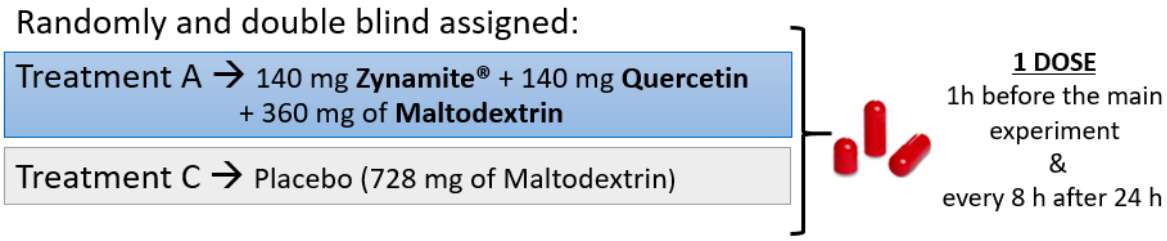

Figure 22. Treatments, doses and protocol of the Study 4.

Fasting blood samples were obtained before the 10 km race and 24 h after. Biomarkers of muscle damage were assessed in serum: myoglobin, high-sensitivity C-reactive protein (hs-CRP), creatine kinase (CK) and alanine aminotransferase (ALT). Race performance, muscle pain (analogic scale) and countermovement vertical jump performance (force plate, Kistler) were assessed

before and after competition. In consequence, a single dose of Zynamite® combined with quercetin, taken one hour before exercise eliciting muscle damage, accelerated the recovery of muscle performance and attenuated the muscle pain reported at the end of the race. Nevertheless, this polyphenol combination in a single dose pre-race did not improve running performance during a 10 km competition.

Potential mechanisms of action of these polyphenols during exercise

In *vitro* studies indicate that the antioxidant capacity of polyphenol mixtures exceeds that of their constituents [149, 150]. This is probably due to the fact that each polyphenolic compound has unique chemical properties which determine some specific actions in different cellular compartments [2, 151]. Although in *vivo* experimental evidence is lacking, a combination of polyphenols likely counteracts more efficiently the RONS produced during exercise in different subcellular compartments of the skeletal muscle fibers than single compounds [152]. In this case, these supplements enhance exercise performance during sprint exercise and facilitates muscle oxygen extraction. In addition, this polyphenolic combination improves muscle performance after ischemia-reperfusion by three main mechanisms. Firstly, it facilitates muscle oxygen extraction as demonstrated by the greater reduction of the muscle oxygenation index during the first five seconds of total occlusion of the circulation at exhaustion. Secondly, it reduces oxygen consumption during the sprints preceded by ischemia. Thirdly, it may have facilitated ATP production through additional recruitment of the glycolysis, as indicated by the higher levels of blood lactate concentration observed in the sprints performed after ischemia-reperfusion. Importantly, mangiferin + luteolin enhanced mean power output during prolonged sprints (30

s Wingate test) carried out after 30 min of recovery following an incremental exercise test [153]. This improvement in prolonged sprint performance was accompanied by enhanced brain oxygenation and larger muscle oxygen extraction during the sprints.

In these investigations, we have shown that these polyphenols allow the skeletal muscle to reach lower levels of tissue oxygenation during sprint exercise and post-exercise ischemia. This effect could be explained by a better microvascular distribution of perfusion (prioritizing the active skeletal muscle fibers) [154, 155] and enhanced mitochondrial O_2 extraction.

Oxygen extraction depends on muscle oxygen diffusing capacity, oxygen delivery, and the partial pressure of oxygen (PO_2) gradient from the muscle capillaries to the mitochondria [156]. Muscle O_2 diffusing capacity does not limit VO_2 during 30 s all-out sprints, because there is a large functional reserve in muscle O_2 diffusing capacity [157]. Muscle blood flow during sprint exercise is determined by cardiac output [157] and vascular conductance [158]. Increasing muscle blood flow may enhance the mean capillary PO_2 and hence, the gradient for O_2 diffusion, which could improve O_2 extraction. At maximal exercise, skeletal muscle vascular conductance is assumed to be maximal. This has been shown by experiments in which no increase of vascular conductance was observed in subjects exercising maximally with the intra-arterial infusion of maximal doses of ATP (one of the most potent vasodilators) [154]. An increase of skeletal muscle blood flow at maximal exercise is unlikely since this requires a higher cardiac output. The mean HR during the sprints was almost identical in the different conditions, suggesting unchanged cardiac output after the polyphenol administration. Thus, by assuming that muscle O_2 diffusing capacity does not limit muscle VO_2 during sprint exercise, and that muscle blood flow was likely similar in all conditions, the only mechanism that could explain an improvement in muscle O_2 extraction is an increase of the gradient driving

diffusion. This gradient may be increased by reducing the mitochondrial P50 [159] or by improving mitochondrial bioenergetics facilitating a higher muscle VO_2. The mitochondrial respiratory rate and ATP production depend, among other factors, on the mitochondrial concentrations of adenosine diphosphate (ADP) [160] and Ca^{2+} [161, 162]. Flavonoids may increase mitochondrial Ca^{2+} concentration by acting on the mitochondrial Ca^{2+} uniporter [144]. Mangiferin improves skeletal muscle mitochondrial ATP production and upregulates several enzymes of the glycolysis, facilitating a higher glycolytic rate in rodent skeletal muscle [163]. Moreover, cell experiments have shown that mangiferin reduces lactate accumulation by improving pyruvate dehydrogenase activity [151].

The greater level of muscle deoxygenation during the first 5 s of ischemia in the experiments performed with polyphenols, suggests that when the PO_2 is very low, as expected when ischemia is applied after maximal exercise [164], mitochondrial bioenergetics is likely enhanced by the administration of polyphenol mixture. This observation concurs with animal studies showing that luteolin [118-120, 122, 165] quercetin [106, 107] and mangiferin [91] attenuate the ischemia-reperfusion injury in different tissues. This protective effect of these polyphenols has been attributed to their potent direct free-radical scavenging properties and their inhibitory action on the superoxide-generating enzymes XO and NOX, which are activated during sprint exercise [28] and ischemia-reperfusion [91, 122, 166, 167]. During high-intensity exercise as well as during ischemia, NO is produced in skeletal muscle from nitrite by the action of nitrite reductases such as myoglobin [168, 169], deoxyhaemoglobin [170] and XO [166, 171]. Xanthine oxidoreductase usually reduces molecular oxygen to superoxide, but at low oxygen tensions and pH, as observed during prolonged sprints [94, 172], repeated sprints [173] and post-exercise ischemia [164], this enzyme can also reduce nitrite to NO [166]. The NO formed can bind to cytochrome c oxidase of

the mitochondrial electron transport chain, reducing electron flow and oxygen utilization [174]. Thus, the potential inhibitory action of mangiferin + luteolin on XO might have been beneficial during high-intensity exercise, ischemia and ischemia-reperfusion by reducing superoxide and secondary RONS generation and attenuating NO production from nitrite in skeletal muscle. Moreover, cell experiments have shown that mangiferin reduces lactate accumulation by improving pyruvate dehydrogenase activity [151]. During high-intensity exercise hemoglobin [157] and myoglobin [175] deoxygenate, with this effect exacerbated in ischemia [168]. Both deoxyhemoglobin and deoxymyoglobin have nitrite reductase activity resulting in the production of NO from nitrite, and oxidation of heme-ferrous ion to heme-ferric ion (heme-Fe^{2+} to heme-Fe^{3+}) [174]. This reaction is facilitated by H+, which increases during both high-intensity exercise and ischemia [164]. Mangiferin and quercetin could facilitate the nitrite reductase activity of deoxyhemoglobin and deoxymyoglobin by preventing the oxidation of Fe^{2+} to Fe^{3+} [176]. The NO released or produced within the muscle fibers can bind to cytochrome c oxidase of the mitochondrial electron transport chain, reducing electron flow and oxygen utilization [174], increasing oxidative phosphorylation efficiency in a redox-sensitive manner by decreasing the slipping in the proton pumps [177].

Consequently, this combination could have facilitated mitochondrial respiration and aerobic energy production during the sprints and ischemia periods, as indicated by the lower levels of muscle oxygenation observed when the ingestion of polyphenols preceded the sprints. At the same time, these polyphenols could have facilitated mitochondrial bioenergetics, improving muscle efficiency during high-intensity exercise [178] (Fig 23).

Given the high sensitivity of the brain to hypoxia [179], any small reduction of brain oxygen delivery could potentially alter brain functioning and contribute to fatigue. Moreover, reduced brain oxygenation may facilitate local

production of RONS, which may combine with circulating RONS released by contracting muscles, particularly during high-intensity exercise [180]. This could also deteriorate cognitive and executive function during exercise, reducing performance in complex tasks [181, 182]. Thus, it is not surprising that the reduction in brain oxygenation has often been argued as a mechanism lowering exercise performance [172, 183-186]. Moreover, fatigue can be swiftly relieved by raising the fraction of inspired oxygen (FiO_2), during exercise in severe acute hypoxia [184]. The ingestion of these polyphenolic combinations improved frontal lobe oxygenation during the prolonged sprints. This effect may be related to a better distribution of blood flow between tissues or enhanced cerebral vasodilation facilitated by the polyphenols [151] (Fig. 23). During sprint exercise, the partial pressure of carbon dioxide ($PaCO_2$) is markedly reduced what may cause vasoconstriction in the brain circulation [187]. The latter combined with the increased production of RONS during sprint exercise, which may hamper endothelial NO production and NO bioavailability, could contribute to reducing brain perfusion and oxygenation. Mangiferin and luteolin supplementation could have improved brain oxygenation during sprint exercise likely through its antioxidant properties, inhibitory action on endothelial NOX [167], suppressive effects on the endoplasmic reticulum-induced stress [151], and increasing the bioavailability of vascular NO [188] (Fig. 23). Moreover, mangiferin can cross the blood-brain barrier, modulate neurotransmission, K^+ channels and nociception [92], and attenuate sensory feedback. Although the improvement in performance reported here may seem small it is superior to that reported for caffeine during repeated Wingate tests [189].

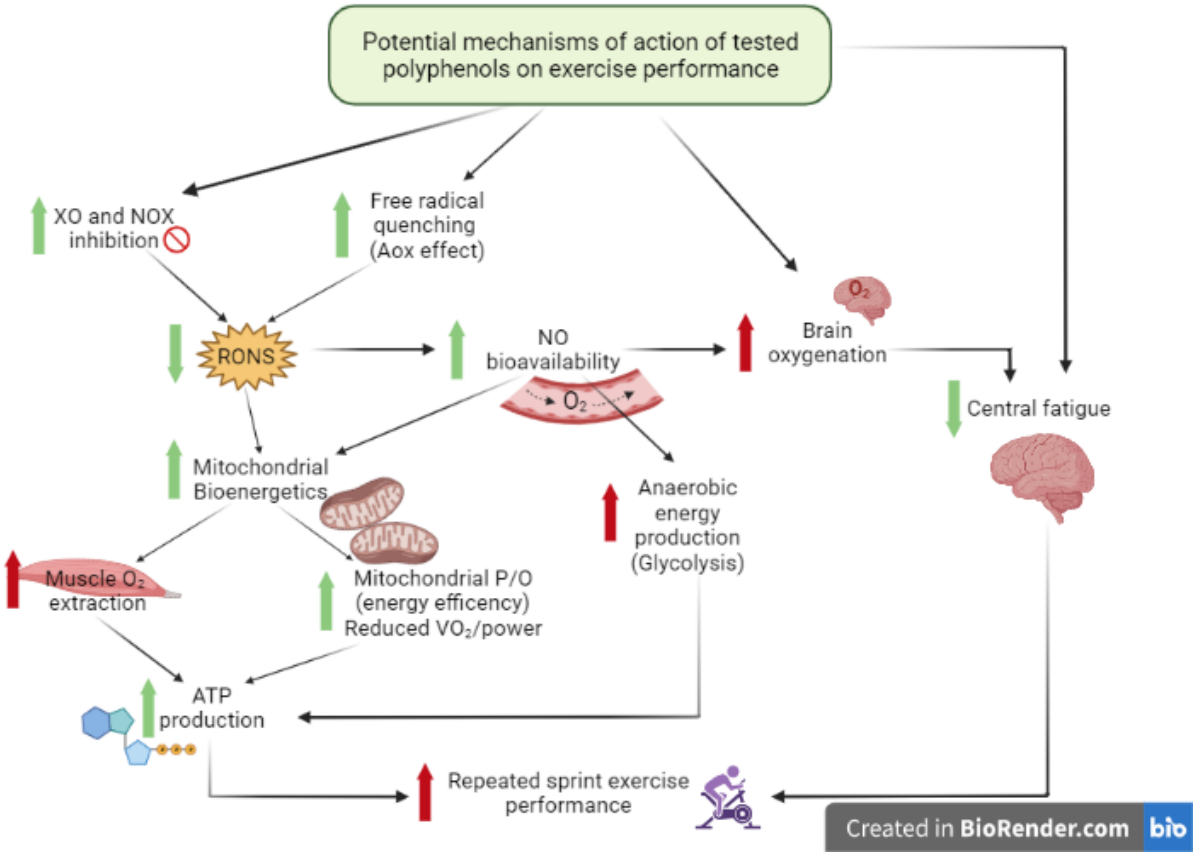

Figure 23. Potential mechanisms of action of polyphenolic supplementation tested on exercise performance.

These polyphenolic formulations can also reduce EIMD and muscle pain. Exercise-induced muscle damage is initially caused by mechanical disruption of the ultrastructure of muscle [190], which affects several sarcomere proteins [191]. This causes one-half sarcomere nonuniformity and overstretching of sarcomeres beyond filament overlap, reducing the number of myosin-actin cross-bridges and hence, causing a reduction in the capacity to produce force and an overload of the sarcolemma and T-tubule structures [192]. This is followed by the opening of stretch-activated Ca^{2+} channels, membrane ruptures, and excitation-contraction coupling dysfunction. The increase of sarcoplasmic Ca^{2+} may stimulate calpain proteases with loss of contractile proteins prolonging the loss of force [193-195].

The muscle pain is likely triggered by inflammation of the extracellular matrix [196], by neurotrophic factors released by the muscle fibers and satellite cells, as well as by invading polymorphonuclear cells in the following days [192]. This causes a mild inflammatory response in which reactive oxygen species are involved [43, 44]. Likely, several mechanisms act conjointly to elicit pain, but the nature may differ in the immediate post-exercise phase from the subacute phase (48–72 h after the exercise bout). The protective effects of polyphenols after prolonged administration may depend more of the stimulation of the endogenous antioxidant systems through nuclear factor erythroid 2-like 2 (NRF2) and antioxidant response element pathway signalling [197-199], rather than on a direct free radical-scavenging effect [41].

In the case of the combination of Zynamite® with quercetin, the antioxidant / anti-inflammatory effect due to the inhibition of the ROS-producing enzymes XO and NADP oxidase may explain the attenuation of muscle damage and pain, even though only a single dose was administered before exercise. In fact, a trend to lower pain during post-exercise ischemia was observed for this polyphenolic combination, although given at larger doses [135]. This analgesic effect could have been mediated by the free radical-scavenging properties of Zynamite® and quercetin since free radicals have been implicated in nociception [200]. In addition, adenosine accumulation due to XO inhibition by Zynamite® and quercetin may have partly attenuated nociception reducing the pain felt by the subjects that received the polyphenol mixture, as suggested by animal experiments with allopurinol [201]. Analgesic effects counteracting exercise-induced muscle pain have been reported in previous studies with polyphenol supplementation during the days preceding the exercise [202]. Lastly, although EIMD is similar between men and women, the inflammatory response is more marked in men [203, 204]. This sex dimorphism has been attributed to the anti-inflammatory and antioxidant properties of oestrogens [205, 206]. In the women

studied here, polyphenols accelerated the recovery of performance and muscle pain, regardless of the intake of oral contraceptives.

V

JUSTIFICATION

JUSTIFICATION

In recent decades, polyphenols have caused great interest not only in the scientific community, but also in the general population, primarily because of their contribution to the potential development of functional foods or cosmetic products, and their use as food supplements or natural additives in food. Given the great interest observed in the population regarding the consumption and development of natural products, the investigation of how natural polyphenols of plant origin can improve physical performance and human health is of great relevance.

This thesis will allow to add new evidence to the literature on how some polyphenolic combinations may influence on sports performance after sprinting and strenuous exercises, as well as recovery after EIMD. Investigating how these natural compounds affect different types of exercise can be a decisive step for elite athletes, for whom a small improvement in performance can mean winning a medal or an important competition.

Advances in this area will allow a more in-depth understanding of the relationship of these antioxidants in sports performance as well as in weight loss. In addition, they will help to elucidate the molecular mechanisms involved, the metabolic pathways related, as well as the attenuation or increase of relevant specific proteins that eventually may arise as potential therapeutic targets.

This will allow the creation of new, more specific and detailed training models, where the benefits of polyphenols can be observed both in performance and in recovery from very intense, strenuous or very fatigable exercise. Besides, these models could be combined with other dietary guidelines to promote weight loss.

On the other hand, these studies are necessary to advance the knowledge on the specific polyphenols alone or in combination and identify their most effective dosages to achieve improvements in physical performance and health-related outcomes.

Finally, the findings of the present thesis can promote biotechnological and environmental advances, optimizing and improving the extraction processes used to date or developing and creating new ones.

VI

OBJECTIVES AND HYPOTHESES

OBJECTIVES AND HYPOTHESES

The overall aim of this thesis was to determine how different combinations of natural polyphenols improve physical performance and recovery after different types of exercise.

More specifically, the objectives and hypotheses of each study were:

Study 1

Objective:

- To determine the acute and prolonged effects of oral supplementation with botanical extracts containing mangiferin and luteolin on exercise performance, muscle metabolism, and brain and muscle oxygenation in healthy young men.

Hypothesis:

- The ingestion of these compounds before exercise, in acute and prolonged supplementation, might help to enhance exercise performance by facilitating mitochondrial respiration.

Study 2

Objective:

- To test whether a mango leaf extract (MLE) (60% weight mangiferin) administered in two different formulations: one with quercetin and tigernut extract, and another with luteolin, has a performance-enhancing effect in young men and women.

- To test whether the combinations mangiferin-luteolin and mangiferin-quercetin protect skeletal muscle from the negative effects of ischemia-reperfusion applied immediately at the end of sprint exercise.

Hypothesis:

- Both mangiferin-containing supplements will enhance sprint performance.

- MLE in combination with quercetin or luteolin might facilitate the recovery of exercise performance after ischemia-reperfusion in humans.

Study 3

Objective:

- To determine whether a single dose of Zynamite® administered in combination with a small amount of quercetin, or with quercetin combined with sunflower lecithin, increases exercise performance during repeated sprint exercise.

- To determine whether the ergogenic effects of the combination of Zynamite® with quercetin can be improved by adding sunflower lecithin.

Hypothesis:

- A single dose of the combination of Zynamite® with quercetin will improve muscle contractile capacity and muscle oxygenation during repeated sprint exercise to exhaustion.

- These effects will be further augmented by adding sunflower phospholipids to the Zynamite®-quercetin mixture.

Study 4

Objective:

- To determine whether Zynamite® administered in combination with a small amount of quercetin, facilitates recovery after repeated damaging exercise.

- To determine whether this polyphenol combination attenuates exercise induced muscle damage and pain.

Hypothesis:

- The combination of Zynamite® with quercetin will accelerate the recovery of muscle performance.

- The combination of Zynamite® with quercetin will reduce the muscle damage caused by the exercise and will attenuate the muscle pain reported at the end of the race.

VII

PUBLISHED ARTICLES

nutrients

MDPI

Article

Enhancement of Exercise Performance by 48 Hours, and 15-Day Supplementation with Mangiferin and Luteolin in Men

Miriam Gelabert-Rebato [1,2], Julia C. Wiebe [2], Marcos Martin-Rincon [1], Victor Galvan-Alvarez [1], David Curtelin [1], Mario Perez-Valera [1], Julian Juan Habib [1], Alberto Pérez-López [1,3], Tanausú Vega [2], David Morales-Alamo [1] and Jose A L Calbet [1,*]

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Abstract: The natural polyphenols mangiferin and luteolin have free radical-scavenging properties, induce the antioxidant gene program and down-regulate the expression of superoxide-producing enzymes. However, the effects of these two polyphenols on exercise capacity remains mostly unknown. To determine whether a combination of luteolin (peanut husk extract containing 95% luteolin, PHE) and mangiferin (mango leave extract (MLE), Zynamite®) at low (PHE: 50 mg/day; and 140 mg/day of MLE containing 100 mg of mangiferin; L) and high doses (PHE: 100 mg/day; MLE: 420 mg/day; H) may enhance exercise performance, twelve physically active men performed incremental exercise to exhaustion, followed by sprint and endurance exercise after 48 h (acute effects) and 15 days of supplementation (prolonged effects) with polyphenols or placebo, following a double-blind crossover design. During sprint exercise, mangiferin + luteolin supplementation enhanced exercise performance, facilitated muscle oxygen extraction, and improved brain oxygenation, without increasing the VO_2. Compared to placebo, mangiferin + luteolin increased muscle O_2 extraction during post-exercise ischemia, and improved sprint performance after ischemia-reperfusion likely by increasing glycolytic energy production, as reflected by higher blood lactate concentrations after the sprints. Similar responses were elicited by the two doses tested. In conclusion, acute and prolonged supplementation with mangiferin combined with luteolin enhances performance, muscle O_2 extraction, and brain oxygenation during sprint exercise, at high and low doses.

Keywords: sports nutrition; ergogenic aids; polyphenols; performance; O_2 extraction; ischemia; reperfusion; metabolism; exercise

1. Introduction

Excessive production of reactive oxygen and nitrogen species (RONS) during exercise may cause damage to the cellular structures resulting in maladaptation to exercise [1,2], inflammation [3], muscle [4–

6] and cardiac fatigue [7], and impairment of executive and cognitive functions [8]. Although some antioxidants may enhance mechanical efficiency (e.g., acetylcysteine) and improve performance [9], they may also counteract some of the signaling processes necessary for the adaptive responses to exercise [10–12]. This has prompted the search for products alternative to classical antioxidants capable of modulating redox responses without blunting some beneficial exercise adaptations [13].

Hundreds of natural polyphenols present in edible plants and plant products contribute to the health effects attributed to the consumption of certain foods [14–17]. Most polyphenols have free radical-scavenging capacity [18], while others act as signaling molecules, or have interesting properties as anti-ageing [19,20], anti-mutagenic [14,21,22] and anti-obesogenic [15,23,24] compounds. After ingestion, some polyphenols can cross the blood-brain barrier and exert specific effects on the central nervous system acting on brain metabolism, neurotransmission, and oxygenation with positive effects on neurogenesis, neurocognitive functions, and mood state [25–27]. Some polyphenols may enhance sports performance [28] and facilitate the adaptation to regular exercise by reducing exercise-induced muscle damage [29].

During exercise, reactive oxygen and nitrogen species are continuously produced by mitochondrial respiration, but xanthine oxidase (also called xanthine oxidoreductase; XO) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase, also called NOX) are also important sources of RONS during exercise [10,30,31]. The classical approach to counteract RONS during exercise has been the administration of antioxidants, i.e., compounds with free radical-quenching properties. This approach has been criticized due to the potential interference with some critical signaling events that depend exclusively on free radicals [32–34]. Nevertheless, it has been reported that supplementation with some polyphenolic compounds could avoid some of the adverse effects on performance observed with the intake of antioxidant vitamins, like vitamin C during training [33,34]. Besides, pharmacological inhibition of XO seems to reduce exercise-induced muscle damage both in animals [35] and athletes [36,37].

Natural polyphenols like mangiferin and luteolin are potent antioxidants and inhibitors of XO [23,38,39] and NOX [40,41]. A previous study has shown ergogenic effects after acute supplementation (48 h) with a mango leave extract (MLE, Zynamite®) combined with either quercetin or luteolin [27]. No data is available regarding the effects of prolonged mangiferin or luteolin supplementation on exercise performance. Chronic ingestion of either of these two polyphenols could stimulate the antioxidant gene program through up-regulation of the nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2) transcription factor [39,42] and could elicit an up-regulation of the natural antioxidant enzymes, increasing the capacity of the cells to face the burden of RONS produced during exercise. Mangiferin may also be an excellent bioactive to prevent mitochondrial lipid peroxidation [43], which may occur during prolonged and unaccustomed exercise [30,44]. Moreover, animal experiments indicate that luteolin may down-regulate the expression of the genes (Cyba, Cybb, Ncf1, Ncf4, and Rac2) encoding the enzymatic subunits of NADPH oxidase [40,41]. The selective action of these two polyphenols on XO and NOX is particularly interesting since other sources of free radicals would not be inhibited, permitting the signaling events necessary for the normal adaptation to exercise [34].

Therefore, we hypothesized that the ingestion of these compounds before exercise might help to enhance exercise performance by facilitating mitochondrial respiration through its antioxidant and XO-inhibitory properties, enhancing muscle O_2 extraction and improving brain oxygenation as previously reported in young women [27].

Consequently, this investigation aimed at determining the acute and prolonged effects of oral supplementation with mangiferin and luteolin botanical extracts on exercise performance, muscle metabolism, and brain and muscle oxygenation in healthy young men. Given the fact that these two polyphenols may have ergogenic effects through several mechanisms, a specific exercise protocol was designed, including phases of low-intensity, high-intensity, and repeated sprinting exercise combined with ischemia-reperfusion episodes.

2. Materials and Methods

2.1. Subjects

Twelve healthy male physical education students (age = 21.3 ± 2.1 years, height = 176.6 ± 5.8 cm, body mass = 75.7 ± 9.9 kg, body fat = 20.4 ± 5.3%, VO_2max: 3.69 ± 0.47 L/min and 49.4 ± 8.2 mL/kg/min) agreed to participate in this investigation. The inclusion criteria for participation in the study were: age from 18 to 35 years old; male without chronic diseases or recent surgery; non-smoker; normal resting electrocardiogram; body mass index below 30 and above 18; no history of disease requiring medical treatments lasting more than 15 days during the preceding six months; no medical contraindications to exercise testing; and lack of allergies to peanuts or mango fruit. All volunteers applying for participation met the inclusion criteria. Subjects were requested to avoid intense physical activity 48 h before laboratory tests and to refrain from carbonated, caffeinated and alcohol-containing beverages during the 24 h period preceding the tests. They were also requested to record the dinner preceding the first experimental day and reproduce the same dinner the night before the subsequent experimental days.

All subjects received written and oral information about the experimental procedures before providing their written consent to participate. The study was performed by the Helsinki Declaration and approved by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2016-02). The sample size required to allow detecting a 5% improvement of performance with a statistical power of 0.8 ($\alpha = 0.05$), assuming a coefficient of variation for the ergometric test below 5%, was eight subjects. To account for potential dropouts and technical difficulties twelve subjects were finally recruited.

2.2. General Procedures

After inclusion, a medical history, resting electrocardiogram, a blood analysis including the assessment of a basic hemogram and general clinical biochemistry tests were carried out to verify the health status of participants. The clinical tests were repeated 24 h and 14 days after the start of supplementation. Subjects were randomly assigned to a placebo (P) or treatment group (T) in a double-blind, counterbalanced crossover design. The placebo group received microcrystalline cellulose capsules of identical aspect containing 500 mg of maltodextrin, while the treatment group received similar capsules containing luteolin and mangiferin. Three subjects were provided with 50 mg/day of peanut husk extract containing 95% luteolin and 140 mg/d of MLE (Zynamite®) containing 100 mg/day of mangiferin (low-dose treatment group; L), while the remaining three subjects of the treatment group received 100 mg/day of peanut husk extract containing 95% luteolin and 420 mg/day MLE containing 300 mg/day of mangiferin (high-dose treatment group; H). A detailed description of the composition of the two supplements can be found elsewhere [27]. Subjects ingested the supplements every eight hours during 15 days, then after 3–4 weeks of washout, treatment groups received placebo, and the placebo group was again split into low and high-dose treatment subgroups, also for 15 days. The low dose of mangiferin was based on a pharmacokinetic study by Hou et al. [45] showing oral absorption and mean residence time close to 7 h, after the ingestion of 0.1 g of pure mangiferin in humans. The high dose of luteolin was based on human pharmacokinetic data obtained following the ingestion of an artichoke leaf extract rich in luteolin [46], and 100 mg of encapsulated luteolin [47], as previously reported [27].

Subjects reported to the laboratory early in the morning after a 12 h fast, 48 h after the start of the supplementation, and received an extra dose of the assigned supplements. After that, their body composition was determined using dual-energy X-ray absorptiometry (Lunar iDXA, General Electric, WI, USA), followed by the assessment of their resting metabolic rate (RMR) by indirect calorimetry (Vyntus CPX; Jaeger-CareFusion, Hoechberg, Germany) during 20 min lying supine and motionless on a comfortable stretcher while a quiet environment was maintained. Then near-infrared spectroscopy (NIRS) optodes were placed on the frontal lobe and the musculus vastus lateralis and medialis as previously reported [48,49]. With the subjects resting supine a 10 cm wide cuff connected to a rapid cuff inflator (SCD10, Hokanson, Bellevue, DC, USA) was placed around the right thigh, as proximal as possible, as previously reported [49]. After an initial 3 min period with legs elevated on a cushion, the cuff was inflated at 300 mmHg at maximal speed, resulting in full occlusion of the legs' circulation within less than 2 s,

which was maintained for 8 min. At the end of the occlusion period, the cuff was released and the hyperemic response measured during the next 2 min.

2.3. Exercise Protocol

The exercise protocol (Figure 1) started with a warm-up consisting of 8 s of isokinetic sprint on a cycle ergometer (Excalibur Sport 925900, Lode, Groningen, The Netherlands) (Figure 1). This was followed by a 5 min recovery period during which the subjects pedaled at low speed (~40 rpm) with no load. Next, an incremental exercise test was performed to determine the maximal fat oxidation capacity (MFO) (see below). The MFO test was followed by 2 min of unloaded pedaling, and then the load was increased to the same intensity reached at the end of the MFO test and increased 15 W every min until exhaustion to determine the VO_2max. Immediately upon exhaustion, the cuffs were instantaneously inflated at maximal speed and pressure (i.e., 300 mmHg) to completely occlude the circulation (ischemia) for 60 s, as previously reported [49]. The subjects remained seated and quiet on the cycle ergometer without pedaling during the periods of ischemia. At the 50th second of ischemia, a 10 s countdown was started while the subjects got ready to sprint as fast and hard as possible for 15 s. At the start of the sprint, following the 60 s of ischemia, the cuff was instantaneously deflated such that the sprint was carried out with the circulation opened. At the end of the 15 s sprint, a second occlusion was started for 30 s, which was followed by 10 s of free circulation. At the end of the 15 s sprint, a second occlusion was started for 30 s, then the cuff was released and the subjects pedaled slowly at 20 W while a 10 s countdown towards a second 15 s sprint was started. Thus, the second 15 s sprint was carried out after a cycle of ischemia (30 s) followed by 10 s reperfusion. Then, after 2.5 min of passive recovery on the bike, a blood sample was obtained from the earlobe to measure blood lactate concentration (Lactate Pro 2, Arkray, Kyoto, Japan). After the second 15 s sprint, the volunteers rested for 30 min. During the first 20 min they rested lying on a stretcher; then, they moved back to the ergometer for unloaded pedaling at low speed while the instruments were reconnected. At the completion of the 30 min recovery, a Wingate test (sprint lasting 30 s) was performed followed by a 4 min recovery period during which the subjects pedaled at low speed with the cycle ergometer unloaded. At the end of this short recovery, a second Wingate test was performed. The second Wingate was followed by a 10 min recovery with slow pedaling at 20 W. After 2.5 min of slow unloaded pedaling on the cycle ergometer, a blood sample was obtained from the earlobe to measure blood lactate concentration. At the completion of the 10 min recovery period, a submaximal constant-intensity time trial to exhaustion was started at 70% of the intensity reached in the incremental exercise test (Wmax). In control experiments, with the subjects rested before the time trial, our volunteers were able to sustain this intensity for 20–60 min, depending on their fitness status. This test was used to assess the effects of the supplements on endurance capacity, since the test likely started with very low glycogen levels, replicating the conditions of the final stages of most endurance competitions. At the end of the endurance test (exhaustion), the circulation of both legs was occluded again for 60 s. At the 50th second of ischemia, a 10 s countdown was started while the subjects prompted to perform a final Wingate (30 s) sprint. At the end of this sprint, the subjects remained seated on the bike while pedaling at low speed with the cycle ergometer unloaded. After 2.5 min of recovery, another blood sample was obtained from the earlobe to measure blood lactate. Then the subjects moved to the stretcher and rested until reaching 30 min of recovery. Strong verbal encouragement was provided throughout the entire exercise protocol and particularly approaching task failure and during the sprints.

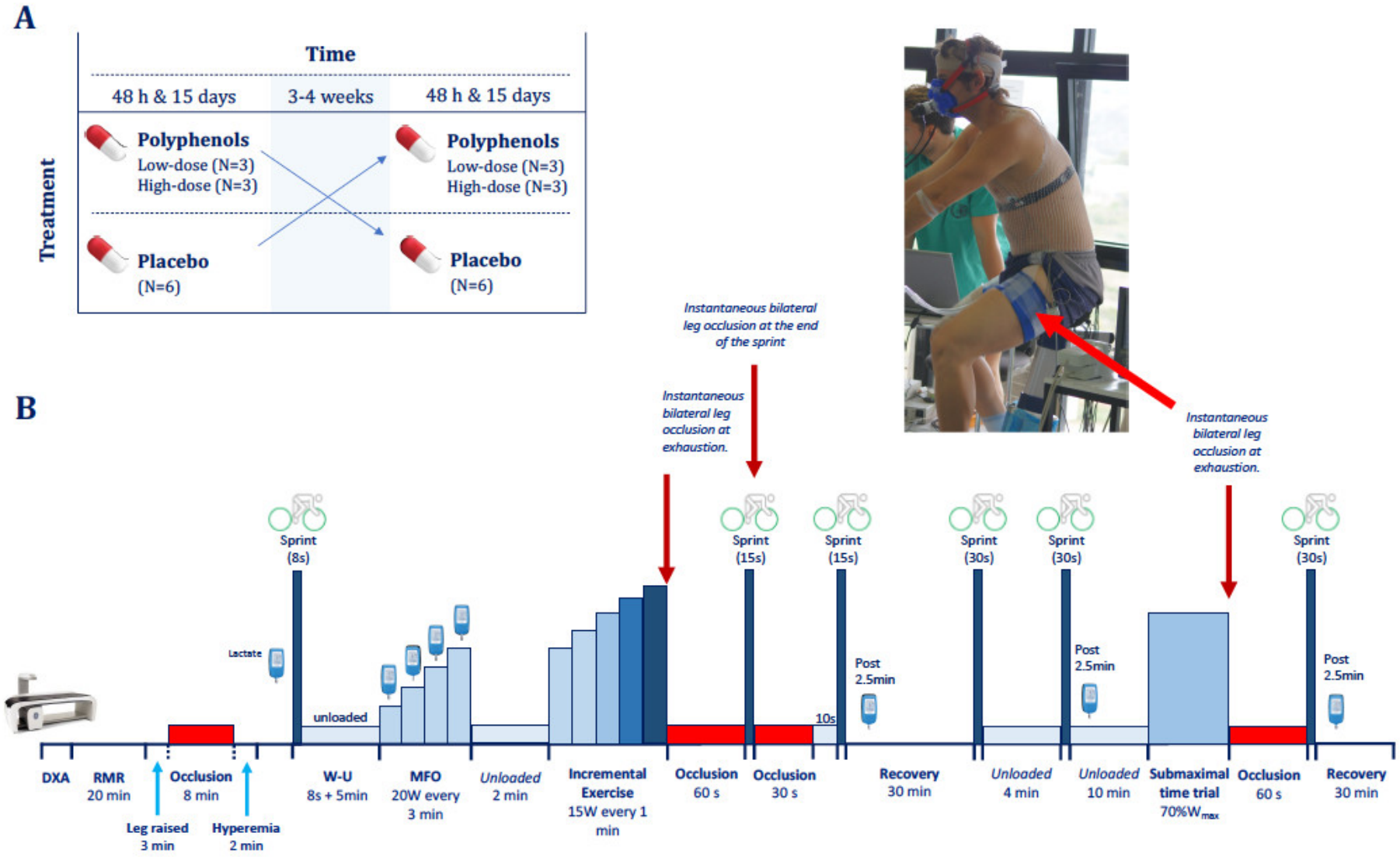


Figure 1. Experimental protocol. (**A**) Botanical extracts of mangiferin and luteolin were administered during following a double-blind, counterbalanced crossover

design. (**B**) Exercise protocol. Forty-eight hours after the start of the supplementation subjects reported to the laboratory and their body composition and resting metabolic rate (RMR) were determined. The exercise protocol started with a warming-up 8 s isokinetic sprint on a cycle ergometer, followed by 5 min of unloaded pedaling (~40 rpm) and an incremental exercise test (20 W/3 min) to determine their maximal fat oxidation (MFO). This was followed by 2 min of unloaded pedaling and an incremental exercise test (15 W/min) until exhaustion to determine the VO_2max. At exhaustion, ischemia was applied to both legs for 60 s. Then the cuff was released, and the subjects sprinted maximally for 15 s. At the end of the 15 s sprint, a second occlusion was started for 30 s, which was followed by 10 s of free circulation with unloaded pedaling and a second 15 s sprint. Then the subjects rested for 30 min and after that performed two 30 s Wingate tests interspaced by 4 min of unloaded pedaling. After 10 min of unloaded pedaling a submaximal constant-intensity time trial to exhaustion was started at 70% of the intensity reached at exhaustion in the incremental exercise test (Wmax). At the end of the endurance test, ischemia was instantaneously applied for 60 s, followed by the last Wingate test with open circulation and 30 min of recovery on a stretcher. Blood samples for blood lactate assessment were obtained as indicated in the figure. This protocol was repeated after 15 days of supplementation.

This exercise protocol was repeated after 15 days of supplementation, to determine potential effects due to prolonged supplementation. After 3–4 weeks of washout, the acute and chronic phases were repeated following the crossover counterbalanced design described above.

2.4. Power Output and VO_2max

All sprints were performed with the cycle ergometer set in isokinetic mode and results reported as instantaneous peak power (PPO) and mean power output (MPO) [49]. Oxygen uptake was measured with a calibrated metabolic cart (Vyntus CPX; Jaeger-CareFusion, Hoechberg, Germany). Respiratory variables were analyzed breath-by-breath and averaged every 5 s during the sprints. During maximal exercise 15-breath, rolling averages were generated starting from 120 s before the end of the exercise, and the highest 15-breath averaged value was taken as the VO_2max.

2.5. Maximal Fat Oxidation

This test started at 20 W for 3 min, followed by 20 W increases every 3 min until the respiratory exchange ratio (RER) was ≥1.0 [50,51]. The VO_2 and VCO_2 data averaged during the last min of each load, and was used to determine the maximum rate of fat oxidation as previously reported [50,51]. Blood lactate concentrations were determined from earlobe samples obtained after 90 s after each increase in intensity.

2.6. Exercise Efficiency, Supramaximal Exercise O_2 Demand, and Oxygen Deficit

The O_2 demand during the sprints was calculated from the linear relationship between the last 60 s averaged VO_2 of each load, measured during the MFO and the exercise intensity. The accumulated oxygen deficit (AOD), representing the difference between O_2 demand and the actual VO_2, was determined as previously reported [52,53]. The delta energy efficiency of exercise was determined as the slope of the linear relationship between work and energy expenditure [54], using the data collected during the MFO tests.

2.7. Cerebral and Musculus Vastus Lateralis Oxygenation

Cerebral oxygenation was assessed using near-infrared spectroscopy (NIRS, NIRO-200 NX, Hamamatsu, Hamamatsu City, Japan) employing spatially resolved spectroscopy to obtain the tissue oxygenation index (TOI) using a pathlength factor of 5.92 [55]. The first NIRS optode was placed on the right frontoparietal region at 3 cm from the midline and 2–3 cm above the supraorbital crest, to avoid the sagittal and frontal sinus areas [56]. This optode placement allows recording the tissue oxygenation of the superficial frontal cerebral cortex, which may influence exercise performance [57,58]. A second optode was placed in the lateral aspect of the thigh at middle length between the patella and the anterosuperior iliac crest, over the middle portion of the musculus vastus lateralis and an additional optode was placed on the vastus medialis at 1/8 distance between the iliac spine and the joint space in front of the medial ligament. The quadriceps muscle oxygenation index (TOI) was obtained from the average of the mean TOI of the two vastus.

2.8. Diet Analysis

Subjects' dietary information was collected using dietary logs during four days, including one weekend day, on two occasions: before the start of the supplementation, and after one week into each supplementation period, using dietary logs. For this purpose, subjects were provided with a dietary diary and a kitchen scale (1 g precision from 0 to 5000 g, calibrated in our laboratory with Class M1 calibration weights, Schenk) and instructions to report in grams all food and drinks ingested. The information recorded was later analyzed with specific software for the Spanish diet (Dial, Alce Ingeniería, Madrid, Spain [59]).

2.9. Statistics

Variables were checked for normal distribution by using the Shapiro-Wilks test. When necessary, the analysis was carried out on logarithmically transformed data. A three-way repeated-measures ANOVA test with time (two levels: 48 h and 15 days), treatment (two levels: placebo and polyphenol treatment) and polyphenols dose (two levels: low and high) as between-subjects factors was first applied. Pairwise comparisons were carried using the least significant post hoc test (LSD). The relationship between variables was determined using linear regression analysis. Values are reported as the mean ± standard error of the mean (unless otherwise stated). $p \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

Polyphenols had no significant effects on the clinical blood biochemistry and hemogram tests (Tables S1 and S2). The diet was not significantly altered by the treatment regarding total energy, macronutrients, vitamins, dietary fiber, and plant sterols intakes. Likewise, no significant alterations were observed in body weight or resting metabolic rate, resting blood pressure, blood lactate concentration or heart rate after polyphenols administration (Table S3). The level of deoxygenation reached during the occlusion performed at rest was similar in all conditions, as well as the increase in tissue oxygenation index elicited by the post-ischemic hyperemia.

3.1. Incremental Exercise Test

All respiratory variables responded similarly to the placebo and the polyphenol treatments. As reflected in Table 1, the subjects exercised to a similar extent in all tests. Neither the VO_2max nor the load reached at exhaustion (Wmax) were affected by the treatment. There was a slight 2 mmHg improvement in $P_{ET}O_2$ in the second test which was also accompanied by a small reduction in $P_{ET}CO_2$ (~2 mmHg), without differences due to the supplementation administered.

Lactate responses to submaximal exercise were almost identical. Although blood lactate concentration at 200 W was 11% lower after the polyphenol treatment, this effect did not reach statistical significance ($p = 0.11$) (Table 1). Delta efficiency was transiently improved 48 h after the start of polyphenols in the group receiving the lower dose (compared to placebo, $p = 0.002$, ANOVA treatment × time × dose interaction $p = 0.001$). Polyphenols supplementation did not alter the MFO nor peak HR (Table 1).

Table 1. (a) Effects of mangiferin and luteolin botanical extracts on muscle energy efficiency, heart rate, performance, and pulmonary gas exchange during incremental exercise to exhaustion. (b) Effects of mangiferin and luteolin botanical extracts on muscle energy efficiency, heart rate, performance, and pulmonary gas exchange during incremental exercise to exhaustion and the final time trial.

(a)

		Placebo (48 h)	Placebo (15 Days)	MA + Luteolin (48 h)	MA + Luteolin (15 Days)	Treatment	Pre-Post	T × t	T × t × d
Delta Efficiency (%)	L	27.0 ± 2.5	29.2 ± 4.6	30.0 ± 2.1	27.2 ± 1.9	0.74	0.73	0.46	<0.001
	H	28.9 ± 2.1	28.0 ± 1.9	27.1 ± 2.1	29.7 ± 3.4				
MFO (mg/min)	L	392.2 ± 40.0	347.3 ± 53.6	393.7 ± 100.9	370.9 ± 52.9	0.81	0.35	0.50	0.84
	H	399.8 ± 129.0	367.3 ± 107.4	377.7 ± 143.6	385.8 ± 178.4				
MFO VO_2 (mL/min)	L	1377 ± 282	1260 ± 136	1260 ± 173	1313 ± 205	0.58	0.17	0.75	0.33
	H	1455 ± 406	1389 ± 301	1478 ± 385	1387 ± 455				
Wmax (W)	L	277 ± 30	282 ± 25	288 ± 25	271 ± 24	0.87	0.11	0.16	0.03
	H	291 ± 48	286 ± 42	291 ± 48	291 ± 47				
HRmax (beats/min)	L	192 ± 8	187 ± 14	187 ± 12	192 ± 8	0.20	0.33	0.13	0.08
	H	193 ± 8	189 ± 10	198 ± 10	194 ± 12				
VO_2max (mL/min)	L	3568 ± 513	3660 ± 318	3649 ± 387	3623 ± 240	0.87	0.11	0.16	0.026
	H	3821 ± 456	3742 ± 566	3770 ± 590	3681 ± 567				
RERmax	L	1.17 ± 0.09	1.16 ± 0.05	1.18 ± 0.06	1.14 ± 0.03	0.2	0.33	0.13	0.08
	H	1.11 ± 0.03	1.14 ± 0.04	1.13 ± 0.07	1.12 ± 0.05				
$V_E$max (L/min)	L	148 ± 35	161 ± 24	153 ± 27	167 ± 38	0.78	0.61	0.47	0.54
	H	161 ± 21	167 ± 25	164 ± 20	160 ± 17				
BFmax (breaths/min)	L	56 ± 13	63 ± 11	60 ± 10	64 ± 15	0.90	0.67	0.026	0.86
	H	62 ± 9	64 ± 11	63 ± 8	64 ± 8				
$P_{ET}CO_2$ (mmHg)	L	37.1 ± 2.9	33.3 ± 4.8	37.3 ± 3.3	34.2 ± 3.5	0.69	0.07	0.63	0.57
	H	33.5 ± 2.6	31.8 ± 4.6	32.8 ± 2.7	33.1 ± 2.2				
$P_{ET}O_2$ (mmHg)	L	117 ± 5	119 ± 4	117 ± 4	120 ± 6	0.47	0.08	0.61	0.91
	H	119 ± 3	119 ± 3	119 ± 3	118 ± 2				

(b)

		Placebo (48 h)	Placebo (15 days)	MA + Luteolin (48 h)	MA + Luteolin (15 days)	Treatment	Pre-Post	T x t	T x t x d
Lact at 100 W (mM)	L	1.9 ± 0.5	1.8 ± 0.5	1.7 ± 0.3	1.9 ± 0.4	0.55	0.94	0.41	0.92

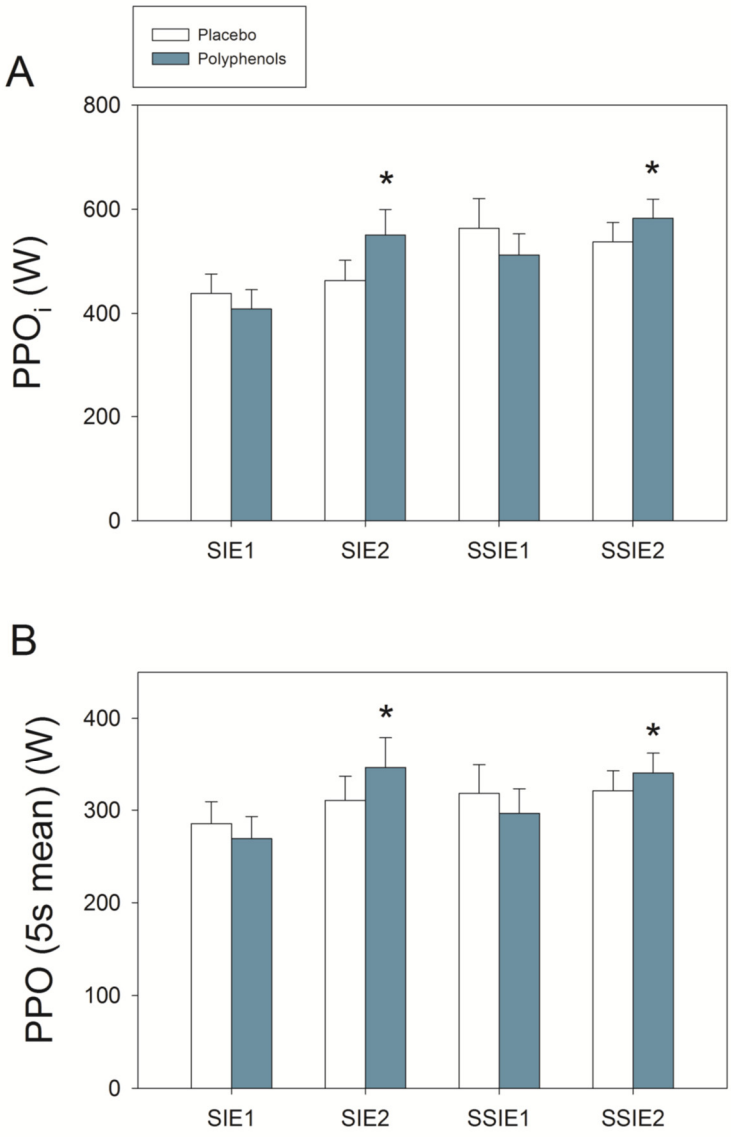
	H	2.1	±	1.1	2.1	±	1.3	2.1	±	1.0	2.1	±	1.6				
Lact at 200 W (mM)	L	5.8	±	2.6	6.0	±	1.6	5.2	±	1.2	5.7	±	0.5	0.11	0.59	0.44	0.69
	H	6.4	±	3.9	5.9	±	3.2	5.0	±	1.9	5.4	±	3.7				
LT 4 mM (W)	L	177	±	29	173	±	26	181	±	19	170	±	8	0.78	0.40	0.84	0.39
	H	180	±	58	177	±	63	181	±	48	182	±	68				
Lact Peak Post-Ischemia (mM)	L	9.1	±	2.2	10.2	±	1.5	8.6	±	2.4	11.2	±	1.2	0.53	0.02	0.29	0.88
	H	10.5	±	3.2	10.6	±	2.7	10.4	±	2.4	11.7	±	2.3				
RPE (post Incremental exercise)	L	7.5	±	0.6	7.8	±	1.0	6.8	±	2.2	7.8	±	1.9	0.89	0.12	0.60	0.88
	H	7.3	±	1.6	7.3	±	2.3	7.7	±	1.5	8.1	±	0.5				
Time trial total work (kJ)	L	81.7	±	54.9	124.5	±	73.6	96.1	±	48.2	124.1	±	74.3	0.78	0.07	0.99	0.60
	H	94.5	±	63.8	118.8	±	71.7	88.5	±	83.5	126.5	±	100.0				

(a) MA: mangiferin, Pre-Post: comparison of main effects between 48 h and 15 days, T × t: treatment by time interaction; T × t × d: Treatment × time × dose interaction, L: 50 mg of luteolin and 100 mg mangiferin; H: 100 mg of luteolin and 300 mg mangiferin; MFO: maximal fat oxidation, VO_2: oxygen uptake, Wmax: power output reached at exhaustion during the incremental exercise, HRmax: maximal heart rate during the incremental exercise, VO_2max: maximal oxygen uptake, RERmax: respiratory exchange ratio at maximal exercise, $V_E$max: pulmonary ventilation at maximal exercise, BFmax: breathing frequency at maximal exercise, $P_{ET}CO_2$: end-tidal carbon dioxide pressure, $P_{ET}O_2$: end-tidal oxygen pressure, (n = 12 for all variables). (b) MA: mangiferin, Pre-Post: comparison of main effects between 48 h and 15 days, T × t: treatment by time interaction; T × t × d: Treatment × time × dose interaction, L: 50 mg of luteolin and 100 mg mangiferin; H: 100 mg of luteolin and 300 mg mangiferin; Lact: blood lactate concentration, LT 4 mM: Power output at the Lactate threshold of 4 mM, RPE: rate of perceived exertion, (n = 12 for all variables, except the final time trial n = 11).

3.2. Sprint Exercise after Ischemia-Reperfusion

The PPO was not altered by the acute administration of polyphenols (Figure 2A). Following fifteen days of supplementation, PPO in the sprints preceded by ischemia was 500.0 ± 120.1 and 566.4 ± 141.9 W, in the placebo and polyphenol trials, respectively (p = 0.11). Nevertheless, from the first (48 h) to second trial (15 days), PPO was enhanced by 22% when the subjects were taken polyphenols ($p < 0.05$), being this effect more marked in the first (+31%) than the second sprint (+14%) (first sprint compared with the second sprint, $p < 0.05$; ANOVA sprint × trial × treatment × dose interaction p = 0.026). There were no significant differences between the higher and lower doses of polyphenols on PPO.

In the sprints post-ischemia performed with polyphenols, the MPO developed during the first 5 s was increased by 23% from 48 h to 15 days (272.5 ± 63.8 and 333.8 ± 93.2 W, respectively, p = 0.01). In contrast, no significant changes were observed from 48 h to 15 days in the placebo conditions (Figure 2B).



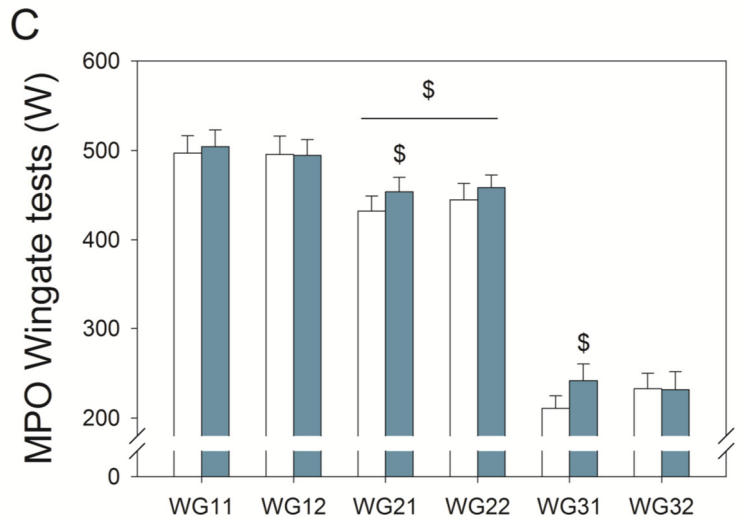


Figure 2. Performance during the sprint exercise after the ingestion of polyphenols (mangiferin + luteolin) or placebo. (**A**) Peak power output in 15 s sprints performed after ischemia. (**B**) Mean power output during the first 5 s during the sprints performed after ischemia. SIE: first sprint after incremental exercise, SSIE: second sprint after incremental exercise. Number 1 indicates after 48 h and 2 after 15 days of supplementation. (**C**) Mean power output during the 30 s Wingate test. WG: Wingate test, the first number represents the Wingate order number (1, 2, or 3), the second number (1 or 2) indicates after 48 h and 2 after 15 days of supplementation, respectively. * $p < 0.05$ compared with 48 h test in the same condition. \$ $p < 0.05$ for treatment effect. ANOVA Wingate × time × treatment interaction, $p = 0.027$. N = 12.

Despite the fact that the mean power output remained at the same level (256 ± 56 and 268 ± 75 W, in the placebo and mangiferin + luteolin condition, respectively, $p = 0.45$), the mean VO_2 during the sprints post-ischemia was reduced by 5.7% after the administration of mangiferin + luteolin (from 666 ± 98 to 628 ± 77 mL, in the placebo and mangiferin + luteolin conditions, respectively, $p = 0.010$) (Table 2). Although the O_2 deficit was 23% larger after the ingestion of mangiferin + luteolin, this difference was not statistically different ($p = 0.245$). The peak blood lactate measured 2.5 min after the last sprint postischemia was unchanged in the placebo experiments (9.8 ± 2.7 and 10.4 ± 2.1 mM, $p = 0.35$), but increased from 9.5 ± 2.5 to 11.4 ± 1.8 mM (48 h and 15 days, respectively) after the ingestion of polyphenols ($p = 0.04$) (Table 1).

Table 2. Effects of mangiferin and luteolin botanical extracts on heart rate and pulmonary gas exchange during 15 s all-out sprint performed after ischemia/reperfusion, immediately after the incremental exercise to exhaustion.

		First 15 s sprint				Second 15 s Sprint							
		Placebo (48 h)	**Placebo (15 Days)**	**MA + Luteolin (48 h)**	**MA + Luteolin (15 Days)**	**Placebo (48 h)**	**Placebo (15 Days)**	**MA + Luteolin (48 h)**	**MA + Luteolin (15 Days)**	**Sprint**	**Treat**	**Pre-Post**	**Sprint × Treat**
HR (beats/min)	L	170 ± 12	171 ± 13	168 ± 12	170 ± 13	168 ± 12	172 ± 11	165 ± 11	170 ± 14	0.36	0.79	0.011	0.74
	H	180 ± 15	182 ± 10	180 ± 14	184 ± 13 *	178 ± 16	182 ± 9	177 ± 14	186 ± 14 *				
VO_2 (mL)	L	530 ± 121	542 ± 101	515 ± 105	554 ± 127	728 ± 160	749 ± 98	655 ± 76	729 ± 94	<0.001	0.010	0.038	0.99
	H	585 ± 85	577 ± 93	461 ± 124	553 ± 69	823 ± 112	799 ± 131	740 ± 85	821 ± 120				
O_2 Deficit (mL)	L	164 ± 163	219 ± 233	130 ± 70	195 ± 74	28 ± 158	51 ± 136	38 ± 119	41 ± 121	<0.001	0.19	0.42	0.94
	H	306 ± 87	338 ± 106	399 ± 175	466 ± 58	129 ± 142	139 ± 90	232 ± 249	184 ± 53				
V_E (L/min)	L	95 ± 36	102 ± 39	101 ± 25	107 ± 42	121 ± 39	123 ± 35	115 ± 32	126 ± 49	<0.001	0.74	0.025	0.74
	H	119 ± 32	122 ± 18	104 ± 24	119 ± 18	138 ± 23	150 ± 21	134 ± 13	155 ± 11				
BF (breaths/min)		48 ± 13	50 ± 14	49 ± 8	42 ± 9	53 ± 13	55 ± 13	53 ± 11	49 ± 11	0.029	0.61	0.52	0.72
		52 ± 11	58 ± 11	55 ± 12	55 ± 13	57 ± 11	61 ± 7 *	59 ± 10	62 ± 8				
$P_{ET}CO_2$ (mmHg)	L	29 ± 3	30 ± 7	28 ± 4	29 ± 8	31 ± 6	24 ± 10	30 ± 5	28 ± 9	0.77	0.91	0.046	0.25
	H	27 ± 5	25 ± 6	25 ± 4	25 ± 5	29 ± 3	26 ± 4 *	27 ± 4	27 ± 3				
$P_{ET}O_2$ (mmHg)	L	119 ± 4	118 ± 7	121 ± 4	119 ± 8	116 ± 6	122 ± 8	116 ± 5	117 ± 10	0.057	0.72	0.101	0.178
	H	121 ± 4	122 ± 6	124 ± 5	123 ± 6	118 ± 3	120 ± 3	120 ± 4	120 ± 4				

MA: mangiferin, Sprint: differences between sprints, Treat: treatment effect, Pre-Post (time effect): comparison of main effects between 48 h and 15 days, Sprint × treat: Sprint × treatment interaction, L: 50 mg of luteolin and 100 mg mangiferin; H: 100 mg of luteolin and 300 mg mangiferin, VO_2: oxygen uptake, HR: heart rate, VO_2: total O_2 uptake during the sprint, V_E: pulmonary ventilation, BF: breathing frequency, $P_{ET}CO_2$: end-tidal carbon dioxide pressure, $P_{ET}O_2$: end-tidal oxygen pressure, (n = 10 for all variables). Two subjects were eliminated from the statistical analysis due to missing values. * $p < 0.05$ compared with 48 h test in the same condition.

3.3. Wingate Tests

Compared to placebo, polyphenol intake resulted in 4.0% greater MPO (48 h and 15 days assessments combined, $p = 0.017$; ANOVA Wingate × time × treatment $p = 0.027$). Acutely, compared to placebo, polyphenol administration enhanced MPO by 5% in the second Wingate test ($p = 0.009$) (Figure 2C). This was accompanied by enhanced brain oxygenation (Figure 3) (ANOVA treatment effect $p = 0.02$), being this response greater for the higher dose (ANOVA, treatment × dose interaction $p = 0.047$). Quadriceps muscle oxygenation index during sprint exercise was significantly lower, reflecting enhanced O_2 extraction, after the ingestion of polyphenols both after 48 h (59.7 ± 6.0 and 57.9 ± 6.4%, $p = 0.007$) and 15 days (60.1 ± 3.9 and 57.0 ± 6.1%, $p = 0.007$) supplementation (ANOVA, treatment × dose interaction $p = 0.01$) (Figure 4). Oxygen uptake during the sprints was 6.0% lower after the ingestion of mangiferin + luteolin ($p = 0.010$) (Table 3). Neither the heart rate nor respiratory variables were significantly altered by the ingestion of polyphenols during the two Wingate tests (Table 3).

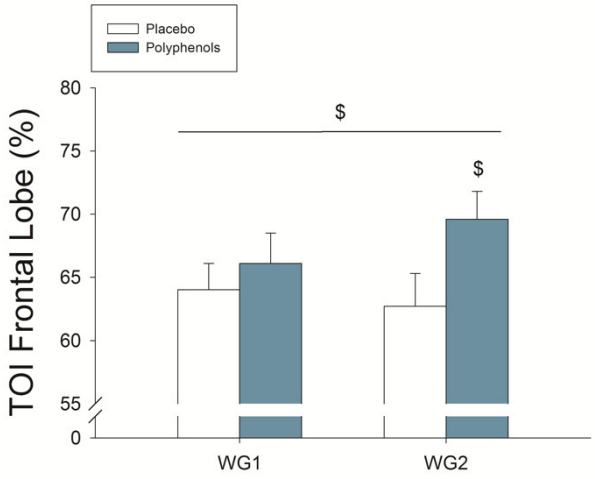


Figure 3. Frontal lobe oxygenation index (TOI) during the first two 30 s Wingate tests after the ingestion of polyphenols (luteolin + mangiferin) or placebo. Number 1 indicates after 48 h and 2 after 15 days of supplementation. $ $p < 0.05$ for treatment effect. N = 12.

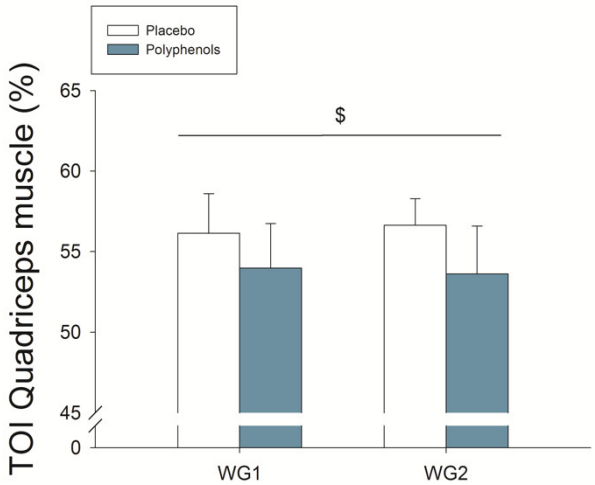


Figure 4. Quadriceps muscle oxygenation index (TOI, mean of the *musculus vastus lateralis* and *vastus medialis*) during the first two 30 s Wingate tests after the ingestion of polyphenols (mangiferin + luteolin) or placebo. Number 1 indicates after 48 h and 2 after 15 days of supplementation. $ $p< 0.05$ for treatment effect. N = 12.

Table 3. Effects of mangiferin and luteolin botanical extracts on heart rate and pulmonary gas exchange during the 30-s all-out sprint (Wingate tests) performed after a 30 min recovery period and interspaced by 4 min of unloaded pedaling.

		First 30 s Sprint				Second 30 s Sprint							
		Placebo (48 h)	Placebo (15 Days)	MA + Luteolin (48 h)	MA + Luteolin (15 Days)	Placebo (48 h)	Placebo (15 Days)	MA + Luteolin (48 h)	MA+ Luteolin (15 Days)	Sprint	Treat	Pre-Post	Sprint × Treat
HR (beats/min)	L	164 ± 6	164 ± 7	161 ± 2	162 ± 6	165 ± 6	165 ± 7	164 ± 4	166 ± 8	0.011	0.92	0.62	0.058
	H	169 ± 10	170 ± 9	170 ± 9	171 ± 13	171 ± 8	171 ± 9	172 ± 8	172 ± 10				
VO_2 (mL)	L	1321 ± 238	1292 ± 239	1182 ± 181	1337 ± 240	1393 ± 196	1392 ± 293	1379 ± 183	1442 ± 203	<0.001	0.59	0.58	0.27
	H	1230 ± 246	1287 ± 143	1264 ± 209	1226 ± 181	1435 ± 234	1414 ± 202	1428 ± 257	1377 ± 177				
O_2 Deficit (mL)	L	1566 ± 307	1415 ± 275	1573 ± 358	1421 ± 348	1212 ± 332	1091 ± 269	1141 ± 256	1167 ± 290	<0.001	0.55	0.58	0.55
	H	1783 ± 358	1683 ± 276	1794 ± 314	1610 ± 362	1211 ± 295	1291 ± 228	1364 ± 229	1258 ± 277				
V_E (L/min)	L	86 ± 23	83 ± 26	78 ± 19	91 ± 40	115 ± 33	124 ± 44	119 ± 29	122 ± 34	<0.001	0.82	0.49	0.71
	H	98 ± 23	102 ± 24	101 ± 24	99 ± 18	134 ± 16	135 ± 20	137 ± 10	136 ± 15				
BF (breaths/min)	L	49 ± 11	49 ± 12	48 ± 8	43 ± 10	52 ± 12	51 ± 9	53 ± 10	49 ± 12	0.001	0.88	0.34	0.108
	H	49 ± 10	50 ± 11	48 ± 15	52 ± 16	56 ± 11	55 ± 9	61 ± 11	58 ± 10				
$P_{ET}CO_2$ (mmHg)	L	28 ± 2	26 ± 4	26 ± 6	27 ± 5	26 ± 4	24 ± 6	25 ± 5	26 ± 5	0.101	0.73	0.71	0.57
	H	23 ± 7	23 ± 5	24 ± 7	24 ± 5	23 ± 5	23 ± 5	22 ± 5	23 ± 4				
$P_{ET}O_2$ (mmHg)	L	112 ± 4	113 ± 5	113 ± 9	111 ± 8	117 ± 5	120 ± 6	119 ± 5	117 ± 6	<0.001	0.626	0.783	0.548
	H	119 ± 8	118 ± 8	117 ± 11	117 ± 8	122 ± 6	122 ± 4	122 ± 6	122 ± 4				

MA: mangiferin, Sprint: differences between the first and the second sprints, Treat: treatment effect, Pre-Post (time effect): comparison of main effects between 48 h and 15 days, Sprint × treat: Sprint × treatment interaction, L: 50 mg of luteolin and 100 mg mangiferin; H: 100 mg of luteolin and 300 mg mangiferin, HR: heart rate, VO_2: total O_2 uptake during the sprint, V_E: pulmonary ventilation, BF: breathing frequency, $P_{ET}CO_2$: end-tidal carbon dioxide pressure, $P_{ET}O_2$: end-tidal oxygen pressure, (n = 10 for all variables). Two subjects were eliminated from the statistical analysis due to occasional missing values. * $p < 0.05$ compared with 48 h test in the same condition.

The last sprint was performed after a time trial to exhaustion followed by a 60 s of ischemia, in a situation of extreme fatigue and low-availability of energy resources. After 48 h of supplementation, MPO was 15% higher in the group receiving polyphenols than in the placebo group ($p = 0.04$). No significant differences were observed neither in brain oxygenation index during the last Wingate test (65.8 ± 8.6 and 68.5 ± 7.2%, for the placebo and polyphenols trials, respectively, $p = 0.38$) nor in quadriceps muscle oxygenation index (57.1 ± 6.7 and 55.8 ± 9.0%, for the placebo and polyphenols trials, respectively, $p = 0.22$). Neither there was a significant difference in the mean lactate responses after incremental exercise nor after the three Wingate tests (10.3 ± 2.4 and 11.1 ± 2.3 mM, for the placebo and polyphenols trials, respectively, $p = 0.15$).

3.4. Final Time Trial

No significant effects were observed in the total work performed during the final time trial (101.3 ± 56.6 and 103.5 ± 61.6 kJ, for the placebo and polyphenol trials, respectively, $p = 0.85$). Although the brain oxygenation index was higher after the ingestion of polyphenols, this difference did not reach statistical significance (64.6 ± 6.5 and 68.0 ± 6.0%, for the placebo and polyphenol trials, respectively, $p = 0.18$). The quadriceps muscle oxygenation index was not significantly altered during the final time trials (61.3 ± 6.3 and 60.6 ± 8.5%, for the placebo and polyphenol trial, respectively $p = 0.34$).

3.5. Quadriceps Muscle O_2 Extraction during Ischemia

During the first five seconds of the occlusion, the quadriceps muscle oxygenation index was reduced to lower levels after the ingestion of polyphenols ($p = 0.04$) (Figure 4).

4. Discussion

This study shows that a mango leaf extract rich in mangiferin in combination with luteolin enhances exercise performance during sprint exercise and facilitates muscle oxygen extraction. In addition, this polyphenolic combination improves muscle performance after ischemia-reperfusion by three main mechanisms. Firstly, it facilitates muscle oxygen extraction as demonstrated by the greater reduction of the muscle oxygenation index during the first five seconds of total occlusion of the circulation at exhaustion. Secondly, it reduces oxygen consumption during the sprints preceded by ischemia. Thirdly, it may have facilitated ATP production through additional recruitment of the glycolysis, as indicated by the higher levels of blood lactate concentration observed in the sprints performed after ischemia-reperfusion. Importantly, mangiferin + luteolin enhanced mean power output during prolonged sprints (30 s Wingate test) carried out after 30 min of recovery following an incremental exercise test. This improvement in prolonged sprint performance was accompanied by enhanced brain oxygenation and larger muscle oxygen extraction during the sprints.

4.1. A Combination of Mangiferin and Luteolin Botanical Extracts Improves Muscle O_2 Extraction

Although it is well established that increasing O_2 delivery enhances performance during whole body incremental exercise to exhaustion as well as during submaximal aerobic exercise [60–62], performance is not limited by muscle oxygen delivery during a single sprint exercise, at least in healthy humans exercising at sea level [63]. Although O_2 delivery has not been measured during repeated sprint exercise in humans, muscle biopsy metabolite data [64–66] and whole body VO_2 assessments [52,67] indicate a greater dependency on aerobic metabolism during high-intensity intermittent exercise to exhaustion. Therefore, reducing the need for O_2 may be advantageous for performance during repeated sprint exercise.

In the present investigation, we have shown that mangiferin + luteolin supplementation allows the skeletal muscle to reach lower levels of tissue oxygenation during sprint exercise and post-exercise ischemia. This effect could be explained by a better microvascular distribution of perfusion (prioritizing the active skeletal muscle fibers) [68,69] and enhanced mitochondrial O_2 extraction. The most plausible mechanism by which mangiferin + luteolin supplementation could have enhanced O_2 extraction is by improving mitochondrial bioenergetics [70], which could be impaired by the high

levels of reactive oxygen and nitrogen species (RONS) produced during repeated sprint exercise [2,10,71].

Lower muscle perfusion after the administration of mangiferin + luteolin is unlikely because the polyphenols effects on muscle extraction were greater during the second Wingate test, i.e., when skeletal muscle blood flow is expected to increase faster and to a higher level [52,72]. Moreover, the fact that the heart rate response was not different with supplementation also argues against a different cardiovascular regulation between conditions. The matching between tissue perfusion and VO_2 at the microvascular level cannot be assessed with current technology during whole body exercise in humans [73] and will not be further discussed here.

4.2. A Combination of Mangiferin and Luteolin Botanical Extracts Enhances Sprint Performance after Ischemia-Reperfusion

In agreement with our previous study, performance was improved in the sprints carried out immediately after ischemia (first 15 s sprint). The effect was less marked during the second 15 s sprint, which was preceded by 30 s of ischemia and 10 s of active recovery with reoxygenation [27]. The latter, combined with the greater level of muscle deoxygenation during the first 5 s of ischemia in the experiments performed with polyphenols (Figure 4), suggests that when the PO_2 is very low, as expected when ischemia is applied after maximal exercise [49], mitochondrial bioenergetics is likely enhanced by the administration of mangiferin + luteolin. This observation concurs with animal studies showing that luteolin [74–78] and mangiferin [79] attenuate the ischemia-reperfusion injury in different tissues. This protective effect of both polyphenols has been attributed to their potent direct free-radical scavenging properties and their inhibitory action on the superoxide-generating enzymes XO and NOX, which are activated during sprint exercise [10] and ischemia-reperfusion [76,79–81].

During high-intensity exercise as well as during ischemia, nitric oxide (NO) is produced in skeletal muscle from nitrite by the action of nitrite reductases such as myoglobin [82,83], deoxyhemoglobin [84] and XO [80,85]. Xanthine oxidoreductase usually reduces molecular oxygen to superoxide, but at low oxygen tensions and pH, as observed during prolonged sprints [48,86], repeated sprints [66] and post-exercise ischemia [49], this enzyme can also reduce nitrite to NO [80]. The NO formed can bind to cytochrome c oxidase of the mitochondrial electron transport chain, reducing electron flow and oxygen utilization [87]. Thus, in this investigation, the potential inhibitory action of mangiferin + luteolin on XO might have been beneficial during high-intensity exercise, ischemia and ischemia-reperfusion by reducing superoxide and secondary RONS generation, and attenuating NO production from nitrite in skeletal muscle. Consequently, mangiferin + luteolin could have facilitated mitochondrial respiration and aerobic energy production during the sprints and ischemia periods, as indicated by the lower levels of muscle oxygenation observed here when the ingestion of polyphenols preceded the sprints. At the same time, mangiferin + luteolin could have facilitated mitochondrial bioenergetics, improving muscle efficiency during high-intensity exercise [88].

4.3. A Combination of Mangiferin and Luteolin Botanical Extracts Increases Frontal Lobe Oxygenation during Repeated Sprint Exercise

Given the high sensitivity of the brain to hypoxia [89], any small reduction of brain oxygen delivery could potentially alter brain functioning and contribute to fatigue. Moreover, reduced brain oxygenation may facilitate local production of RONS, which may combine with circulating RONS released by contracting muscles, particularly during high-intensity exercise [90]. This could also deteriorate cognitive and executive function during exercise, reducing performance in complex tasks [91,92]. Thus, it is not surprising that the reduction in brain oxygenation has often been argued as a mechanism lowering exercise performance [48,58,93–95]. Moreover, fatigue can be swiftly relieved by raising the FiO_2, during exercise in severe acute hypoxia [94].

In agreement with our previous study [27], the ingestion of mangiferin + luteolin improved frontal lobe oxygenation during the prolonged sprints. This effect may be related to a better

distribution of blood flow between tissues or enhanced cerebral vasodilation facilitated by the polyphenols [96]. During sprint exercise, the $PaCO_2$ is markedly reduced what may cause vasoconstriction in the brain circulation [97]. The latter combined with the increased production of RONS during sprint exercise, which may hamper endothelial NO production and NO bioavailability, could contribute to reducing brain perfusion and oxygenation. Mangiferin + luteolin supplementation could have improved brain oxygenation during sprint exercise likely through its antioxidant properties, inhibitory action on endothelial NOX [81], suppressive effects on the endoplasmic reticulum-induced stress [96], and increasing the bioavailability of vascular NO [98].

Although the improvement in performance reported here may seem small it is superior to that reported for caffeine during repeated Wingate tests [99]. Moreover, the smallest yet meaningful change in performance for elite male cyclists is as little as 1%, which is difficult to detect in single studies because of the typical measurement error (i.e., 0.7–4.7% [100]. Thus, the improvements elicited by mangiferin + luteolin in peak and mean power output may be critical in sports disciplines where sprint performance in state of fatigue may decide the winner [101].

4.4. Limitations

Although the effects on performance, O_2 extraction, and cerebral oxygenation were robust, this study is limited by the relatively small sample and lack of oxidative stress biomarkers assessment. Although women were not recruited in this investigation, we have previously shown improvement of sprint performance and brain oxygenation in men and women after 48 h supplementation with mangiferin combined with either luteolin or quercetin [27].

Excessive RONS production may cause muscle damage [1,2], fatigue [30] and maladaptation. However, it is thought that exercise-induced RONS act like a hormetic signal necessary for an optimal adaptation to exercise training [102]. According to the hormesis theory, ingestion of antioxidants before exercise may blunt RONS-mediated signaling needed for adaptation [32,102]. However, the use of antioxidants during high-intensity training sessions could allow withstanding high-stress training sessions [1,67], displacing the bell-shaped hormesis curve to higher intensities [102]. Although we have identified some physiological mechanisms, whether the ingestion of mangiferin combined with luteolin could facilitate the adaptive response to high-intensity training remains unknown. Future studies using muscle biopsies are needed to examine whether mangiferin and luteolin modulate RONS induced signaling or prevent oxidative stress.

5. Conclusions

Supplementation with the combination of two botanical extracts of mangiferin and luteolin enhances exercise sprint performance, likely by improving brain oxygenation and allowing a higher muscle extraction of oxygen. These effects were observed following 48 h and 15 days of supplementation without significant differences between the two doses tested.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Effects of mangiferin and luteolin botanical extracts on blood biochemistry tests, Table S2: Effects of mangiferin and luteolin botanical extracts on blood hematology tests, Table S3: Effects of mangiferin and luteolin botanical extracts on body mass and cardiorespiratory variables measured at rest.

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Mangifera indica L. Leaf Extract in Combination With Luteolin or Quercetin Enhances VO_2peak and Peak Power Output, and Preserves Skeletal Muscle Function During Ischemia-Reperfusion in Humans

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It remains unknown whether polyphenols such as luteolin (Lut), mangiferin and quercetin (Q) have ergogenic effects during repeated all-out prolonged sprints. Here we tested the effect of *Mangifera indica* L. leaf extract (MLE) rich in mangiferin (Zynamite®) administered with either quercetin (Q) and tiger nut extract (TNE), or with luteolin (Lut) on sprint performance and recovery from ischemia-reperfusion. Thirty young volunteers were randomly assigned to three treatments 48 h before exercise. Treatment A: placebo (500 mg of maltodextrin/day); B: 140 mg of MLE (60% mangiferin) and 50 mg of Lut/day; and C: 140 mg of MLE, 600 mg of Q and 350 mg of TNE/day. After warm-up, subjects performed two 30 s Wingate tests and a 60 s all-out sprint interspaced by 4 min recovery periods. At the end of the 60 s sprint the circulation of both legs was instantaneously occluded for 20 s. Then, the circulation was re-opened and a 15 s sprint performed, followed by 10 s recovery with open circulation, and another 15 s final sprint. MLE supplements enhanced peak (Wpeak) and mean (Wmean) power output by 5.0–7.0% ($P < 0.01$). After ischemia, MLE+Q+TNE increased Wpeak by 19.4 and 10.2% compared with the placebo ($P < 0.001$) and MLE+Lut ($P < 0.05$), respectively. MLE+Q+TNE increased Wmean post-ischemia by 11.2 and 6.7% compared with the placebo ($P < 0.001$) and MLE+Lut ($P = 0.012$). Mean VO_2 during the sprints was unchanged, suggesting increased efficiency or recruitment of the anaerobic capacity after MLE ingestion. In women, peak VO_2 during the repeated sprints was 5.8% greater after the administration of MLE, coinciding with better brain oxygenation. MLE attenuated the metaboreflex hyperpneic response post-ischemia, may have improved O_2 extraction by the *Vastus Lateralis* (MLE+Q+TNE vs. placebo, $P = 0.056$), and reduced pain during ischemia ($P = 0.068$). Blood lactate, acid-base balance, and plasma electrolytes responses were not altered by the supplements. In conclusion, a MLE extract rich in mangiferin combined with either quercetin and tiger

nut extract or luteolin exerts a remarkable ergogenic effect, increasing muscle power in fatigued subjects and enhancing peak VO_2 and brain oxygenation in women during prolonged sprinting. Importantly, the combination of MLE+Q+TNE improves skeletal muscle contractile function during ischemia/reperfusion.

Keywords: sprint exercise, polyphenols, antioxidants, fatigue, recovery

INTRODUCTION

Fatigue is a complex process which may originate in any structure intervening in the production and control of muscle contractions. Performance-enhancing compounds may exert their effects by facilitating energy supply and utilization, easing central command and motor control and reducing the negative effects caused by energy depletion, shortage of O_2, metabolite accumulation, and reactive oxygen and nitrogen species (RONS) on force generation, muscle contraction activation and afferent feedback. Among the natural substances that may have performance-enhancing properties (ergogenic effects) several polyphenols have been investigated (González-Gallego et al., 2010; Braakhuis and Hopkins, 2015). Most polyphenols may act as antioxidants (Sandoval-Acuña et al., 2014), signaling molecules, or hold anti-inflammatory (Luczkiewicz et al., 2014), anti-aging (Khurana et al., 2013; Menendez et al., 2013), neuromodulatory and neuroprotective (Campos-Esparza et al., 2009; Luo et al., 2017) properties, which may confer their ergogenic potential. Most of these effects have been demonstrated in cell culture or animal models, in many instances using supra-physiological doses (González-Gallego et al., 2010). Moreover, studies performed in humans have reported divergent results which have been attributed to differences in the exercise model, fitness level of the study population and the type of polyphenol tested (González-Gallego et al., 2010). The ergogenic potential of some polyphenols such as luteolin and mangiferin remains unknown, and the effects of quercetin on performance during repeated all-out prolonged sprints is yet to be studied in humans.

Mangiferin (2-b-D-glucopyranosyl-1,3,6,7-tetrahydroxy-xanthone) is a xanthone (non-flavonoid polyphenol) originally isolated from *Mangifera indica* L. *(Anacardiaceae)*, present in abundance in mango leaves and other plants (Masibo and He, 2008). Mangiferin is considered a "super antioxidant" capable of specifically protecting against free radical production by the Fenton reaction due to its iron-chelating properties. The Fenton reaction is thought to play an important role as a source of RONS during sprint exercise (Morales-Alamo and Calbet, 2014), due to the marked acidification elicited by the high glycolytic rates attained during this type of exercise. Acidosis accelerates hydroxyl radical production by the Fenton reaction and reduces the activities of the antioxidant enzymes glutathione peroxidase, glutathione S-transferase, and glutathione reductase (Ying et al., 1999).

Mangiferin has powerful free radical scavenging properties and has been shown to attenuate ischemia/reperfusion injuries in diabetic rats (Suchal et al., 2017), but it remains unknown whether mangiferin attenuates the effects of ischemia/reperfusion in humans. Mangiferin can traverse the blood-brain barrier and modulate neurotransmission, K^+ channels and nociception (Rauf et al., 2017). Strong stimulation of type III and IV afferents by metabolite accumulation during sprint exercise (Cheetham et al., 1986; Morales-Alamo et al., 2012), particularly H^+ and lactate (Light et al., 2008) are likely involved in the perception of effort and exercise-induced pain. III/IV muscle afferents discharge inhibits corticospinal drive and could contribute to limit exercise capacity or enhance fatigue sensation (Amann and Dempsey, 2008; Rossman et al., 2012; Sidhu et al., 2014; Kennedy et al., 2015). Mangiferin has properties which may attenuate III/IV muscle afferent discharge during exercise, either by reducing RONS-mediated stimulation of III/IV muscle afferents or by downregulating the glycolytic rate and interstitial K^+ accumulation.

Quercetin is a well-studied flavonoid polyphenol which may improve performance during prolonged exercise (Kressler et al., 2011; Myburgh, 2014), although its effects in athletes are unclear (Braakhuis and Hopkins, 2015). Quercetin is found in several fruit and vegetables, including mangoes. Although quercetin has a low bioavailability due to its poor intestinal absorption (Graefe et al., 2001), this may be improved by an oleaginous vehicle (Tran et al., 2014) such as tiger nut extract. In animal models, quercetin attenuates ischemia/reperfusion injuries in several tissues (Shoskes, 1998; Cho et al., 2006; Annapurna et al., 2009) including skeletal muscle (Ekinci Akdemir et al., 2016). A potential sex dimorphism in the responses to polyphenol supplementation have not been specifically tested, although quercetin (like mangiferin) is a phytoestrogen, capable of binding to and activating estrogen receptors (Wilkinson et al., 2015).

Luteolin (30, 40, 50, 70-tetrahydroxyflavone) is one of the most abundant flavones and, like mangiferin and quercetin, is a potent antioxidant and inhibitor of xanthine oxidase (XO) (Nagao et al., 1999; Pinto et al., 2005; Paredes-Gonzalez et al., 2015; Niu et al., 2016; Nile et al., 2017). Luteolin is also a NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase; NOX) inhibitor (Makino et al., 2013; Xia F. et al., 2014). Both enzyme activities, XO and NOX, play a critical role in RONS generation during intense exercise (Morales-Alamo and Calbet, 2014) and ischemia/reperfusion events (Berry and Hare, 2004). Luteolin mitigates ischemia/reperfusion damage in cell cultures (Tian et al., 2018) and animals (Karakaş et al., 2014; Hong et al., 2017; Liu et al., 2017; Luo et al., 2017; Du et al., 2018).

Flavonoids may facilitate an increase in mitochondrial Ca^{2+} concentration by acting on the mitochondrial Ca^{2+} uniporter (Montero et al., 2004). This may up-regulate the respiratory rate and ATP production and stimulate endothelial nitric oxide synthase (eNOS), increasing nitric oxide (NO) production

(Duarte et al., 2014; Si et al., 2014; Cheng et al., 2018). The vasodilation induced by NO may enhance oxygen delivery to the active muscles also helping to improve performance (Calbet and Lundby, 2012; Si et al., 2014). Although it has been reported that the effects on performance could be enhanced when flavonoids are given in combination (MacRae and Mefferd, 2006), few studies have examined the effects of flavonoid combinations on exercise performance (MacRae and Mefferd, 2006; Deley et al., 2017; Overdevest et al., 2018).

Interestingly, the cytoprotective effects of flavonoids against ischemia/reperfusion may be enhanced by exercise (Chang et al., 2014). However, no single study to date has determined the efficacy of natural polyphenols in mitigating the deterioration of skeletal muscle contractile function after short ischemia/reperfusion in humans. Ischemia/reperfusion is a phenomenon that may occur during isometric contractions (Wigmore et al., 2006; Thompson et al., 2007). Tempol, an antioxidant mimicking superoxide dismutase, preserves skeletal muscle mitochondrial respiratory function during reperfusion in rodents (Charles et al., 2017). Likewise, quercetin has been shown to protect skeletal muscle from ischemia/reperfusion injury in rats (Ekinci Akdemir et al., 2016). Thus, for this study we hypothesized that natural polyphenols might also facilitate the recovery of exercise performance after ischemia/reperfusion in humans by easing mitochondrial O_2 utilization after ischemia.

Therefore, the main aim of this study was to test whether a mango leaf extract (MLE) (60% weight mangiferin) administered in two different formulations: one with quercetin and tiger nut extract, and another with luteolin, has a performance-enhancing effect in young men and women. A secondary aim was to test whether the combinations mangiferin-luteolin and mangiferin-quercetin protect skeletal muscle from the negative effects of ischemia/reperfusion applied immediately at the end of sprint exercise. We hypothesized that both mangiferin-containing supplements would enhance sprint performance.

TABLE 1 | Physical characteristics and ergoespirometric variables (mean ± *SD*).

	Men (n = 17)	Women (13)	P
Age (years)	22.7 ± 2.1	27.0 ± 2.2	0.005
Height (cm)	176.9 ± 4.2	164.4 ± 4.6	0.000
Weight (kg)	71.2 ± 5.2	56.5 ± 5.4	0.000
% body fat	18.4 ± 3.7	26.0 ± 4.9	0.000
Lean mass of both legs (kg)	19.8 ± 2.0	13.6 ± 2.5	0.000
Hemoglobin ($g.dL^{-1}$)	15.0 ± 0.8	13.2 ± 0.9	0.000
HRmax (Beats/min)	191.7 ± 7.5	189.3 ± 0.7	0.567
VO_2max (mL/kg/min)	47.5 ± 6.1	41.2 ± 6.1	0.005
VO_2max (mL/kg LLM/min)	171.1 ± 16.3	170.4 ± 15.7	0.921
Wmax (W)	259.1 ± 32.7	177.7 ± 38.0	0.000
	Constant-intensity test at 120% VO_2max		
Endurance time (s)	150.4 ± 40.1	132.0 ± 40.3	0.168
120% VO_2max intensity (W)	303.8 ± 36.6	216.6 ± 40.5	0.000
Work ($kj.kg^{-1}$ LLM)	2.30 ± 0.60	1.98 ± 0.60	0.086
O_2 deficit (mL)	3362 ± 839	1880 ± 848	0.000
O_2 deficit ($mL.kg^{-1}$ BW)	47.2 ± 11.6	33.4 ± 11.4	0.001
O_2 deficit/LLM	169.3 ± 35.9	137.9 ± 34.1	0.011
% Anaerobic Energy	33.6 ± 6.3	32.1 ± 5.8	0.527
	30 s Wingate test		
$Wpeak_i$	1087.1 ± 86.5	753.0 ± 93.4	0.000
$Wpeak_i$/kg	15.3 ± 1.2	13.4 ± 1.2	0.000
$Wpeak_i$/LLM	55.4 ± 6.0	55.3 ± 6.5	0.979
Wmean	628.0 ± 65.6	417.3 ± 77.0	0.000
Wmean/kg	8.8 ± 0.8	7.4 ± 0.9	0.000
Wmean/kg LLM	31.9 ± 3.1	30.7 ± 3.0	0.270

Wmax, maximal intensity during the incremental exercise test to exhaustion; $Wpeak_i$, instantaneous peak power output during the Wingate test; LLM, lean mass of the lower extremities; Wmean, mean power output during a 30 s Wingate test; Accumulated VO_2, total amount of O_2 consumed); % Anaerobic Energy, percentage of the energy obtained through the anaerobic pathways.

MATERIALS AND METHODS

Subjects

Eighteen men and 17 women, all healthy and physically active agreed to participate in this investigation, but complete data was obtained from 17 men and 13 women (**Table 1**). After a familiarization and a pre-test phase lasting 4 weeks, the main experiment was performed to test the effects of two different combinations of polyphenolic supplement on performance during repeated sprint exercise. The study was carried out in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2017-02). All subjects signed a written informed consent before entering the study. Subjects were requested to avoid strenuous exercise 48 h before the laboratory test and not to drink beverages containing caffeine or taurine during the 24 h preceding the test.

Pre-tests

Body composition was determined by dual-energy x-ray absorptiometry (Lunar iDXA, GE Healthcare, Wisconsin; USA) as described elsewhere (Calbet et al., 1998). Subjects performed two familiarizations visits during which incremental exercise to exhaustion and a 30 s all-out sprint were performed. After familiarization, subjects reported to the laboratory to complete different tests on separate days. First, their peak VO_2 (VO_2peak), maximal heart rate (HRmax) and maximal power output (Wmax) were determined in normoxia (F_IO_2: 0.21, P_IO_2: 143 mmHg) with an incremental exercise test to exhaustion with verification (Poole and Jones, 2017). The test started with 3 min at 20 W, followed by 15 and 20 W increases every 3 min in women and men, respectively, until the respiratory exchange ratio (RER) was >1.0. After completion of the intensity with an RER $\leq$ 1.0, the intensity was increased by 10 and 15 W/min increase (women and men, respectively) until exhaustion. The intensity attained at exhaustion was taken at the maximal power output of the incremental exercise test (Wmax). At exhaustion, the ergometer was unloaded and subjects remained seated on the cycle ergometer pedaling at a slow speed (30–40 rpm) for 3 min. Thereafter, the verification test started at Wmax + 5 W for 1 min,

followed by 4 and 5 W increase (women and men, respectively) every 20 s until exhaustion. Between 1 and 2 weeks later, subjects reported to the laboratory on two occasions separated by at least 1 week, to carry out a constant-intensity supramaximal exercise to exhaustion at 120% of VO_2max. This test was used to determine the anaerobic capacity, as previously described (Morales-Alamo et al., 2015). The constant-intensity supramaximal exercise test with longer endurance time to exhaustion was retained as representative for each subject.

Power Output, Oxygen Uptake, and Supramaximal Exercise O_2 Demand and Deficit

Power output during the sprint was reported as instantaneous peak power output (Wpeak) and mean power output (Wmean) throughout the duration of the sprints. Oxygen uptake was measured with a metabolic cart (Vyntus CPX, Jaeger-Carefusion, Hoechberg, Germany), calibrated according with high-grade certified gases provided by the manufacturer. Respiratory variables were analyzed breath-by-breath and averaged every 20 s during the incremental exercise tests (Calbet et al., 1997) and during the repeated sprints. The highest 20 s averaged VO_2 recorded during the incremental test (i.e., including the verification phase) was taken as VO_2peak.

The O_2 demand during the sprints was calculated from the linear relationship between the last 20 s averaged VO_2 of each load, from 80 W up to 80–90% of VO_2max, while subjects were pedaling at 80 rpm. The accumulated oxygen deficit (AOD), representing the difference between O_2 demand and VO_2, was determined as previously reported (Calbet et al., 1997; Dorado et al., 2004).

Main Experiment

The volunteers were randomly assigned to three treatments, following a double-blind design, using a computer program. Treatment A, was a placebo condition (500 mg of maltodextrin per day); treatment B consisted in 140 mg of MLE (60% mangiferin) and 50 mg of luteolin per day; and treatment C contained 140 mg of MLE (60% mangiferin), 600 mg of quercetin and 350 mg of tiger nut extract per day (**Table 2**). The three treatments were divided in three daily doses administered every 8 h in methylcellulose capsules of identical appearance. The dose of quercetin was based on previous studies (Davis et al., 2010; Myburgh, 2014). The dose of mangiferin was based on a pharmacokinetic study showing oral absorption and mean residence time close to 7 h, after the ingestion of 0.1 g of pure mangiferin (Hou et al., 2012). The dose of luteolin was based on pharmacokinetic data obtained in humans after the administration of artichoke leaf extracts which are rich in luteolin (Wittemer et al., 2005) and the response observed after the administration of 100 mg of encapsulated luteolin in children (Tsilioni et al., 2015). Pilot studies showed no side effects from the administration of Zynamite in twelve volunteers, which had normal kidney, liver, hematological and biochemical variables in blood after two weeks of supplementation.

Subjects started supplement intake 48 h before the main experimental days. On the day of experiment, subjects reported to the laboratory after a 10 h overnight fast, and 60 min before the start of the experiment ingested an additional dose of the supplement (i.e., 1/3 of the daily dose). During the following 60 min subjects were instrumented and a hand vein was catheterized and heated to obtain 10 ml of arterialized blood. Next, subjects were seated on the cycle ergometer and performed two warming-up 8 s sprints in isokinetic mode at 80 rpm, separated by a 2 min interval during which they pedaled with the cycle ergometer unloaded. After a 3 min period of unloaded pedaling, the load was increased to 80 W in women, and 100 W in men for 6 min (80 rpm, ergometer set in rpm-independent mode). This was followed by unloaded pedaling for 4.5 min. Then, subjects stopped pedaling and the ergometer was switched to isokinetic mode. At the 5th minute they performed a Wingate test (30 s all-out sprint in isokinetic mode at 80 rpm). This was followed by another 3.5 min of unloaded pedaling and another 30 s period, during which they stopped pedaling and

TABLE 2 | Chemical composition of the plant extracts used in the supplements.

	***Mangifera indica L.* extract**	***Arachis hypogaea* extract**	***Sophora japonica* extract**	***Cyperus esculentus* extract**
Part of the plant used	Leaves	Shell	Bulbs, skin	Dry tubers
Bioactive compounds (%, w/w)	Mangiferin (≥60%)	Luteolin (≥90%)	Quercetin (≥90%)	HAF[a] (≥5%)
	Homomangiferin (≤2.5%)			Oleic acid glyceryl ester[b] (2:1) (≥91%)
	Isomangiferin (trace levels)			Linoleic acid glyceryl ester[b] (≥7%)
	Sugars (≤10%)			Stigmaesterol[b] (≥0.2%)
				Myricetin[b] (≥0.2%)
				Sucrose (≤30%)
Moisture content (%, w/w)	≤7%	≤7%	≤7%	≤7%
Botanical or native ingredient (%, w/w)	*Mangifera i.* extract (100%)	*Arachis h.* extract (100%)	*Sophora j.* extract (100%)	*Cyperus e.* Extract (≥50%)
Non-botanical ingredient (%, w/w)	None	None	None	Potato maltodextrin (≤25%)
				Arabic gum (≤ 25%)

[a] *High Activity Fraction (HAF): Fraction soluble in ethyl acetate.*
[b] *Relative to the amount of the HAF.*

the ergometer was switched to the isokinetic mode. At the 4th minute, a second 30 s Wingate test was performed, which was also followed by another 3.5 min of unloaded pedaling and another 30 s period of rest. Four min after the end of the second 30 s Wingate test, an all-out 60 s long sprint was carried out. At the end of the 60 s sprint, the circulation of both lower extremities was instantaneously occluded for 20 s by inflating bilateral cuffs at 300 mmHg as previously reported (Morales-Alamo et al., 2015; Torres-Peralta et al., 2016b; **Figure 1**). For this purpose, cuffs were placed around the thighs during the preparation phase, as close as possible to the inguinal crease, and were connected to a rapid cuff inflator before they seated on the cycle ergometer (SCD10, Hokanson E20 AG101, Bellevue, USA).

Ten seconds after the start of the occlusion a reverse countdown was given and the subjects prompted to start pedaling again as fast and hard as possible, with the ergometer in isokinetic mode for 15 s. At the start of the sprint the cuff was deflated to allow full reestablishment of the circulation during the subsequent exercise. At the end of the 15 s sprint, they pedaled slowly for another 5 s and then, stopped for 5 s to get ready for the final 15 s sprint. During the 10 s of recovery that followed the 15 s post-ischemia sprint, as well as during the 15 s final sprint, the circulation was open. A capillary blood sample was drawn from the ear lobe, previously hyperhemized with Finalgon® cream, to measure the concentration of lactate (lactate-Pro 2, Arkray, Valencia, Spain) 1 min after the last sprint.

Blood samples for hemoglobin concentration, blood gases, electrolytes and acid-base balance assessment were obtained from the heated hand vein at rest, 3 min after the second 30 s Wingate test, 1 min after the last sprint, and 5 and 30 min into the recovery period (ABL90, Radiometer, Copenhagen, Denmark).

Cerebral Oxygenation

Cerebral oxygenation was assessed at rest and during exercise using near-infrared spectroscopy (NIRS, NIRO-200, Hamamatsu, Japan) employing spatial resolved spectroscopy to obtain the tissue oxygenation index (TOI) using a path-length factor of 5.92 (van der Zee et al., 1992). The NIRS optodes were placed on the right frontoparietal region at 3 cm from the midline and 2–3 cm above the supraorbital crest, to avoid the sagittal and frontal sinus areas (Curtelin et al., 2018). Using this optode placement the tissue oxygenation of the superficial frontal cerebral cortex is recorded. An additional optode was placed in the lateral aspect of the thigh at middle length between the patella and the anterosuperior iliac crest, over the middle portion of the *musculus Vastus Lateralis*. The rate of muscle deoxygenation upon occlusion was calculated by determining the maximal slope of the linear decay of TOI overtime. For this purpose, data were averaged every second and the slope TOI/time was calculated from the start of the occlusion to the end of occlusion, with a minimum interval of 4 s and a maximum of 20 s. Since the best linear fit was obtained with a 4 s interval, this was applied to all the occlusions.

Middle Cerebral Artery Blood Velocity

The mean blood velocity in the middle cerebral artery ($MCAv_{mean}$), insonated through the trans-temporal window as described elsewhere (Rasmussen et al., 2007; Willie et al., 2011;

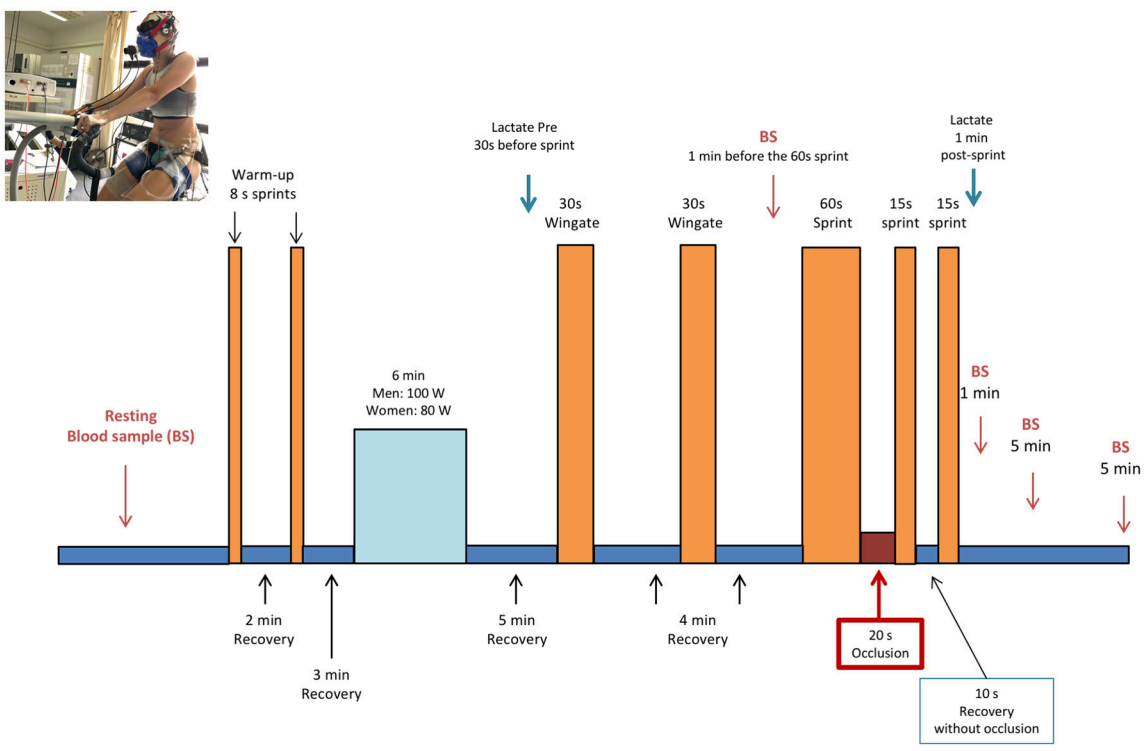

FIGURE 1 | Experimental protocol (see text for details). Written consent was obtained from the volunteer that appears in the picture for the publication of the image.

Curtelin et al., 2018), was determined as an estimate of cerebral blood flow. Two Doppler 2 MHz transducers were applied bilaterally over the middle transtemporal window (Naqvi et al., 2013; Multi Box, DWL, Singen, Germany). Since both Doppler probes yield similar readings these were averaged for further analysis to reduce variability. In some subjects one MCA was insonated due to a mechanical failure of the second probe. A head harness was used to minimize potential movement artifacts. Resting cerebral oxygenation and $MCAv_{mean}$ was calculated as the average of a 2 min collection period, while during exercise 5 s averages were generated and the average for the whole sprint reported. The NIRS and Doppler data were collected using a 16-channel data acquisition system (Power Lab ML880, ADInstruments), sampled at 200 Hz, and stored on a computer for subsequent analysis.

Power Output

All pre-tests were performed on the same cycle ergometer (Lode, Corival, Groningen, The Netherlands), which maintains the exercise intensity constant despite variations in pedaling rate. During all tests subjects were requested to maintain a pedaling rate close to 80 rpm. For the main experiments, an isokinetic ergometer (Excalibur Sport 925900, Lode, Groningen, The Netherlands) was used. This ergometer was operated in a rpm-independent constant load during the warm-up and recovery phases and switched to isokinetic mode during the sprints, with the speed set at 80 rpm. During the isokinetic sprints, the subjects pedaled as fast and hard as possible, exerting as much force on the pedals as they could at each pedal stroke from the start to the end of the sprint, along which subjects were provided with strong verbal support. The servo-control brake system of the cycle ergometer adjusts continuously and almost instantaneously the braking force so the pedaling rate stays at 80 rpm during the whole sprint. In all instances, exhaustion was defined by the incapacity of the subject to maintain a pedaling rate above 50 rpm during 5 s, despite strong verbal encouragement or by a sudden stop in pedaling.

Oxygen Demand and Deficit

The O_2 demand during the supramaximal exercise bouts was estimated from the linear relationship between the last min averaged VO_2 of each load, from 20 to 40 W to the highest intensity with an RER < 1.00 in the incremental exercise test. The accumulated oxygen deficit (AOD), representing the difference between O_2 demand and VO_2, was determined as previously reported (Calbet et al., 1997; Dorado et al., 2004).

Assessment of Pain and Effectiveness of Concealment

Subjects were requested to rate the level of pain felt during the occlusion from 0 to 10, being 10 the highest muscle pain ever suffered during or after exercise in their life. Likewise, at the end of the experiment subjects were asked about the kind of supplement they suspected they had received to check on the effectiveness of concealment. After placebo administration, 7 out of 30 subjects guessed correctly that they had placebo. Following B supplementation, 11 subjects out of 30, guessed correctly that they had polyphenols, and after supplement C, 16 out of 30 guessed correctly that they had polyphenols. Subjects were aware that polyphenols were present in two occasions and that in one occasion there would be placebo. They also knew that it was unknown whether polyphenols may improve or not performance. Notwithstanding, they generally reported to believe that they had taken polyphenols when they felt better during the whole experiment.

Statistics

A sample size between 20 and 28 participants was required to provide adequate power to detect an improvement between 5 and 6% in peak power output ($\alpha = 0.05$, $\beta = 0.80$; G*Power v.3.1.9.2). To allow for potential dropouts, we decided to recruit 36 volunteers. Variables were checked for normal distribution by using the Shapiro–Wilks test. A two-way repeated-measures ANOVA was used with two within-subjects factors: exercise bout (with five levels) and occlusion (with two levels: free or occluded recovery), and with sex as between-subjects factor. The Mauchly's test of sphericity was run before the ANOVA, and in the case of violation of the sphericity assumption, the degrees of freedom were adjusted according to the Huynh and Feldt test. When a significant main effect or interaction was observed, specific pairwise comparisons were carried out with the least significant difference post-hoc test. The relationship between variables was determined using linear regression analysis. Values are reported as the mean ± standard deviation of the mean (unless otherwise stated). $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Men and women had comparable levels of fitness. Although men had a 15% greater VO_2max per kg of body mass, the between-sex difference disappeared when the VO_2max was expressed per kg of lean mass of the lower extremities. Likewise, men had 41% greater anaerobic capacity per kg of body mass, but this difference was reduced to 23% when expressed in relation to the lean mass of the lower extremities. Nevertheless, no significant between-sex differences were observed in the Wingate test when the values were normalized to the lean mass of the lower extremities.

Effects on Performance

Both supplements B and C enhanced performance during the latest sprints, i.e., those carried out with greater level of fatigue and lower functional reserve. Compared with placebo, when all sprints were averaged, supplements B and C increased Wpeak by 5.0 and 6.0%, respectively (**Table 3** and **Figure 2A**). During the 60 s long sprint, supplements B and C increased Wpeak by 12.5 and 10.8%, respectively ($P < 0.05$). In the sprint performed after ischemia, supplement C increased Wpeak by 19.4% compared to placebo ($P < 0.001$) and by 10.2% compared to supplement B ($P < 0.05$).

When all sprints were combined, supplements B and C enhanced Wmean by 6.2 and 6.7%, respectively, compared with placebo ($P < 0.01$; **Table 3** and **Figure 2B**). Consequently, the total amount of work performed was 2.4% higher following

TABLE 3 | Ergoespriometric responses during repeated all-out sprints (mean, upper row, ± *SD*, lower row).

	Sprint 1			Sprint 2			Sprint 3			Sprint 4			Sprint 5			ANOVA effects				
	W1A	**W1B**	**W1C**	**W2A**	**W2B**	**W2C**	**W60A**	**W60B**	**W60C**	**W15A**	**W15B**	**W15C**	**W15FA**	**W15FB**	**W15FC**	**Sprint**	**Sprint × sex**	**Treat**	**Treat × sex**	**Sprint × Treat**
Wpeak (W)[b]	814.9	822.8	798.0	768.1	767.7	790.0	617.9	695.1*	684.4*	288.0	311.9	343.9*¶	385.3	421.0	430.4*	0.001	0.578	0.001	0.728	0.001
	185.8	207.7	202.1	194.2	167.2	204.4	172.6	207.4	167.0	113.3	106.1	111.2	135.0	142.0	125.3					
Wmean (W)[b]	428.5	419.0	414.3	390.4	383.7	383.0	233.4	247.8*	249.0*	165.4	172.5	183.9*¶	201.3	207.7	209.5	0.001	0.698	0.005	0.285	0.001
	101.3	98.8	98.3	94.5	92.1	89.6	62.4	66.9	58.5	65.6	53.2	53.8	62.5	51.7	50.4					
HR (beat.min^{-1})	156.6	155.9	158.7	158.6	158.5	161.0	169.3	168.5	170.1	173.3	174.9	176.2	173.3	175.0	178.1	0.001	0.082	0.92	0.27	0.3
	13.6	15.5	18.4	15.0	16.6	15.5	13.5	14.5	11.4	15.9	14.2	9.9	15.7	16.2	10.1					
VO_2 (mL/min)	1006.3	1011.8	1010.4	1065.7	1063.6	1057.9	2415.1	2426.2	2429.1	540.1	549.2	550.7	635.7	641.9	644.4	0.001	0.001	0.91	0.4	0.99
	227.6	247.4	213.3	254.4	255.9	236.4	578.8	604.6	567.0	160.9	161.7	131.9	185.5	181.5	193.0					
VCO_2 (mL/min)	2012.5	2023.7	2020.8	2131.3	2127.3	2115.7	2415.1	2426.2	2429.1	2160.3	2197.0	2202.8	2542.8	2567.5	2577.5	0.001	0.18	0.88	0.4	0.98
	455.2	494.7	426.7	508.8	511.7	472.9	578.8	604.6	567.0	643.7	646.7	527.7	741.9	725.8	772.2					
O_2 deficit (mL)	1611.5	1601.0	1605.5	1367.1	1378.4	1332.3	810.0	927.2	768.1	57.0	75.9	90.8*	63.5	70.2	76.2	0.001	0.001	0.37	0.62	0.105
	520.6	536.4	495.1	456.8	445.9	440.2	427.2	531.7	432.3	94.6	142.9	108.5	115.7	120.7	123.4					
RER	1.02	1.04	1.02	1.03	1.04	1.03	0.97	0.98	0.96	1.16	1.18	1.16	1.04	1.05	1.07	0.001	0.012	0.574	0.61	0.62
	0.11	0.11	0.11	0.08	0.07	0.09	0.07	0.07	0.05	0.09	0.08	0.07	0.08	0.06	0.12					
V_E (L/min)	78.2	79.2	76.9	98.9	99.6	98.0	112.2	113.2	111.6	118.8	121.9	117.1	121.1	122.5	123.3	0.001	0.027	0.84	0.67	0.98
	22.5	22.1	23.1	23.7	25.7	28.0	29.4	30.3	30.5	35.0	33.4	37.0	36.7	35.3	36.8					
$P_{ET}O_2$ (mmHg)	115.5	114.6	112.6	119.7	119.7	119.3	119.8	120.1	120.0	122.6	122.9	119.5	119.9	119.9	120.5	0.001	0.34	0.21	0.633	0.405
	5.3	4.6	11.9	3.2	3.1	4.7	2.9	2.6	2.8	2.6	2.4	17.1	2.4	2.5	2.7					
$P_{ET}CO_2$ (mmHg)	29.0	30.5	29.9	25.6	25.9	26.3	24.2	24.1	24.4	24.6	24.7	24.3	25.7	25.7	25.7	0.001	0.099	0.6	0.6	0.34
	3.7	3.7	4.3	2.3	2.5	3.5	2.5	2.2	2.5	2.6	2.2	4.5	2.5	2.5	2.7					

Wpeak, peak power output; Wmean, mean power output; HR, heart rate; VO_2, oxygen uptake; VCO_2, CO_2 production; RER, respiratory exchange ratio; V_E, pulmonary ventilation; $P_{ET}O_2$, end-tidal O_2 pressure; $P_{ET}CO_2$, end-tidal CO_2 pressure; W1, first Wingate (30 s sprint); W2, second Wingate (30 s sprint); W60, 60 s sprint; W15, 15 s sprint post-ischemia; W15F, final 15 s sprint; Treat, treatment effect. A, Placebo; B, luteolin + Mangiferin; C, Mangiferin + Quercetin + Tiger nut extract ([b]statistical analysis done with logarithmically transformed data); $^*P < 0.05$ compared with placebo; ¶$P < 0.05$ compared with treatment B.

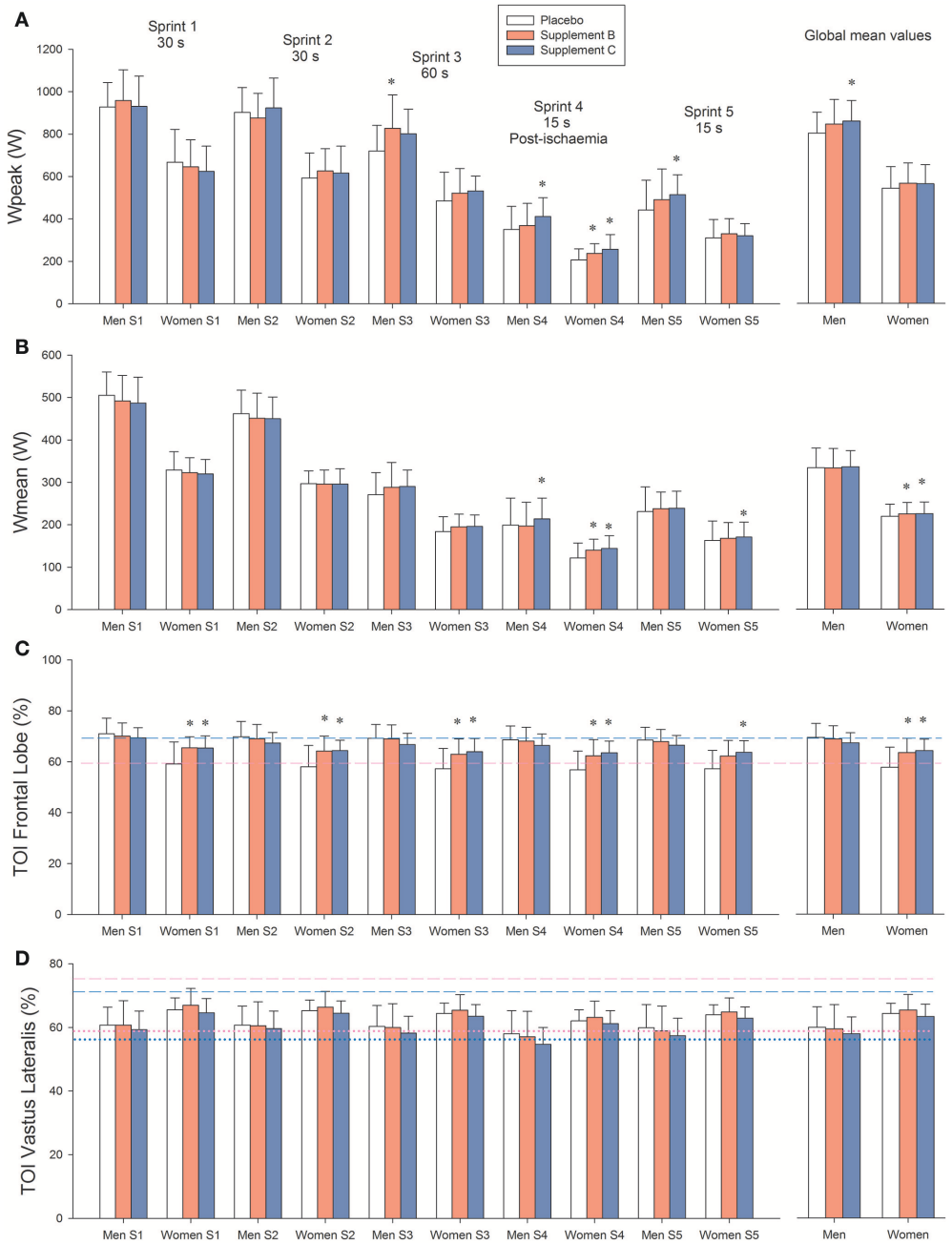

FIGURE 2 | (A) Peak power output (Wpeak). **(B)** Mean power output (Wmean). **(C)** Brain oxygenation (Frontal lobe tissue oxygenation index: TOI). **(D)** *Vastus Lateralis* oxygenation index. Treatment A: placebo (500 mg of maltodextrin per day); treatment B consisted of 140 mg of mango leaves extract and 50 mg of luteolin per day; and treatment C contained 140 mg of mango leaves extract, 600 mg of quercetin, and 350 mg of tiger nut extract per day. Dashed lines in **(C,D)** represent the values recorded at rest. Dotted lines in **(D)** represent the values observed during the last 5 s of the occlusions after sprint 3, i.e., is the TOI value corresponding to "zero oxygenation." Blue color: men, pink color: females. *$P < 0.05$ compared with placebo.

the ingestion of supplements B and C, compared with placebo in women (34.1 ± 4.3, 34.9 ± 4.1, and 34.9 ± 4.0 kJ, for placebo and supplements B and C, respectively, $P < 0.05$). The corresponding values in men were 51.7 ± 6.7, 52.1 ± 7.3, and 52.3 ± 5.8 kJ, respectively ($P > 0.3$). During the sprint performed after ischemia, supplement C enhanced Wmean by 11.2 ($P < 0.001$) compared with the placebo trial and 6.7% compared with supplement B ($P = 0.012$; **Table 3**).

Pulmonary Gas Exchange

During the sprint after ischemia the level of pulmonary ventilation (V_E) was higher than during the preceding (3rd

sprint) and the subsequent sprint (5th sprint) ($P < 0.01$, for both comparisons). The increased V_E during the sprint after ischemia was associated with a higher end-tidal O_2 pressure ($P_{ET}O_2$) compared with the 3rd and 5th sprints, and slightly lower end-tidal CO_2 pressure ($P_{ET}CO_2$) in the 5th than 4th sprint during the placebo and B treatment conditions (all $P < 0.01$). After the administration of supplement C, ischemia did not alter V_E, $P_{ET}O_2$, nor $P_{ET}CO_2$ during the 4th sprint, compared to the 3th and 5th sprints.

In women, the peak VO_2 reached during the repeated sprints was 5.8% greater after the administration of supplements (mean of both trials) compared with the placebo trial (2,189 ± 334 and 2,316 ± 403 mL.min^{-1}, for placebo and supplements, respectively, $P = 0.012$). No such an effect was observed in men (3,265 ± 406 and 3,318 ± 422 mL.min^{-1}, placebo and supplements, respectively, $P = 0.42$).

Neither the accumulated VO_2 nor the O_2 deficit observed during the sprints were significantly altered by any of the treatments, when all sprints were analyzed conjointly. Nevertheless, during the 15 s sprint performed after ischemia, the *m. Vastus Lateralis* oxygenation index tended to be a slightly lower value after the administration of supplement C compared with placebo ($P = 0.056$).

There was a trend for an interaction between sprint and treatment for O_2 deficit ($P = 0.10$). In fact, when the analysis was circumscribed to the sprints performed immediately following ischemia, the O_2 deficit incurred was 2.7-fold greater after the ingestion of supplement C than after placebo in men ($P = 0.001$), while it remained at the same level in women. Pulmonary ventilation and gas exchange were similar in the three conditions (**Table 3**).

Brain Oxygenation

Resting brain oxygenation was lower in women than in men ($P < 0.001$). This was associated with lower $P_{ET}CO_2$ in women than in men (30.7 ± 2.6 and 34.2 ± 2.1 mmHg, in women and men, respectively, $P < 0.001$). In women, both supplements increased frontal lobe oxygenation at rest (59.4 ± 5.7, 64.9 ±3 .8, and 64.9 ± 6.4%, for placebo, supplement B and C, respectively, $P < 0.05$; for the comparisons of supplement B and C against placebo, treatment x sex interaction, $P = 0.013$). In men, brain oxygenation remained unchanged (69.3 ± 5.4, 69.1 ± 4.2, and 68.0 ± 4.4%, for placebo, supplement B and C, respectively, $P > 0.50$, for the comparisons of supplement B and C against placebo).

Brain oxygenation during the sprints was similar to that observed at rest. In women, brain oxygenation during the sprints was greater after the ingestion of supplements B and C than placebo (**Figure 2**). Likewise, during the 20 s ischemic recovery that followed the 60 s long sprint (sprint 3), brain oxygenation was higher after the ingestion of supplements B and C in women than in men (57.7 ± 7.2, 63.1 ± 6.0, and 64.0 ± 4.8%, for placebo, and supplements B and C, respectively, $P < 0.05$; for the comparison of supplement B and C with placebo: treatment x sex interaction $P = 0.005$). The corresponding values in men were not altered by the ingestion of supplements (68.0 ± 3.8, 67.9 ± 5.7, and 66.3 ± 4.3%, for placebo, and supplements B and C, respectively, $P > 0.30$).

Muscle O_2 Extraction

During the occlusion, *Vastus Lateralis* TOI tended to be lower after the ingestion of supplement C compared to placebo ($P = 0.082$). The rate of muscle deoxygenation upon occlusion was similar for the three trials (1.18 ± 0.87, 1.21 ± 0.82, and 1.27 ± 0.71 TOI units.s^{-1}, after the ingestion of placebo, supplement B and supplement C, respectively, $P > 0.64$). The level of TOI corresponding to "zero oxygenation" in the *m. Vastus Lateralis* was attained in about 5 s (5.0 ± 1.6, 5.0 ± 1.1, and 5.5 ± 1.8 s, for placebo and supplement B and C, respectively, $P = 0.24$; for the comparison of supplement C with placebo).

Blood Lactate, Acid Base-Balance, and Electrolytes

No significant differences in capillary blood lactate concentration 1 min after the last sprint were observed (14.1 ± 2.6, 13.9 ± 2.9, and 13.5 ± 3.2 mmol.L^{-1}, after the ingestion of placebo, supplement B and supplement C, respectively). Exercise and recovery plasma Na^+, Cl^-, K^+, Ca^{2+}, glucose, and total bilirubin concentration was not modified by the supplements. Nevertheless, total bilirubin showed a trend for better recovery after the ingestion of supplement C compared to placebo ($P = 0.06$). None of the supplements modified the exercise-induced lactic acidosis in blood, which was similar for the three conditions (**Table 4**).

Pain During Post-sprint Ischemia

When both conditions with polyphenols were averaged, the level of pain reported was lower compared with the placebo condition (7.1 ± 1.8 and 6.7 ± 2.0 arbitrary units, for the placebo and the mean of the B and C conditions, respectively, $P = 0.068$).

DISCUSSION

This study shows that the ingestion of two supplements containing a mango leaf extract rich in mangiferin enhances performance in humans during high intensity exercise. Moreover, the combined MLE/quercetin/tiger nut extract had a remarkable effect increasing peak power output after ischemia/reperfusion, with a similar effect in men and women. In women, MLE-containing supplements improved brain oxygenation at rest and during exercise, and increased peak VO_2 during high-intensity exercise.

Although, the main mechanism eliciting the ergogenic effect of the two MLE-containing supplements remains to be determined, our findings provide indirect evidence for an enhancement of performance without additional consumption of oxygen, suggesting either better muscle energy efficiency and/or enhanced capacity to recruit the exhausted muscle fibers by the central nervous system, with increased production of energy through the anaerobic pathways. Moreover, in agreement with our hypothesis, a trend for better muscular extraction of O_2 was also observed in the sprints performed after ischemia/reperfusion when the subjects had taken the combined MLE/quercetin/tiger nut extract. In agreement with previous findings, ischemia elicited a slightly higher V_E response to sprint, which is consistent with an increased stimulation of the metaboreflex

TABLE 4 | Acid base balance, blood lactate, total bilirubin and electrolytes in arterialized blood ($n = 10$).

		Resting			Three min after Wingate 2			One min after the last sprint			Five min into recovery			Thirty min into recovery		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Lac (mmol/L)	Mean	0.8	0.8	0.8	15.3	14.8	15.4	16.3	16.5	16.6	18.2	18.7	17.4	10.8	11.5	10.9
	SD	0.2	0.2	0.2	4.1	3.6	4.4	4.7	4.0	4.9	3.8	3.2	3.8	4.2	3.5	3.7
pH	Mean	7.411	7.416	7.414	7.190	7.200	7.190	7.143	7.127	7.145	7.130	7.119	7.127	7.291	7.277	7.285
	SD	0.043	0.029	0.031	0.058	0.046	0.058	0.056	0.067	0.075	0.076	0.072	0.077	0.076	0.063	0.065
pCO_2 (mmHg)	Mean	41.0	41.3	37.5	35.7	34.1	33.3	42.2	45.5	37.9	27.0	28.8	30.0	30.4	30.8	31.0
	SD	4.1	3.5	12.9	8.0	7.0	5.9	9.9	13.9	8.7	2.4	4.5	6.4	4.2	4.1	4.7
SBC (mmol/L)	Mean	25.10	25.65	25.56	14.01	14.25	13.90	13.78	13.68	13.40	11.37	11.36	11.81	16.22	15.78	16.22
	SD	1.47	1.16	1.27	1.85	2.27	2.25	2.33	1.99	2.73	1.59	1.53	1.96	3.07	2.18	2.63
cBase (Ecf) (mmol/L)	Mean	1.34	1.99	1.74	−14.65	−14.61	−15.32	−14.46	−14.35	−15.69	−20.08	−19.91	−19.09	−11.59	−12.20	−11.65
	SD	1.56	1.64	1.65	2.99	4.09	4.05	4.27	4.01	5.07	3.00	2.99	3.90	5.02	3.79	4.48
Na^+ (mmol/L)	Mean	140.5	140.4	127.3	145.5	144.8	144.6	147.8	148.1	147.0	144.2	144.5	143.4	140.9	141.1	140.1
	SD	1.2	1.1	42.2	3.7	1.7	1.9	3.1	2.4	1.7	2.5	1.5	1.7	1.1	1.4	1.1
Cl^- (mmol/L)	Mean	106.4	105.9	95.9	108.3	108.0	107.7	109.1	109.2	109.6	107.4	107.1	107.0	106.2	106.5	105.5
	SD	1.5	1.6	31.8	2.5	1.3	1.7	2.7	2.1	1.7	2.4	1.1	1.8	2.2	1.3	1.6
K+ (mmol/L)	Mean	3.9	3.9	3.6	3.9	3.8	3.8	4.7	4.8	5.1	3.5	3.8	4.0	3.8	3.8	3.8
	SD	0.3	0.2	1.2	0.6	0.5	0.4	0.4	0.5	0.9	0.2	0.6	1.1	0.2	0.2	0.2
Ca^{2++} (mmol/L)	Mean	1.21	1.21	1.11	1.26	1.24	1.25	1.30	1.30	1.29	1.26	1.25	1.25	1.21	1.21	1.21
	SD	0.04	0.03	0.37	0.03	0.04	0.03	0.04	0.05	0.05	0.04	0.04	0.04	0.03	0.04	0.03
tBil (μmol/L)	Mean	6.18	4.82	4.80	13.09	13.09	13.33	17.55	15.09	15.88	18.20	14.30	16.00	10.73	8.36	8.10[a]
	SD	7.51	9.34	7.11	8.68	9.14	8.65	9.06	9.90	9.64	9.99	4.64	6.65	7.63	9.99	7.06

Lac, lactate; SBC, standard bicarbonate; cBase (Ecf), Base excess; tBil, Total bilirubin; [a]*P = 0.06 compared with placebo.*

as previously explained (Morales-Alamo et al., 2015; Torres-Peralta et al., 2016b), and as shown by experiments using fentanyl blockade of III/IV muscle afferents (Dempsey et al., 2014). The combined MLE/quercetin/tiger nut extract suppressed this additional hyperpneic response observed during the sprints after ischemia. Interestingly, high interstitial accumulation of K^+ and H^+ may elicit pain via stimulation of III/IV muscle afferents (Kniffki et al., 1978; Mense, 1996). Here, the MLE-containing extract supplement tended to reduce the pain evoked by the occlusions. The attenuation of metaboreflex responses by MLE/quercetin/tiger nut extract is an important effect, since exaggerated responsiveness of III/IV have been shown to limit exercise performance in patients with heart failure (Ives et al., 2016; Keller-Ross et al., 2016) and chronic obstructive pulmonary disease (Gagnon et al., 2012).

Mango Leaves Extract Rich in Mangiferin Enhances Performance During Repeated Prolonged Sprints

The two supplements containing MLE had positive effects on performance, however, our data point toward some superiority of the MLE/quercetin/tiger nut extract over the combination MLE/Luteolin, particularly regarding the effects on ischemia/reperfusion. Although luteolin attenuates the ischemia/reperfusion injury in several tissues (Karakaş et al., 2014; Hong et al., 2017; Liu et al., 2017; Du et al., 2018) it remains unknown whether luteolin prevents the ischemia/reperfusion injury in skeletal muscle. In contrast, at least one study has shown that quercetin protects skeletal muscle from ischemia/reperfusion injury in rodents submitted to ischemia for 3 h (Ekinci Akdemir et al., 2016). Although the present experimental design does not allow partitioning the contribution of each polyphenol to the observed effects, the fact that an increase in performance was observed when MLE was present points toward mangiferin as the main compound responsible for the ergogenic effect. Moreover, quercetin supplementation during 1 week in 12 × 30 m running sprints has been reported to reduce performance (Abbey and Rankin, 2011).

Potential Mechanisms Accounting for the Performance-Enhancing Effects of MLE Formulations

It has been shown in cell cultures that mangiferin activates pyruvate dehydrogenase (PDH) resulting in reduced lactate production and increase carbohydrate oxidation (Apontes et al., 2014). In contrast, no changes in exercise blood lactate responses nor substrate oxidation (data not shown) were observed in the present investigation during submaximal exercise. Although the energy efficiency during submaximal exercise was not significantly altered (data not shown), we cannot rule out an improvement of the contractile efficiency during repeated high intensity exercise after the administration of MLE. Muscle energy efficiency is reduced during high intensity exercise by several mechanisms which include, among others, increased recruitment of less efficient type II muscle fibers, lactic acidosis, electrolyte alterations, and RONS (Fitts, 1994; Westerblad and Allen, 2011; Morales-Alamo and Calbet, 2014). During high intensity exercise RONS are produced due to both the high mitochondrial respiratory rate and the activation of the anaerobic metabolism (Morales-Alamo et al., 2013; Morales-Alamo and Calbet, 2014). RONS may contribute to muscle fatigue by two main mechanisms: by reducing calcium sensitivity and/or reducing calcium release from sarcoplasmic reticulum (Bruton et al., 2008). In cardiac myofilaments, xanthine oxidase reductase inhibition enhances myofilament Ca^{2+} sensitivity, which may result in greater force production if the required energy is available. A similar effect might have been produced by the MLE-containing supplements in the present investigation.

Excessive RONS production could reduce mitochondrial P/O ratio, while antioxidants may favorably influence mitochondrial function improving efficiency (Clerc et al., 2007). Moreover, the ingestion of antioxidants before sprint exercise reduces the level of protein carbonyls in muscle and plasma (Morales-Alamo et al., 2012) and lowers the glycolytic rate (Morales-Alamo et al., 2017) without a detrimental effect on performance (Morales-Alamo et al., 2012, 2017).

The three polyphenols tested here have free radical quenching capacity (González-Gallego et al., 2010; Braakhuis and Hopkins, 2015; Luo et al., 2017; Rauf et al., 2017), being also inhibitors of XO (Pinto et al., 2005; Paredes-Gonzalez et al., 2015; Niu et al., 2016) and NOX (Makino et al., 2013; Xia N. et al., 2014). However, no study in humans has shown that antioxidants, even when administered intravenously are capable of enhancing peak power output during repeated prolonged sprint exercise. Thus, although the antioxidant properties of the supplements tested here may have contributed to enhance performance, other mechanisms must be involved, since a wide variety of antioxidants have previously failed to enhance peak power output in humans and none have shown these properties in the fatigued state.

To boost performance in a fatigued muscle greater calcium release is needed to enhance the number of cross-bridges that can be established, but also a faster calcium reuptake is required to shorten the relaxation phase. *In vitro* caffeine can enhance force in fatigued muscle by boosting Ca^{2+} release, but the dose needed would be lethal for humans (Fredholm, 1995). In this regard, mangiferin, a major component of MLE, shares some common intracellular mechanisms of action with caffeine, which may facilitate calcium release in the fatigued state (i.e., when Ca^{2+} release is depressed). Like caffeine and beta-agonists, mangiferin may increase cAMP, and through the activation of protein kinase A (PKA), stimulate SERCA activity. At rest phospholamban (PLN) inhibits SERCA activity, but when phosphorylated by PKA or Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), PLN dissociates from SERCA, and SERCA activity increases (MacLennan and Kranias, 2003). However, at tolerable doses it is unlikely that caffeine can alter skeletal muscle metabolism in humans (Desbrow et al., 2009) and the main mechanism of the ergogenic action of caffeine is supposed to rely on its effect on the central nervous system, by enhancing muscle activation (Behrens et al., 2015) and decreasing the perception of effort (de Morree et al., 2014). Interestingly, in our experiment the mangiferin-containing supplements reduced the level of

pain perceived during post-exercise ischemia. Whether this may positively influence the corticospinal drive during sprint exercise remains to be determined.

Although caffeine may enhance performance during prolonged exercise and team-sport activities its capacity to enhance power and strength is debated (Davis and Green, 2009; Goldstein et al., 2010). Moreover, there is no evidence supporting an ergogenic effect of caffeine during episodes of ischemia/reperfusion which may occur in some sport disciplines. Unlike caffeine, which may cause hypokalemia in athletes (Eichner, 2011), no effect of mangiferin on plasma potassium was observed here.

Brain Oxygenation and Fatigue

Reduction in brain oxygenation has been associated with fatigue in several studies (Smith and Billaut, 2010; Torres-Peralta et al., 2016a; Santos-Concejero et al., 2017; Curtelin et al., 2018). Moreover, at exhaustion during exercise in hypoxia improving the oxygenation of the brain (and upper body) by swiftly raising the F_IO_2, while maintaining the lower extremities deoxygenated by instantaneously occluding the circulation, was associated with improved performance (Morales-Alamo et al., 2015), which supports a mechanistic link between brain oxygenation and fatigue during sprint exercise in a fatigued state (Torres-Peralta et al., 2016a). Here we show that MLE-containing supplements consumed before sprint exercise may counteract fatigue by improving brain oxygenation, at least in women. The reason why women were more sensitive to this effect should be addressed in new experiments, but may be related to phytoestrogen dependent protection of endothelial function, by an NO-dependent mechanism (Chen et al., 1999), similar to that of resveratrol (Xia N. et al., 2014), which has been shown to enhance brain perfusion in postmenopausal women (Klinge et al., 2005) and young adults (Wightman et al., 2015). In agreement with our results, it has been shown that resveratrol may improve frontal lobe oxygenation despite unchanged Doppler-measured middle cerebral artery velocity at rest in young adults (Wightman et al., 2015).

The Combination of Mango Leaf Extract With Quercetin Has a Strong Protective Effect of Muscle Functional Capacity When Exhausted Skeletal Muscles Are Submitted to Ischemia/Reperfusion

In the present investigation, we have tested for the first time in humans the potential protective effects of an empirical polyphenol combination (MLE + quercetin + tiger nut extract) on functional deterioration induced by ischemia/reperfusion. Estrogens (and phytoestrogens) may protect against ischemia/reperfusion injury through the activation of estrogen receptor (ERα) and the downstream signaling cascade phosphatidylinositol-3-OH kinase (PIK3)/Akt to promote cell survival and through PIK3/eNOS to stimulate endothelial NO release, the latter resulting in vascular protection (Chen et al., 1999). Both luteolin and mangiferin are known inhibitors of xanthine oxidoreductase (XOR), which is considered responsible for part of damage generated by O_2^- during reperfusion, due to the formation of ·OH and $ONOO^-$ radicals by XOR catalytic activity (Berry and Hare, 2004).

In summary, this study shows that the MLE 60% mangiferin (Zynamite) has a remarkable ergogenic effect increasing muscle power in fatigued subjects, without increasing the consumption of oxygen, submaximal exercise efficiency or submaximal and maximal blood lactate concentrations. This type of response is expected for a compound acting on the central nervous system. We have also shown for the first time in humans that MLE combined with quercetin and tiger nut extract assist in maintaining skeletal muscle function during ischemia/reperfusion, strongly suggesting that this combination is also acting directly on the skeletal muscles. Further studies should explore whether MLE/quercetin/tiger nut extract might have clinical application to prevent ischemia-reperfusion damage in patients during surgery or after post-embolism reperfusions.

DISCLOSURES

This study has been partly financed by Nektium Pharma, who kindly provided the phenolic compounds.

AUTHOR CONTRIBUTIONS

JC, JW, and NG: conception and design of the experiments; DC, MG-R, MM-R, MP-V, VG-A, and DM-A: pre-testing, experimental preparation, and data collection; MG-R, MM-R, MP-V, VG-A, DM-A, LL-R, and JC: data analysis. The first draft of the manuscript was written by MG-R and JC. All co-authors edited and proofread the manuscript and approved the final version.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

nutrients

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Article

A Single Dose of The Mango Leaf Extract Zynamite® in Combination with Quercetin Enhances Peak Power Output During Repeated Sprint Exercise in Men and Women

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Abstract: The mango leaf extract rich in mangiferin Zynamite® improves exercise performance when combined with luteolin or quercetin ingested at least 48 h prior to exercise. To determine whether a single dose of Zynamite® administered 1 h before exercise increases repeated-sprint performance, 20 men and 20 women who were physically active were randomly assigned to three treatments following a double-blind cross-over counterbalanced design. Treatment A, 140 mg of Zynamite®, 140 mg of quercetin, 147.7 mg of maltodextrin, and 420 mg of sunflower lecithin; Treatment B, 140 mg of Zynamite®, 140 mg of quercetin, and 2126 mg of maltodextrin and Treatment C, 2548 mg of maltodextrin (placebo). Subjects performed three Wingate tests interspaced by 4 min and a final 15 s sprint after ischemia. Treatments A and B improved peak power output during the first three Wingates by 2.8% and 3.8%, respectively (treatment x sprint interaction, $p = 0.01$). Vastus Lateralis oxygenation (NIRS) was reduced, indicating higher O_2 extraction (treatment × sprint interaction, $p = 0.01$). Improved O_2 extraction was observed in the sprints after ischemia ($p = 0.008$; placebo vs. mean of treatments A and B). Blood lactate concentration was 5.9% lower after the ingestion of Zynamite®with quercetin in men (treatment by sex interaction, $p = 0.049$). There was a higher Vastus Lateralis O_2 extraction during 60 s ischemia with polyphenols (treatment effect, $p = 0.03$), due to the greater muscle VO_2 in men ($p = 0.001$). In conclusion, a single dose of Zynamite® combined with quercetin one hour before exercise improves repeated-sprint performance and muscle O_2 extraction and mitochondrial O_2. consumption during ischemia. No advantage was obtained from the addition of phospholipids.

Keywords: ergogenic aids; polyphenols; high-intensity exercise; ischemia; reperfusion; sports nutrition; metabolism; oxygen extraction; RONS; oxidative stress

1. Introduction

Zynamite®, a mango leaf extract rich in the natural polyphenol mangiferin, increases exercise performance when given in combination with luteolin or quercetin [1,2]. Besides, both combinations of polyphenols enhanced the contractile response of human skeletal muscle to ischemia-reperfusion. Since the combination of Zynamite® with quercetin was administered for 48 h to 15 days before the exercise [1,2], it remains unknown whether a single dose of Zynamite® would also have ergogenic effects.

During intense exercise, fatigue may be caused by a mismatch between oxygen delivery and utilization leading to faster consumption of glycogen stores and activation of the anaerobic metabolism, resulting in H^+ and P_i accumulation, and increased generation of reactive oxygen and nitrogen species (RONS) [3–5]. Increased levels of H^+, P_i, and RONS may reduce Ca^{2+} release from the sarcoendoplasmic reticulum [6] and diminish troponin calcium sensitivity, diminishing peak power [7,8]. Exercise performance also depends on the capacity of the nervous system to provide an adequate activation signal for the prescribed task [9]. The central nervous drive is, in turn, modulated by sensory feedback from group III/IV afferents acting at spinal and supraspinal levels [10]. This is further compounded by the fact that perceived fatigability depends on many factors including core temperature, hydration, brain oxygenation, blood glucose, and several psychological factors (arousal, executive function, expectations, mood, motivation, pain, and performance feedback) [9].

The polyphenolic combination of Zynamite® with quercetin can counteract fatigue by several mechanisms [1,2]. It has been shown that intake of Zynamite® combined with quercetin during the 48 h preceding repeated-sprint exercise attenuates the decline in brain oxygenation normally seen during prolonged sprinting [11], while it facilitates muscle O_2 extraction [1,2]. In addition, natural polyphenols and antioxidants could attenuate sensitive afferent signals generated by group III and IV ergoreceptors [7,8], facilitating muscle activation [12].

The main constituent of Zynamite® is mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), a xanthone (non-flavonoid polyphenol) abundant in Mangifera indica (mango) leaves, bark and pulp, but also in other edible plants [13]. Mangiferin is a potent antioxidant with iron-chelating properties, exceptionally efficient in protecting against the production of free radicals by the Fenton reaction. The mitochondria and the superoxide producing enzymes (xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase or (NOX)) are the primary sources of RONS during sprint exercise [14]. Mangiferin could attenuate RONS production by inhibiting XO [15] and thus maintain calcium sensitivity [7] during sprint exercise, which can contribute to preserve or enhance peak power output during high-intensity exercise.

Quercetin is categorized as a flavonol, a flavonoid subclass highly abundant in a variety fruits and vegetables. This plant metabolite may have ergogenic effects during prolonged exercise [16–18], and during sprint exercise when given in combination with Zynamite® [1]. Quercetin attenuates the damage caused by ischemia-reperfusion in animal models [19], and in combination with Zynamite® improves contractile muscle function after ischemia-reperfusion [1]. However, these effects were observed after repeated pre-exercise supplementation [1,2]. Quercetin also has XO and NOX inhibiting properties [20,21], which could also contribute to enhancing exercise performance during sprint exercise. It has been reported that the intestinal absorption and oral bioavailability of quercetin may be improved by lipidic vehicles [22].

Therefore, the purpose of this study was to determine whether a single dose of Zynamite® administered in combination with a small amount of quercetin (140 mg), or with quercetin combined with sunflower lecithin, increases exercise performance during repeated-sprint exercise. We hypothesized that a single dose of the combination of Zynamite® with quercetin will improve muscle contractile capacity and muscle oxygenation during repeated sprint exercise to exhaustion. We also hypothesized that these effects will be further augmented by adding sunflower phospholipids to the Zynamite®-quercetin mixture.

2. Materials and Methods

2.1. Subjects

In total, 50 subjects (all of them physically active) volunteered to participate in this study (24 women and 26 men) (Table 1). From this pool of volunteers, men and women were recruited randomly to a final experimental group of 40 subjects matched for age and sex. Subjects were accepted in the participation list if they fulfilled the inclusion criteria for the study, i.e., age from 18 to 45 years old; without chronic diseases or recent surgery; non-smoker; normal resting electrocardiogram; body mass index (BMI) below 30 and above 18; no history of disease requiring medical treatments lasting more than 15 days during the preceding 6 months; no medical contraindications to exercise testing and lack of food allergies. All volunteers applying met the inclusion criteria, except for one girl having asthma. Ten subjects were excluded due to medical reasons (five subjects) or time availability (five subjects). All volunteers received oral and written information about the experiments and possible risks before they signed their written consent to participate. The study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2019-02).

A sample size between 20 and 28 participants was required to provide adequate power to detect an improvement between 5 and 6% in peak power output ($\alpha = 0.05$, $\beta = 0.80$; G *Power v 3.1.9.2) in sprint performance. Nevertheless, a total of 40 subjects were included (20 men and 20 women) to increase statistical power and account for potential dropouts and missing values.

Table 1. Physical characteristics, body composition and VO_2max.

	Men			**Women**			***p***
Age (Years)	23.1	±	2.2	23.5	±	2.9	0.64
Height (cm)	174.4	±	5.6	165.1	±	6.5	0.000
Weight (kg)	73.4	±	9.1	59.5	±	8.0	0.000
Body fat (%)	18.5	±	3.7	27.7	±	4.1	0.000
Fat mass (kg)	13.7	±	3.8	16.7	±	4.4	0.03
Lean mass (kg)	56.6	±	6.3	40.3	±	4.2	0.000
Legs lean mass (kg)	20.2	±	2.7	14.1	±	1.8	0.000
VO_2max (mL min^{-1})	3189.7	±	525.4	2167.4	±	279.2	0.000
VO_2max (mL kg^{-1} min^{-1})	43.5	±	5.4	36.6	±	3.9	0.000
LLM VO_2max (mL kg^{-1} min^{-1})	158.7	±	19.3	154.4	±	17.1	0.47
Hemoglobin (g dL^{-1})	15.4	±	0.7	13.3	±	0.7	0.000
Systolic BP (mmHg)	122.4	±	9.4	111.3	±	5.4	0.000
Diastolic BP (mmHg)	66.2	±	7.3	67.9	±	4.2	0.365

LLM: lower extremities lean mass, also legs lean mass; BP: blood pressure; $N = 20$ for men and women.

2.2. General Overview

Subjects were first familiarized with the equipment and experimental protocol with two familiarization visits to perform submaximal and maximal (sprint) tests on the cycle ergometer. In subsequent days, the pre-tests were carried out to determine their body composition, maximal oxygen uptake (VO_2max) and anaerobic capacity (accumulated oxygen deficit). At least one week after the last pre-test visit, the main experiments started, each including four maximal sprints. The minimum wash-out period between the main trials was ten days. Subjects were requested to maintain their usual level of physical activity between treatments to a maximum of two sessions per week of no more than 30 min per session. All subjects were requested not to exercise and to refrain from carbonated, caffeinated and alcohol-containing beverages during the 48 h period preceding the main sprint experiments and the 24 h before pre-tests. Subjects were also instructed to refrain from taking drugs, medications, dietary supplements and the use of any putative recovery treatments during the duration of the study.

2.3. Pre-Tests

Body composition was determined by dual-energy X-ray absorptiometry (Lunar iDXA, GE Healthcare, WI, USA) as described elsewhere [23]. Subjects performed a familiarization visit during which incremental exercise to exhaustion and an all-out sprint were performed. After familiarization, subjects reported to the laboratory to complete different tests on separate days. First, their VO_2max, maximal heart rate (HRmax) and maximal power output (Wmax) were determined in normoxia (F_IO_2: 0.21, P_IO_2: 143 mmHg) with an incremental exercise test to exhaustion with verification [24]. The incremental exercise test started with three min at 20 W, followed by 15 W and 20 W increases every three minutes in women and men, respectively, until the respiratory exchange ratio (RER) was ≥ 1.0. After completion of the load eliciting a RER ≥ 1.0, the intensity was increased by 10 and 15 W/min (women and men, respectively) until subject reached their limit of tolerance (exhaustion). The intensity attained at exhaustion was taken as the maximal power output of the incremental exercise test (Wmax). At exhaustion, the ergometer was unloaded, and subjects remained seated on the cycle ergometer pedaling at a low cadence (30–40 rpm) for 3 min. This was followed by the verification test starting at Wmax + 5 W for 1 min, with increases of 4 and 5 W (women and men, respectively) every 20 s until exhaustion. During the incremental tests to exhaustion and the main sprint experiments, gas exchange was continuously recorded using a Vyntus CPX (Jaeger-Carefusion, Hoechberg, Germany) metabolic cart in breath-by-breath mode. The metabolic cart was calibrated prior to each test following manufacturer instructions with high-grade calibration gases provided by the manufacturer of Vyntus CPX. Respiratory variables were averaged every 20 s during the incremental test and the highest averaged value was reported as the VO_2max.

2.4. Main Sprint Experiments

The volunteers were randomly assigned to three different treatments (A, B and C) which were administered in a single dose 1 h before the repeated-sprint protocol, following a double-blind cross-over and counterbalanced experimental design. Treatment A consisted of 140 mg of Zynamite® (standardized to 60% mangiferin), 140 mg of quercetin (in the form of 280 mg *Sophora japonica* extract, standardized to 50% quercetin), 147.7 mg of maltodextrin, and 420 mg of sunflower lecithin. Treatment B consisted of 140 mg of Zynamite®, 140 mg of quercetin (in the form of 280 mg *Sophora japonica* extract, standardized to 50% quercetin), and 2126 mg of maltodextrin. Treatment C consisted of 2548 mg of maltodextrin (placebo). All treatments were administered in methylcellulose capsules with identical appearance.

On the days of the main experiments, subjects reported to the laboratory with a 4 h to 10 h period of fasting, depending on the testing time of the day. Each subject thoroughly recorded the last dinner and the last meal preceding the main experiment. Subjects performed all three trials at the same time of the day (±1 h) and were asked to reproduce precisely the fasting period and the preceding meals for each of the trials. Sixty minutes before the start of the experiment, they ingested the supplement tested with 300 mL of water. During the following 60 min, subjects were instrumented and prepared for the exercise, while their hemoglobin concentration was measured in capillary blood (HemoCue, Ängelholm Sweden). After that, subjects sat on the cycle ergometer and performed a 6 min warm-up pedaling at 80 or 100 W (women and men, respectively) keeping the pedaling cadence at 80 rpm (±3 rpm).

After a 4.5 min period of unloaded pedaling, they stopped pedaling, and the ergometer was switched to isokinetic mode. At the 5th minute, they performed a Wingate test (30 s all-out sprint in isokinetic mode at 80 rpm). This was followed by 3.5 min of unloaded pedaling and another 30 s period, during which they stopped pedaling, and the ergometer was switched to the isokinetic mode. At the 4th minute, a second 30 s Wingate test was performed, which was also followed by another 3.5 min of unloaded pedaling and another 30 s period of rest. A third 30 s Wingate was then performed. Immediately at the end of the third 30 s sprint, the circulation of both lower extremities was instantaneously occluded for 60 s by inflating bilateral cuffs at 300 mmHg as previously reported [25,26]

(Figure 1). For this purpose, bilateral cuffs were placed around the thighs during the preparation phase, as close as possible to the inguinal crease, and were connected to a rapid (0.3 s) cuff inflator system (SCD10, Hokanson E20 AG101, Bellevue, WA, USA) before they were seated on the cycle ergometer.

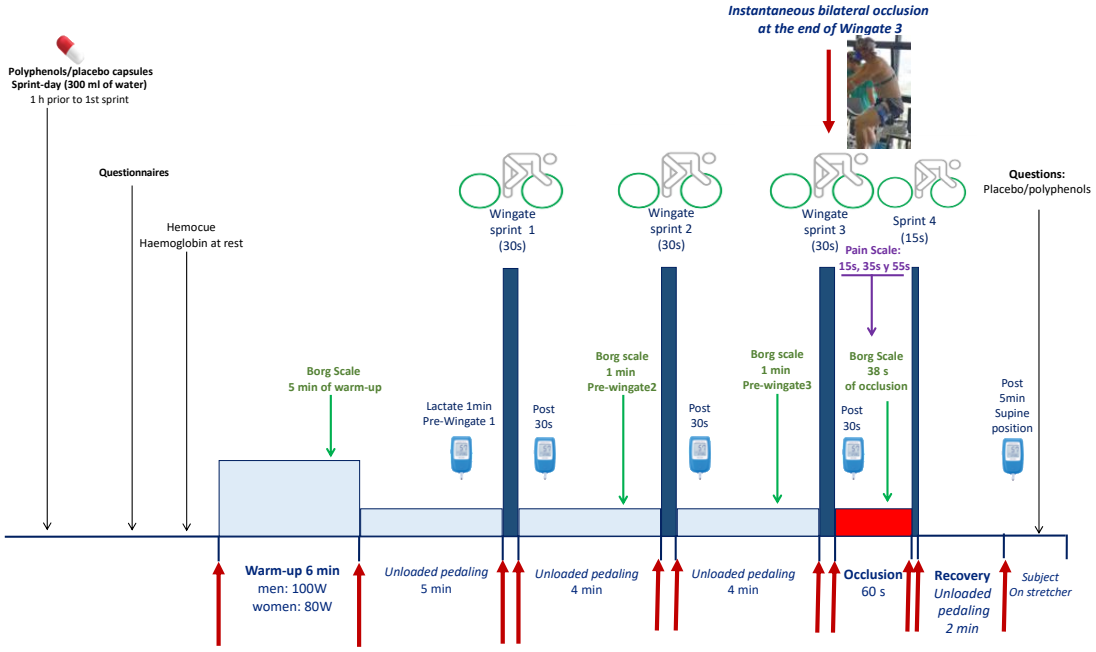

Figure 1. Experimental protocol.

Fifty seconds after the start of the occlusion, a reverse countdown was started, and the subjects re-started pedaling as fast and hard as possible for 15 s, with the ergometer set in the isokinetic mode. At the beginning of the sprint, the cuff was deflated to allow for a full reestablishment of the circulation during the last sprint. This was followed by two minutes of unloaded pedaling. After that, the subjects rested supine on a laboratory stretcher. One min before the first Wingate test, 30 s after each Wingate test and five min after the end of the final 15 s sprint, a capillary blood sample was drawn from the earlobe, previously hyperhemized with Finalgon® cream, to measure the concentration of lactate (Lactate-Pro 2, Arkray, Kyoto, Japan). Before the start and at the end of the experiments, the subjects were asked whether they could figure out what kind of supplement they had. They were previously informed that they would be ingesting polyphenols in two of the three experiments, and placebo in the remaining experiment (Figure 1).

2.5. Power Output

During the incremental exercise protocol (pre-test) subjects were requested to maintain the pedaling rate at 80 rpm (±3 rpm) while the cycle ergometer (Excalibur Sport 925900, Lode, Groningen, The Netherlands) was set in rpm-independent mode. Exhaustion was defined by the incapacity to maintain a pedaling rate above 50 rpm during 5 s, despite strong verbal encouragement, or by a sudden stop in pedaling. For the main sprint experiments, the cycle ergometer was set on isokinetic mode at 80 rpm for all sprints and in an rpm-independent mode during the warm-up and recovery phases. During the isokinetic sprints, the volunteers pedaled as fast and hard as possible, exerting as much force on the pedals as they could at each pedal stroke from the start to the end of the sprint. The servo-control brake system of the cycle ergometer adjusts continuously and almost instantaneously the braking force, so the pedaling rate stays at 80 rpm during the whole sprint. Data from all isokinetic sprints were reported as instantaneous peak power (PPO) and mean power output (MPO). Strong verbal encouragement was provided throughout the entire protocol.

2.6. Oxygen Demand and Deficit

The O_2 demand during the supramaximal exercise bouts was estimated from the linear relationship between the averaged VO_2 for the last minute of each load, from 20–40 W to the highest intensity with an RER < 1.00 in the incremental exercise test. The accumulated oxygen deficit (AOD), representing the difference between O_2 demand and VO_2, was determined as previously reported [27].

2.7. Vastus Lateralis Muscle and Cerebral Oxygenation

Cerebral oxygenation was assessed at rest and during exercise using near-infrared spectroscopy (NIRS, NIRO-200, Hamamatsu, Japan) employing spatially-resolved spectroscopy to obtain the tissue oxygenation index (TOI) using a path-length correction factor of 5.92 [28]. The NIRS optodes were double-sided taped in the lateral aspect of the right thigh at middle length between the patella and the anterosuperior iliac crest, over the middle portion of the musculus Vastus Lateralis. An additional optode was placed on the right frontoparietal region at 3 cm from the midline and 2–3 cm above the supraorbital crest, to avoid the sagittal and frontal sinus areas [11]. This optode placement examines the tissue oxygenation of the superficial frontal cerebral cortex is recorded. The probes were secured in place by double-sided tape and elastic bandages were used to avoid the entrance of external light and minimize movement artifacts.

2.8. Assessment of Pain and Effectiveness of Concealment

Subjects were requested to rate the level of pain felt during the occlusion from 0 to 10, being 10 the highest muscle pain ever suffered during or after exercise in their life. Likewise, at the end of the experiment, subjects were asked about the kind of supplement they thought they had received to check on the effectiveness of concealment.

2.9. Statistical Analysis

Variables were checked for normal distribution by using the Shapiro-Wilks test. A two-way repeated-measures ANOVA was used with two within-subjects factors: treatment (with three levels) and exercise bout (with three levels), and with sex as a between-subjects factor. The Mauchly's test of sphericity was run before the ANOVA, and in the case of violation of the sphericity assumption, the degrees of freedom were adjusted according to the Huynh and Feldt test. When a significant main effect or interaction was observed, specific pairwise comparisons were carried out with the Least Significant Difference (LSD) post-hoc test. Separate analyses were carried out to test for the effects of treatment in measurements performed during the phases of ischemia and the 15 s post-ischemia sprint. For these purposes, a repeated-measures ANOVA was used with one within-subjects factor: treatment (with three levels), and with sex as a between-subjects factor. Values are reported as the mean ± standard deviation of the mean (unless otherwise stated). $p \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects on Sprint Performance

Compared to placebo, treatments A and B improved peak power output during the first three sprints by 2.8 ($p = 0.04$) and 3.8% ($p = 0.01$), respectively (ANOVA treatment x sprint interaction, $p = 0.01$) (Table 2) (Figure 2). This effect was accompanied by reduced Vastus Lateralis oxygenation, indicating higher O_2 extraction (treatment x sprint interaction, $p = 0.01$) (Table 3). This enhanced O_2 extraction was only observed in men (treatment by sex interaction, $p = 0.037$). Improved O_2 extraction was also seen in the sprints performed after ischemia ($p = 0.008$; placebo compared with the mean of treatments A and B), due to the effect elicited in men (A vs. B, $p = 0.04$, and A vs. C, $p = 0.005$) (Figure 3). Consequently, a trend to a higher VO_2peak during the repeated-sprint protocol was observed after the

administration of treatment A compared to placebo (+3.7%, $p = 0.096$) and treatment B compared to placebo in men (+1.7%, $p = 0.065$) (Table 4).

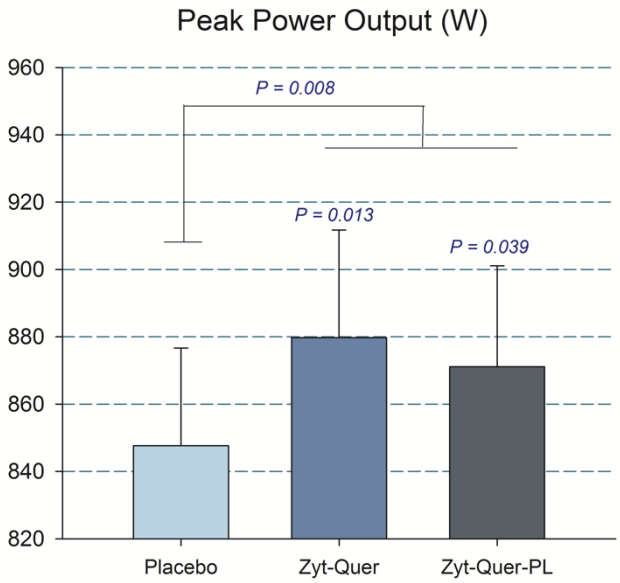

Figure 2. Peak Power output. The bars correspond to the mean of the three sprints, after the ingestion of placebo, Zynamite® (Zyt) combined with quercetin (Quer) or Zynamite® combined with quercetin and phospholipids (PL). Error bars represent the standard error of the mean. p values: comparison with placebo. $N = 40$.

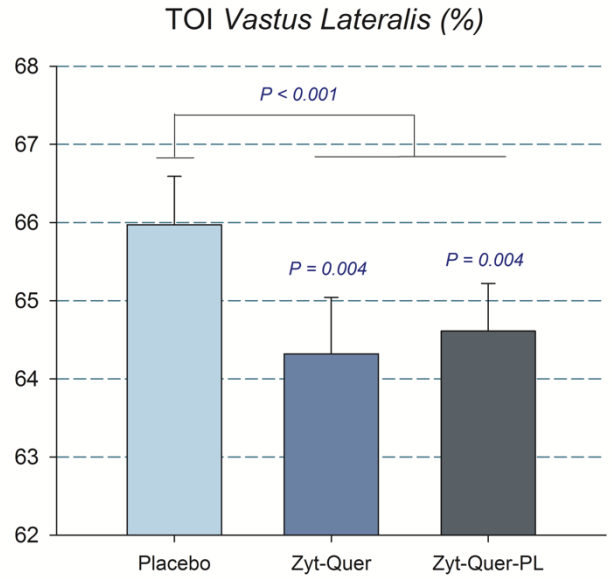

Figure 3. Peak Power output. The bars correspond to the mean of the three sprints, after the ingestion of placebo, Zynamite® (Zyt) combined with quercetin (Quer) or Zynamite® combined with quercetin and phospholipids (PL). Error bars represent the standard error of the mean. p values: comparison with placebo. $N = 40$.

In agreement with a slightly higher reliance on the aerobic metabolism in the sprint performed after the intake of Zynamite® with quercetin, blood lactate concentration tended to be 7.4% lower in men after the administration of treatment B compared to placebo ($p = 0.03$, treatment by sex interaction for the first three sprints, $p = 0.059$) (Figure 4).

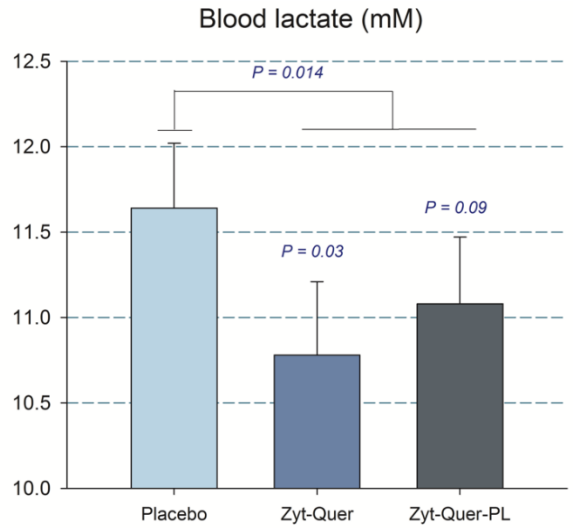

Figure 4. Capillary blood lactate concentration in 20 men. The bars correspond to the mean of the three sprints, after the ingestion of placebo, Zynamite® (Zyt) combined with quercetin (Quer) or Zynamite® combined with quercetin and phospholipids (PL). Treatment by sex interaction for the first three sprints $p = 0.059$. Error bars represent the standard error of the mean. p values: comparison with placebo. $N = 20$.

A similar result was observed when the blood lactate obtained 5 min after the sprint performed following ischemia was included in the ANOVA analysis, i.e., the blood lactate concentration was 5.9% lower after the ingestion of Zynamite® with quercetin (11.9 ± 1.7 and 12.8 ± 1.6 mM, in B and placebo, respectively, $p = 0.017$; treatment by sex interaction, $p = 0.049$). The mean rate of perceived exertion (RPE) during the protocol was similar in the three trials (Table 3) and no significant differences were found in the pain felt at different timepoints nor the mean pain during the occlusion (Table 4). Compared to placebo, during the whole 60 s of ischemia, there was a higher O_2 extraction by the Vastus Lateralis after the ingestion of polyphenols (ANOVA treatment effect, $p = 0.03$) (Figure 5).

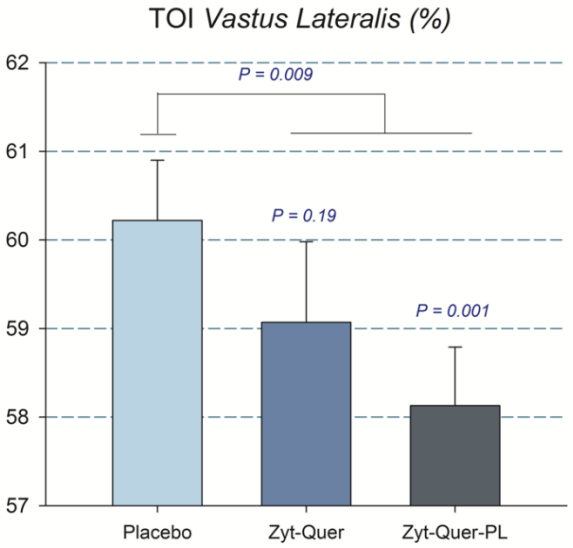

Figure 5. Tissue oxygenation index of the musculus Vastus Lateralis during the 60 s ischemia applied at the end of the third Wingate test. The bars correspond to the mean values after the ingestion of placebo, Zynamite® (Zyt) combined with quercetin (Quer) o Zynamite® combined with quercetin and phospholipids (PL). Error bars represent the standard error of the mean. p values: comparison with placebo. $N = 40$.

Table 2. Power output and cardiorespiratory variables during the first three 30 s Wingate tests (mean ± SD).

		A			B			C			Treatment	Treat × Sex	Treat × Wing	Treat × Wing × Sex
PPO (W)	All	871	±	193	880	±	203	848	±	184	0.01	0.048	0.96	0.79
	M	1025	±	133	1015	±	171	981	±	152				
	W	717	±	93	738	±	120	714	±	95				
MPO (W)	All	482	±	107	479	±	109	478	±	109	0.80	0.39	0.70	0.84
	M	570	±	75	564	±	81	567	±	80				
	W	394	±	39	390	±	41	389	±	34				
VO_2 (mL)	All	1069	±	245	1054	±	228	1064	±	225	0.69	0.24	0.38	0.60
	M	1269	±	177	1226	±	175	1246	±	153				
	W	870	±	88	874	±	103	882	±	104				
VCO_2 (mL)	All	1102	±	278	1093	±	273	1108	±	276	0.43	0.61	0.28	0.84
	M	1336	±	181	1310	±	177	1340	±	166				
	W	869	±	104	864	±	129	876	±	128				
RER	All	1.03	±	0.06	1.02	±	0.07	1.03	±	0.08	0.20	0.81	0.49	0.45
	M	1.05	±	0.06	1.05	±	0.06	1.08	±	0.06				
	W	1.00	±	0.06	0.99	±	0.07	0.99	±	0.07				
V_E (L min^{-1})	All	49	±	12	50	±	12	50	±	13	0.41	0.64	0.14	0.15
	M	57	±	10	58	±	10	58	±	10				
	W	41	±	8	42	±	9	41	±	9				
$P_{ET}O_2$ (mmHg)	All	119	±	4	119	±	4	119	±	4	0.59	0.53	0.17	0.26
	M	119	±	3	119	±	3	119	±	3				
	W	119	±	4	120	±	4	119	±	4				
$P_{ET}CO_2$ (mmHg)	All	26	±	3	26	±	4	26	±	3	0.29	0.56	0.12	0.37
	M	27	±	3	27	±	3	27	±	3				
	W	26	±	3	24	±	4	25	±	3				
Heart rate (bpm)	All	163	±	10	165	±	11	164	±	10	0.21	0.31	0.32	0.17
	M	165	±	8	166	±	10	164	±	8				
	W	162	±	12	164	±	12	164	±	11				

M: men; W: women; PPO: peak power output; MPO: mean power output; VO_2: oxygen uptake; VCO_2: CO_2 production; RER: respiratory exchange ratio; V_E: pulmonary ventilation; $P_{ET}O_2$: end-tidal O_2 pressure; $P_{ET}CO_2$: end-tidal CO_2 pressure; HR: heart rate. N = 39–40 for all variables.

Table 3. Oxygen deficit, brain and muscle oxygenation, VO_2 per watt and rate of perceived exertion for the first three 30 s Wingate tests (mean ± SD).

		A			B			C			Treatment	Treat × Sex	Treat × Wing	Treat × Wing × Sex
Oxygen deficit (mL)	All	1625	±	542	1632	±	622	1612	±	565				
	M	1962	±	556	1982	±	660	1969	±	576	0.81	0.82	0.80	0.72
	W	1289	±	232	1263	±	278	1256	±	238				
Lactate (mM) [L]	All	11.1	±	1.9	11.3	±	2.0	11.5	±	1.9				
	M	11.1	±	1.8 [&]	10.8	±	1.9 *	11.6	±	1.7	0.46	0.059	0.83	0.16
	W	11.2	±	2.0	11.9	±	2.1	11.3	±	2.2				
TOI Frontal Lobe (%)	All	66	±	5	65	±	7	67	±	5				
	M	68	±	6	67	±	7	69	±	5	0.20	0.62	0.32	0.33
	W	65	±	3	63	±	6	64	±	4				
TOI Vastus Lateralis (%)	All	64.6	±	3.8	64.3	±	4.5	66.0	±	3.9				
	M	63.4	±	3.7 *	62.3	±	3.9 *	65.2	±	4.5	0.01	0.04	0.60	0.63
	W	65.8	±	3.7	66.5	±	4.1	66.7	±	3.3				
VO_2/W (mL W^{-1}) [a, L]	All	2.27	±	0.20	2.29	±	0.22	2.29	±	0.19				
	M	2.28	±	0.25	2.28	±	0.26	2.26	±	0.23	0.96	0.72	0.73	0.86
	W	2.25	±	0.12	2.30	±	0.18	2.31	±	0.13				
VO_2/W/kg LLM (mL W^{-1} kg^{-1}) [b]	All	0.139	±	0.034	0.140	±	0.038	0.141	±	0.035				
	M	0.116	±	0.023	0.114	±	0.027	0.115	±	0.025	0.93	0.50	0.42	0.72
	W	0.162	±	0.026	0.167	±	0.027	0.166	±	0.022				
RPE	All	4.5	±	1.6	4.7	±	1.5	4.7	±	1.5				
	M	4.8	±	1.5	5.0	±	1.4	5.0	±	1.3	0.51	0.77	0.57	0.72
	W	4.2	±	1.7	4.3	±	1.6	4.4	±	1.6				

M: men; W: women; TOI: Tissue oxygenation index; VO_2: oxygen uptake; LLM: lower extremities lean mass; RPE: rate of perceived exertion. [a] Oxygen uptake per watt during the sprints; [b] Oxygen uptake per watt and kg of lower extremities lean mass during the sprints; [L] analysis done with logarithmically transformed data; * $p < 0.05$ compared to placebo (C); [&] $0.05 > P < 0.10$ compared to placebo. $N = 39$–40 for all variables.

Table 4. Peak VO_2 during the sprints, pain felt during the 60 s occlusions and hemoglobin concentration before the sprints (mean ± SD).

		A			B			C			Treatment	Treat × Sex
Sprints VO_2peak (mL min^{-1})	All	2668	±	597	2650	±	581	2624	±	566	0.290	0.110
	M	3160	±	391	3099	±	425	3046	±	453		
	W	2177	±	265	2178	±	252	2202	±	279		
Pain 15 s [L]	All	7.9	±	1.5	8.1	±	1.7	7.6	±	2.0	0.11	0.29
	M	7.6	±	1.6	7.9	±	1.8	7.0	±	2.4		
	W	8.3	±	1.3	8.3	±	1.6	8.2	±	1.5		
Pain 35 s [L]	All	8.6	±	1.2	8.6	±	1.9	8.4	±	1.7	0.12	0.35
	M	8.5	±	1.3	8.6	±	1.4	8.1	±	2.1		
	W	8.7	±	1.2	8.6	±	2.4	8.8	±	1.2		
Pain 55 s [L]	All	9.1	±	1.1	9.3	±	1.1	9.2	±	1.0	0.20	0.27
	M	9.0	±	1.2	9.1	±	1.3	9.1	±	1.1		
	W	9.3	±	0.9	9.6	±	0.8	9.3	±	0.9		
Mean Pain [L]	All	8.5	±	1.2	8.7	±	1.3	8.4	±	1.5	0.12	0.61
	M	8.4	±	1.3	8.5	±	1.4	8.1	±	1.8		
	W	8.7	±	1.1	9.0	±	1.1	8.7	±	1.1		
Hemoglobin (g dL^{-1})	All	14.3	±	1.3	14.4	±	1.3	14.4	±	1.2	0.70	0.09
	M	15.4	±	0.7	15.5	±	0.7	15.3	±	0.6		
	W	13.2	±	0.7	13.3	±	0.8	13.4	±	0.7		

M: men; W: women; Pain 15 s: pain reported at the 15th s of the occlusion; Pain 35 s: pain reported at the 35th s of the occlusion; Pain 55 s: pain reported at the 55th s of the occlusion; [L] analysis done with logarithmically transformed data. N = 39–40 for all variables.

3.2. Efficiency of Concealment

Over a total of 120 tests, in 66 instances subjects reported not to be able to figure out whether they had a placebo or an active polyphenolic substance. In 11 cases of a total of 40 trials with placebo, the subjects guessed correctly when they had been on a placebo.

4. Discussion

In agreement with previous reports, Zynamite® enhances peak power output when given in combination with quercetin [1,2]. In the present study, the quercetin dose was about 25–50% lower than that associated with quercetin ergogenic effects in previous studies [16], suggesting that this polyphenolic combination may have synergistic effects. Here, we have shown that even a single dose of Zynamite® combined with quercetin administered one hour before exercise improves repeated-sprint exercise performance and muscle O_2 extraction. As expected with greater reliance on the aerobic energy production, blood lactate concentration was reduced. We have also confirmed that the combination of Zynamite® with quercetin improves exercise performance and muscle O_2 extraction capacity in the sprint performed after ischemia.

Previous studies reporting ergogenic effects after quercetin administration have used supplementation regimes characterized by higher doses (>600 mg), administered during several days [16]. In contrast with our findings, a previous study has reported no effect of supplementation with quercetin (1000 mg/day for one week) on repeated-sprint performance (12 × 30 m maximal effort sprints) in team-sport athletes [29]. Others have concluded that the effect of quercetin in athletes is unclear [18].

4.1. The Combination of Zynamite® with Quercetin Improves Peak Power Output During Repeated Maximal Sprints

Sprint exercise elicits high glycolytic rates, lactate accumulation, and reduction of muscle pH [30]. In turn, acidification facilitates the production of hydroxyl radicals by the Fenton reaction and reduces the activities of the antioxidant enzymes glutathione reductase, glutathione peroxidase, and glutathione S-transferase [31]. Quercetin [32,33] and its colonic catecholic metabolites [34] possess potent free radical-scavenging capacity. Mangiferin, the main polyphenol present in mango leaf extract, is also a potent antioxidant [35,36]. In vitro studies indicate that the antioxidant capacity of polyphenol mixtures exceeds that of their constituents [36,37]. However, each polyphenolic compound has unique chemical properties which determine some specific actions in different cellular compartments [38,39]. Although *in vivo* experimental evidence is lacking, a combination of polyphenols likely counteracts more efficiently the RONS produced during exercise in different subcellular compartments of the skeletal muscle fibers than single compounds [40].

RONS may reduce Ca^{2+} release from the sarcoendoplasmic reticulum [6] and troponin calcium sensitivity, lowering peak power [7,8]. However, excessive antioxidant capacity may also limit force generation [41]. This could also explain why in the present investigation, an ergogenic effect was seen at a rather small dose of quercetin, which has not been tested previously in humans. The impact of RONS on muscle force follows a bell-shaped curve [42], and therefore, antioxidants at high dose may be detrimental [29]. Nevertheless, it has been reported that 12 weeks of quercetin supplementation at doses of 500 to 1000 mg/day, combined with 125 or 250 mg of vitamin C/day, respectively, had no effect on oxidative stress and antioxidant capacity [43].

An alternate mechanism by which the combination of quercetin and mangiferin may have limited RONS production during exercise is through the inhibition of XO and NOX [15,20,21], which play a crucial role as sources of RONS during sprint exercise [14,44]. Although mangiferin can cross the blood-brain barrier, modulate neurotransmission, K^+ channels and nociception [45], and attenuate sensory feedback, no significant effects on RPE were observed in the present investigation.

4.2. The Combination of Zynamite® with Quercetin Improves O_2 Extraction and Reduces Peak Blood Lactate Concentration During Repeated Sprint Exercise

Oxygen extraction depends on muscle oxygen diffusing capacity, oxygen delivery, and the PO_2 gradient from the muscle capillaries to the mitochondria [46]. Muscle O_2 diffusing capacity does not limit VO_2 during 30 s all-out sprints, because there is a large functional reserve in muscle O_2 diffusing capacity [47]. Muscle blood flow during sprint exercise is determined by cardiac output [47] and vascular conductance [48]. Increasing muscle blood flow may enhance the mean capillary PO_2 and hence, the gradient for O_2 diffusion, which could improve O_2 extraction. At maximal exercise, skeletal muscle vascular conductance is assumed to be maximal. This has been shown by experiments in which no increase of vascular conductance was observed in subjects exercising maximally with the intra-arterial infusion of maximal doses of ATP (one of the most potent vasodilators) [49]. An increase of skeletal muscle blood flow at maximal exercise is unlikely since this requires a higher cardiac output. The mean heart rate during the sprints was almost identical in the three conditions, suggesting unchanged cardiac output after the polyphenol administration. Thus, assuming that muscle O_2 diffusing capacity does not limit muscle VO_2 during sprint exercise, and that muscle blood flow was likely similar in the three conditions, the only mechanism that could explain an improvement in muscle O_2 extraction is an increase of the gradient driving diffusion. This gradient may be increased by reducing the mitochondrial P_{50} [50] or by improving mitochondrial bioenergetics facilitating a higher muscle VO_2.

The mitochondrial respiratory rate and ATP production depend, among other factors, on the mitochondrial concentrations of ADP [51] and Ca^{2+} [52,53]. Flavonoids may increase mitochondrial Ca^{2+} concentration by acting on the mitochondrial Ca^{2+} uniporter [54]. Mangiferin improves skeletal muscle mitochondrial ATP production and upregulates several enzymes of the glycolysis, facilitating a higher glycolytic rate in rodent skeletal muscle [55]. Moreover, cell experiments have shown that mangiferin reduces lactate accumulation by improving pyruvate dehydrogenase activity [56]. During high-intensity exercise hemoglobin [47] and myoglobin [57] deoxygenate, with this effect exacerbated in ischemia [58]. Both deoxyhemoglobin and deoxymyoglobin have nitrite reductase activity resulting in the production of NO from nitrite, and oxidation of heme-Fe^{2+} to heme-Fe^{3+} [59]. This reaction is facilitated by H^+, which increases during both high-intensity exercise and ischemia [26]. Mangiferin and quercetin could facilitate the nitrite reductase activity of deoxyhemoglobin and deoxymyoglobin by preventing the oxidation of $Fe_2{}^+$ to Fe^{3+} [60]. The NO released or produced within the muscle fibers can bind to cytochrome c oxidase of the mitochondrial electron transport chain, reducing electron flow and oxygen utilization [59], increasing oxidative phosphorylation efficiency in a redox-sensitive manner by decreasing the slipping in the proton pumps [61].

In the present investigation we have shown that supplementation with Zynamite®combined with quercetin was associated with lower muscle oxygenation, and this was accompanied by reduced capillary blood lactate concentration and a trend for a higher peak VO_2 during the sprints. Moreover, during ischemia, muscle O_2 was reduced to a larger extent after supplementation with Zynamite® combined with quercetin. Since ischemia was applied instantaneously after three maximal sprints, the biochemical environment is thought to be inhibitory for mitochondrial respiration due to lack of O_2 and inhibition of mitochondrial respiration by acidosis [26,62]. Nevertheless, a reduction of muscle tissue oxygenation was observed during the ischemia, to a larger extent after the administration of Zynamite® combined with quercetin. This implies that our polyphenol mixture improved mitochondrial bioenergetics during the sprints and ischemia. These findings agree with animal experiments showing that mangiferin protects mitochondrial function from the action of several noxious agents [63,64]. Mangiferin and quercetin supplementation could have improved the match between O_2 demand and perfusion by facilitating vasodilation [65], and hence, contributing to enhancing O_2 extraction.

In contrast with our previous studies [1,2] mean power output was not improved in the present investigation. While the study population and the methods applied were essentially similar between

the current investigation and our previous studies [1,2], the dose of quercetin and the duration of the supplementation was longer in our previous studies. Thus, repeated administration of Zynamite® combined with quercetin or a larger single dose could elicit even higher ergogenic effects.

5. Conclusions

A single dose of Zynamite® combined with quercetin administered one hour before exercise improves muscular performance and O_2 extraction. Interestingly, the dose of quercetin used in the present investigation was 25–50% lower than that associated with quercetin ergogenic effects in previous studies, what is compatible with a synergistic or additive effect of this polyphenolic mixture. We have also confirmed that Zynamite® combined with quercetin facilitates mitochondrial O_2 consumption during ischemia, a situation which is observed during prolonged isometric contractions in many sports disciplines. This effect may have clinical applications worth exploring in future studies. Finally, adding sunflower phospholipids to the Zynamite®-quercetin mixture had no additional beneficial effects

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Supplementation with a Mango Leaf Extract (Zynamite®) in Combination with Quercetin Attenuates Muscle Damage and Pain and Accelerates Recovery after Strenuous Damaging Exercise

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Abstract: Prolonged or unusual exercise may cause exercise-induced muscle damage (EIMD). To test whether Zynamite®, a mango leaf extract rich in the natural polyphenol mangiferin, administered in combination with quercetin facilitates recovery after EIMD, 24 women and 33 men were randomly assigned to two treatment groups matched by sex and 5 km running performance, and ran a 10 km race followed by 100 drop jumps to elicit EIMD. One hour before the competition, and every 8 h thereafter for 24 h, they ingested placebo (728 mg of maltodextrin) or 140 mg of Zynamite® combined with 140 mg of quercetin (double-blind). Although competition times were similar, polyphenol supplementation attenuated the muscle pain felt after the competition (6.8 ± 1.5 and 5.7 ± 2.2 a.u., $p = 0.035$) and the loss of jumping performance (9.4 ± 11.5 and 3.9 ± 5.2%, $p = 0.036$; $p = 0.034$) and mechanical impulse ($p = 0.038$) 24 h later. The polyphenols attenuated the increase of serum myoglobin and alanine aminotransferase in men, but not in women (interaction $p < 0.05$). In conclusion, a single dose of 140 mg Zynamite® combined with 140 mg of quercetin, administered one hour before competition, followed by three additional doses every eight hours, attenuates muscle pain and damage, and accelerates the recovery of muscle performance.

Keywords: ergogenic aids; polyphenols; antioxidant supplementation; eccentric exercise; DOMS; EIMD; sports nutrition; inflammation; human subjects; muscle function

1. Introduction

Prolonged or unusual exercise, particularly when involving eccentric muscle contractions, may cause muscle damage [1]. Exercise-induced muscle damage (EIMD) is characterized by muscle

soreness, structural disruption, and local inflammation, and it is accompanied by a temporary reduction of muscle force and exercise performance [2]. Although the administration of antioxidants to prevent muscle damage is controversial [3–5], some recent studies indicate that ingestion of polyphenol-rich extracts for at least three days before exercise and during the following hours/days may improve recovery [6]. Zynamite®, a mango leaf extract rich in the natural polyphenol mangiferin, and quercetin have antioxidant and anti-inflammatory properties [7–10], which may prevent EIMD and facilitate recovery [11]. Nevertheless, these two polyphenols have not been studied in combination and the effect of quercetin has been only assessed after chronic administration and with large daily doses (~1000 mg), which could impair exercise performance or blunt part of the adaptations to training [12].

In the following hours and days after EIMD, the range of motion is reduced, and some swelling appears in the affected limbs [13]. The risk and magnitude of EIMD is exacerbated when muscle contractions are performed at longer muscle length, faster angular velocity, and with higher forces [2]. Although a mechanical disruption of muscle fibers is thought to be the main mechanism starting EIMD, this is followed by a mild inflammatory response, in which reactive oxygen species (ROS) are involved [14,15]. However, administration of N-acetylcysteine (a thiol-base antioxidant) may be counterproductive, as it has been associated with lower recovery of strength eight days after EIMD [16].

It has been shown that Zynamite®, a mango leaf extract rich in the natural polyphenol mangiferin, enhances sprint exercise performance when given in combination with luteolin or quercetin [17,18]. Furthermore, both polyphenolic combinations improved the response of human skeletal muscle to ischemia-reperfusion [17–19]. Mangiferin (2-bD-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) is a xanthone abundant in the leaf and pulp of mangoes, which is also present in other edible vegetables [20]. Mangiferin has iron-chelating properties, free-radical scavenging capacity and inhibits some of the oxidases involved in oxidative stress and inflammation [7,9,21–24]. For these reasons, a natural extract rich in mangiferin may mitigate or prevent EIMD.

Quercetin is a flavonoid polyphenol that can improve performance during prolonged exercise [25] and sprint exercise when given in combination with Zynamite® [17]. Quercetin can be found in elderberries, onions, cranberries, kale, sophora japonica, apple and many other vegetables, including mangoes. Analogous to mangiferin, quercetin protects against the injury caused by ischemia-reperfusion [26]. Quercetin inhibits xanthine oxidase (XO) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) [27,28]. Thus, conjointly mangiferin and quercetin may counteract, even at low doses, some of the biochemical processes causing EIMD.

Therefore, the primary aim of this study was to determine whether Zynamite® administered in combination with a small amount of quercetin, which should not interfere with muscle performance, facilitates recovery after repeated damaging exercise. A secondary aim was to determine whether this polyphenol combination attenuates exercise-induced muscle damage and pain.

2. Materials and Methods

2.1. Subjects

The students of Sports Sciences (third year, total population $N = 114$) were invited to take part in this research. Fifty-seven volunteered to participate in the study (24 women and 33 men), but only 48 finished the competition and completed the follow-up assessments (18 women and 30 men) (Table 1). Subjects were informed about the inclusion criteria, risks, and benefits of participation and signed a written consent before participation. The investigation was conducted according to the Helsinki Declaration, after approval by the Ethical Committee of the University of Las Palmas de Gran Canaria (CEIH-2019-02).

Table 1. Physical characteristics, body composition and VO_2max.

	Men	Women	*p*
Age (years)	23.1 ± 2.5	23.3 ± 3.4	0.75
Height (cm)	176.6 ± 5.8	165.7 ± 5.2	0.000
Weight (kg)	74.7 ± 6.8	61.0 ± 6.2	0.000
Body fat (%)	18.7 ± 4.1	26.9 ± 4.5	0.000
Fat body mass (kg)	14.1 ± 3.8	16.5 ± 4.2	0.04
Lean body mass (kg)	57.4 ± 5.3	41.9 ± 3.8	0.000
Legs lean mass (kg)	20.2 ± 2.1	14.8 ± 1.6	0.000
VO_2max (mL min^{-1})	3246 ± 358	2360 ± 415	0.000
VO_2max (mL kg^{-1} min^{-1})	43.6 ± 3.8	38.6 ± 4.5	0.000
LLM VO_2max (mL kg^{-1} min^{-1})	161.3 ± 15.4	159.4 ± 18.1	0.71

LLM: lower extremities lean mass, also legs lean mass. N = 18 and 30 for women and men, respectively.

The inclusion criteria for participation were: age from 18 to 45 years old; without chronic diseases or recent surgery; non-smoker; normal resting electrocardiogram; body mass index below 30 and above 18; no history of disease requiring medical treatments lasting more than 15 days during the preceding 6 months; no medical contraindications to exercise testing and lack of food allergies. All volunteers applying met the inclusion criteria, except one girl having asthma, which was excluded. All subjects were physically active and exercised regularly.

Subjects were requested to avoid strenuous exercise 48 h preceding all laboratory tests and to refrain from caffeinated, carbonated and alcohol-containing beverages during the 24 h preceding the pre-tests and 48 h prior to the main experiment with supplementation. The subjects were asked to abstain from the consumption of drugs, medications, dietary supplements and the usage of any putative recovery treatments along the whole duration of the study.

A sample size between 20 and 28 participants was required to provide adequate power to detect an improvement between 5% and 6% in running and jumping performance ($\alpha = 0.05$, $\beta = 0.80$; G*Power v 3.1.9.2). Fifty-seven subjects were recruited to increase the power of our study and account for potential dropouts and missing values. The final sample was reduced to 48 subjects because nine volunteers could not finalize the running race, due to injuries or exhaustion.

2.2. General Overview

Subjects first reported to the laboratory for body composition assessment and familiarization with exercise testing. In subsequent days, the pre-tests were carried out to determine their maximal oxygen uptake (VO_2max) and vertical jump performance. At least one week after the pre-tests, they performed a 5 km running competition on a standard 400 m running track at the university stadium. Subjects competed in small groups matched by performance (5–10 subjects). At least two weeks after the 5 km run, the final competition (main experiment with supplementation) was carried out. All participants had monetary rewards depending on the performance achieved, resulting in a highly competitive environment. The competition consisted of a 10 km race, in the same 400 m track. Two days before the 10 km race, a resting 12 mL blood sample was obtained after a 12-h overnight fast. The 10 km running competition was followed by 100 drop jumps to elicit additional muscle damage. One hour before the 10 km race subjects were administered a placebo or Zynamite® combined with quercetin supplement, as explained below. Following the 10 km race, the volunteers ingested the supplementation dose assigned for three more times, every eight hours until the next day in the morning, when a second 12 mL blood sample was obtained in similar fasting conditions and their vertical jumping performance re-assessed. Five women on the nine assigned to the placebo group and three on the nine assigned to the polyphenol group were taking oral contraceptives.

2.3. Pre-Tests and Familiarization

Body composition was determined by dual-energy x-ray absorptiometry (Lunar iDXA, GE Healthcare, Wisconsin; USA) as described elsewhere [29]. Subjects attended two familiarization visits during which an incremental exercise to exhaustion and an all-out sprint were performed. After familiarization, subjects reported to the laboratory to complete different tests on separate days. First, their VO_2max, maximal heart rate (HRmax) and maximal power output (Wmax) were determined (F_IO_2: 0.21, P_IO_2: 143 mmHg) with an incremental exercise test to exhaustion with verification [30]. The incremental exercise test started with three min at 20 W, followed by 15 W and 20 W increases every three minutes in women and men, respectively, until the respiratory exchange ratio (RER) was ≥ 1.0. After completion of the intensity with a RER ≥ 1.0, the intensity was increased by 10 and 15 W min^{-1} (women and men, respectively) until volitional exhaustion. The intensity attained at exhaustion was taken at the maximal power output of the incremental exercise test (Wmax). At exhaustion, the ergometer was unloaded, and subjects remained seated on the cycle ergometer pedaling at low cadence (30–40 rpm) for 3 min. After that, the verification test started at Wmax + 5 W for 1 min, followed by increases of 4 and 5 W (women and men, respectively) every 20 s until exhaustion. During all pre-tests, subjects were requested to maintain a pedaling rate close to 80 rpm, while the cycle ergometer (Excalibur Sport 925900, Lode, Groningen, The Netherlands) was set in an rpm-independent mode.

2.4. Main Experiments and Supplement administration

The main competition consisted on a 10 km race run in the same standard 400 m track used for the 5 km run. Participants were randomly assigned to a placebo or polyphenols group. Both groups were matched by running times in the preceding 5 km race, and well as by sex. To elicit EIMD, subjects participated in a 10 km running competition on the athletic track at the university stadium. Race times were registered by an electronic race chip-timing system (Top Time Eventos, Las Palmas de Gran Canaria, Spain) with the runners' chest-wearing a small lightweight-chip and a number that uniquely identified them when crossing the timing mat at the finish line. Prior to the race, all subjects were instructed to perform their best and that financial incentives would be provided based on their individual performance and compared with other subjects with similar performance in the 5 km race. Verbal encouragement was provided throughout the competition. At the end of the race, capillary blood lactate concentration (earlobe) was assessed at 30–60 s after arrival, followed by a 30–minutes recovery period. During the race and the recovery period, exclusively plain water was provided to drink ad libitum. Then, they performed 100 drop jumps from a 59 cm step in height, consisting of 5 sets of 20 repetitions with a 10-s interval between jumps and interspaced by a 2-min recovery period between sets. Upon landing, the downward movement was stopped allowing the knees to bend up to ~90°, which was immediately followed by a maximal vertical jump and again at landing stopping at ~90° of knee flexion. Preceding the drop jumps, all subjects were demonstrated the required technique, and coaching and verbal encouragement were provided during the protocol to ensure that adequate technique and maximal effort were maintained. Two days before and 24 h after the competition, their counter-movement jump performance was assessed on a force platform. During the execution of the counter-movement jumps, the subjects were instructed to keep their hands on the hips and to try to minimize lateral and horizontal displacements.

To compete during the race, subjects were divided in two groups according to their performance levels. The first group reported to the running track at 7.30 A.M. and the second at 9.30 A.M, having ended their breakfast at least 1 h prior to the scheduled time. Participants were instructed to have a light pre-competition breakfast with their habitual composition consisting in 4–5 kcal kg^{-1} and all subjects thoroughly recorded their breakfast and the last dinner. All subjects were requested not to drink caffeine-containing beverages, taurine or alcohol the 48 h preceding the tests and the competitions. Only mild exercise lasting for no more than 30 min was permitted during the two days preceding the tests and competitions. One hour before the start of the 10 km race, subjects ingested the treatment assigned. The placebo was delivered in the form of two capsules, each containing

364 mg of maltodextrin. The polyphenols were also provided in two capsules each containing 70 mg of Zynamite® (*Mangifera indica* leaf extract, standardized to 60% mangiferin) combined with 70 mg of quercetin in the form of 140 mg of *Sophora japonica* extract standardized to 50% quercetin, and 153 mg of maltodextrin. Three additional doses were ingested every 8 h after the race at lunch, dinner time and in the next morning before the vertical jump test, to a total of 420 mg of Zynamite® combined with 420 mg of quercetin. Both treatments (polyphenols and placebo) were orally administered in opaque and non-distinguishable methylcellulose capsules ingested with 300 mL of water.

2.5. Assessment of Pain and Effectiveness of Concealment.

Before, immediately after the 10 km race and 24 h later, subjects were requested to rate the level of pain in their thighs with a visual numerical rating scale from 0 to 10, being 10 the highest muscle pain ever experienced during or after exercise. Participants were inquired while standing with hands on hips and during knee flexion while performing a 90° squat movement. At similar time points, subjects reported their rate of perceived exertion (10-point Borg scale) [31]. All subjects were fully accustomed to rate their level of leg pain and fatigue with the same rating scales due to previous participations in research studies in our laboratory. During the first minute after the race and 24-h later, subjects were asked about the kind of supplement they suspected they had received to check on the effectiveness of concealment.

2.6. Vertical Jump Performance

The forces generated during vertical jumps were measured with a force plate (Kistler, Winterthur, Switzerland), as reported elsewhere [32]. Counter-movement jump (CMJ) performance was tested pre and 24-h post-main experiments, as previously described [33]. Subjects were first familiarized with the correct technique. The execution began from an erect standing position with feet shoulder apart and, when prompted, rapidly descended to reach about 90° of knee flexion and jumped vertically with maximal force. They were aware that the jumps had to be executed explosively to achieve maximum height. During the execution, subjects were instructed to keep their hands on the hips, try to minimize lateral and horizontal displacements, keep their knees straight during the flight phase of the jump and to land in an upright position. On testing days, after a standardized warm-up, participants performed 5 maximal CMJ trials interspaced by 1-min passive rest periods. The vertical velocity at take-off (VT) was determined from the integration of the force applied during the positive phase of the vertical jump [34]. The average jumping height and mechanical impulse developed were used in the subsequent statistical analysis.

2.7. Blood Sampling and Assessment of Biomarkers of Muscle Damage

After a 12-h overnight fast, blood venous samples from an antecubital vein were obtained at baseline and 24-h post-exercise via phlebotomy by a medical doctor. From the total twelve milliliters of blood drawn, 6 mL were placed into serum vacutainer tubes (1 × 8.5 mL) with coagulation enhancer and splitting gel (Ref: 10061, Vacutest Kima, Piove di Sacco, Italy). After ~30 min at room temperature to allow for clotting, blood samples were centrifuged at 2000× g at 4 °C for 10 min. The serum supernatant was aspirated into a series of aliquots and immediately stored at −80 °C and thawed only once before analysis.

The serum concentration of myoglobin was measured with a chemiluminescent immunoassay (Access Myoglobin, Cat. No. 973243, Beckman Coulter, Brea, California, USA) exhibiting a sensitivity of < 0.1 ng mL^{-1}. The intra- and inter-assay coefficients of variation for this assessment were 1.9% and 3.0%, respectively. The serum high-sensitivity C-reactive protein (hs-CRP) concentration was determined spectrophotometrically using an enzyme-linked immunosorbent assay (ELISA) kit commercially available, which was used according to manufacturer´s instructions (catalogue No.: E-EL-H5134) from Elabscience (Houston, TX, USA) with a sensitivity of 9.38 pg mL^{-1} and an intra-assay coefficient of variation of 5.6%. The enzymatic creatine kinase (CK) activity in serum was measured

spectrophotometrically by an enzymatic rate method by assaying the rate of NADPH formation using the hexokinase and glucose-6-phosphate dehydrogenase procedure. The sensitivity was 10 U L^{-1} (153 nkat L^{-1}) and the intra- and inter-assay coefficients of variation were 1.2% and 3.1%, respectively.

The serum alanine aminotransferase (ALT) activity was determined spectrophotometrically by an enzymatic rate method, by assaying the rate of oxidation of NADH to NAD in the presence of pyruvate and lactate dehydrogenase. In this case, the sensitivity was 3.1 U L^{-1} (0.05 µkat L^{-1}), displaying intra- and inter-assay coefficients of variation of 1.1% and 2.4%, respectively.

2.8. Statistical Analysis

The values reported are means ± standard deviations. Variables were checked for normal distribution by using the Shapiro–Wilks test. Variables that were not normally distributed were logarithmically transformed. A repeated-measures ANOVA was used with one within-subjects factor: treatment (with two levels: placebo vs. polyphenols) and with sex as a between-subjects factor. The Mauchly's test of sphericity was run before the ANOVA, and in case of violation of the sphericity assumption, the degrees of freedom were adjusted according to the Huynh and Feldt test. In addition, the changes in percentage were also calculated and compared using a paired two-tailed *t*-test when the ANOVA interaction was statistically significant. Statistical significance was set at a $p \leq 0.05$. The statistical analyses were performed using IBM SPSS v.21.0 for Apple Computers (IBM, New York, NY, USA).

3. Results

3.1. Effects on 10 km Running Time, Blood Lactate, RPE and Leg Pain

Both groups had similar physical characteristics, with almost identical VO_2max and performance in the 5 km run (Tables 2 and 3). Polyphenol supplementation had no significant effects on running time in the 10 km race (3392 ± 561 and 3409 ± 479 s, for the placebo and polyphenol group, respectively, $p = 0.91$) (Table 4), even after accounting for the 5 km mark recorded before the competition ($p = 0.24$). The response was similar in men and women (treatment by sex interaction $p = 0.58$). Likewise, the ratio in time between the 10 km and 5 km marks was not altered by the intake of polyphenols (2.10 ± 0.12 and 2.07 ± 0.10, for the placebo and polyphenol group, respectively, $p = 0.40$) (Table 4).

Table 2. Physical characteristics, body composition and VO_2max by treatment group.

	Placebo			Polyphenols			*p*
Age (years)	23.9	±	3.2	22.5	±	2.2	0.08
Height (cm)	172.3	±	8.3	172.6	±	7.2	0.90
Weight (kg)	69.2	±	10.0	69.8	±	9.4	0.85
Body fat (%)	21.0	±	6.7	22.5	±	4.8	0.37
Fat body mass (kg)	14.3	±	4.5	15.6	±	3.7	0.29
Lean body mass (kg)	52.0	±	9.8	51.2	±	8.2	0.77
Legs lean mass (kg)	18.2	±	3.4	18.1	±	3.2	0.91
VO_2max (mL min^{-1})	2898	±	621	2928	±	539	0.86
VO_2max (mL kg^{-1} min^{-1})	41.6	±	4.6	41.9	±	4.9	0.83
LLM VO_2max (mL kg^{-1} min^{-1})	159	±	11	163	±	20	0.40
5-km run (s)	1611	±	212	1642	±	201	0.60

LLM: lower extremities lean mass, also legs lean mass. $N = 23$ and 25 for placebo and polyphenols group, respectively.

Table 3. Physical characteristics, body composition, VO_2max and 5 km running performance.

	Placebo						Polyphenols					
	Men (N = 14)			Women (N = 9)		p	Men (N = 16)			Women (N = 9)		p
Age (years)	23.9	±	2.5	24.0	± 4.3	0.91	22.4	±	2.2	22.7	± 2.4	0.76
Height (cm)	177.4	±	4.8	164.4	± 6.0	0.000	175.9	±	6.5	166.9	± 4.3	0.001
Weight (kg)	74.3	±	8.0	61.3	± 7.4	0.001	74.9	±	6.6	60.7	± 6.2	0.000
Body fat (%)	16.9	±	4.0	27.3	± 4.9	0.000	20.2	±	3.5 *	26.4	± 4.2	0.001
Fat body mass (kg)	12.7	±	3.8	16.9	± 4.4	0.02	15.3	±	3.6	16.2	± 4.2	0.55
Lean body mass (kg)	58.4	±	6.0	42.0	± 4.5	0.000	56.5	±	4.5	41.9	± 3.3	0.000
Legs lean mass (kg)	20.3	±	2.3	15.0	± 1.8	0.000	20.1	±	2.0	14.6	± 1.3	0.000
VO_2max (mL min^{-1})	3285	±	416	2297	± 334	0.000	3211	±	310	2424	± 495	0.000
VO_2max (mL kg^{-1} min^{-1})	44.2	±	3.5	37.4	± 2.4	0.00	43.0	±	4.1	39.8	± 5.8	0.12
LLM VO_2max (mL kg^{-1} min^{-1})	162	±	11	153	± 8	0.06	161	±	19	166	± 23	0.58
5-km run (s)	1509	±	144	1770	± 209	0.002	1558	±	159	1792	± 186	0.003

LLM: lower extremities lean mass, also legs lean mass. p-values for the comparison between men and women into each treatment group; * $p < 0.05$ compared with the placebo group.

Capillary blood lactate concentration at arrival was 36% higher in men than women (6.1 ± 2.6 and 4.5 ±1.4 mM, respectively, $p = 0.007$), without significant differences between treatment groups (Table 4). The RPE at arrival was 12.6% higher in women than men (8.7 ± 1.0 and 7.7 ± 1.3, respectively, $p = 0.01$), without significant differences between treatment groups (Table 4).

Table 4. Performance and pain after the 10 km race.

	Placebo							Polyphenols						
	Men ($N = 14$)			Women ($N = 9$)			p	Men ($N = 16$)			Women ($N = 9$)			p
10-km race (s)	3128	±	357	3802	±	590	0.003	3209	±	351	3763	±	487	0.003
Ratio 10km/5Km	2.07	±	0.12	2.14	±	0.10	0.18	2.06	±	0.10	2.10	±	0.10	0.43
Lactate (mM)	5.9	±	2.4	4.8	±	1.2	0.21	6.2	±	2.8	4.1	±	1.6	0.05
RPE Post 10 Km	7.9	±	1.2	8.7	±	1.1	0.11	7.6	±	1.5	8.6	±	0.9	0.06

Ratio 10 km/5 km: the ratio between the performance time in the 10 and 5 km-competitive races; Lactate: capillary blood lactate concentration at the 10 km race arrival; RPE: rate of perceived exertion at arrival. p-value for the comparison between men and women into each group. No significant differences were observed between the placebo and the polyphenols group.

The change in leg pain at a 90° angle of knee flexion elicited by the 10 km competition was lower after the ingestion of polyphenols (6.8 ± 1.5 and 5.7 ± 2.2, respectively, $p = 0.035$) (Figure 1).

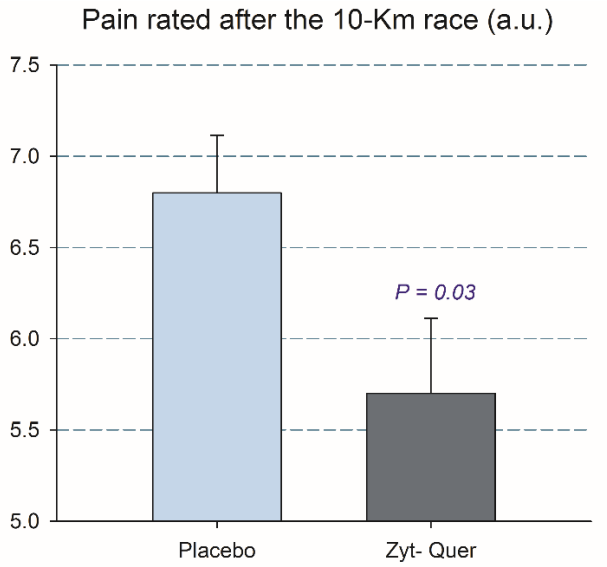

Figure 1. Pain reported at a 90° angle of knee flexion 30 s after arrival from the 10 km race. The error bars represent the standard error of the mean. Placebo, $N = 23$; polyphenols, $N = 25$.

3.2. Effects on Vertical Jump Performance

Twenty-four hours after the race and the drop jumps, CMJ performance was reduced by 6.9% ($p < 0.001$). The ingestion of polyphenols attenuated the loss of jumping performance (9.4 ± 11.5 and 3.9 ± 5.2%, respectively, $p = 0.036$; time by treatment interaction, $p = 0.034$) (Figure 2). This effect was explained by a lower deterioration of the positive vertical mechanical impulse after the ingestion of polyphenols (4.7 ± 5.6 and 1.9 ± 2.8 %, respectively, $p = 0.033$; time by treatment interaction, $p = 0.038$) (Figure 3).

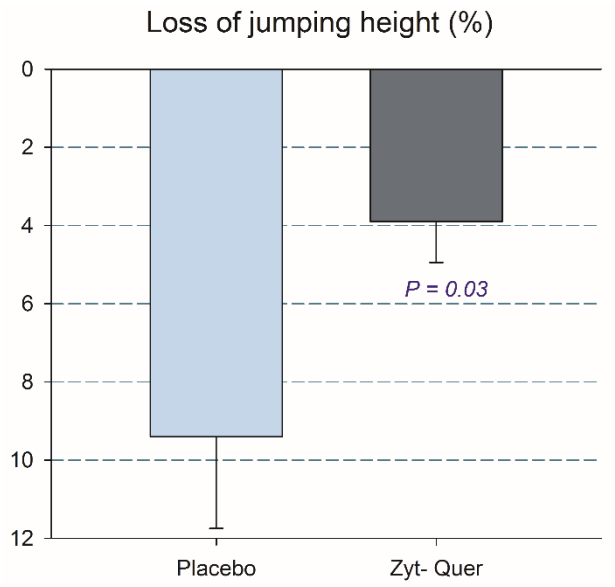

Figure 2. Jumping height loss 24 h after the 10 km race followed by 100 drop jumps. The error bars represent the standard error of the mean. Placebo, $N = 23$; polyphenols, $N = 25$.

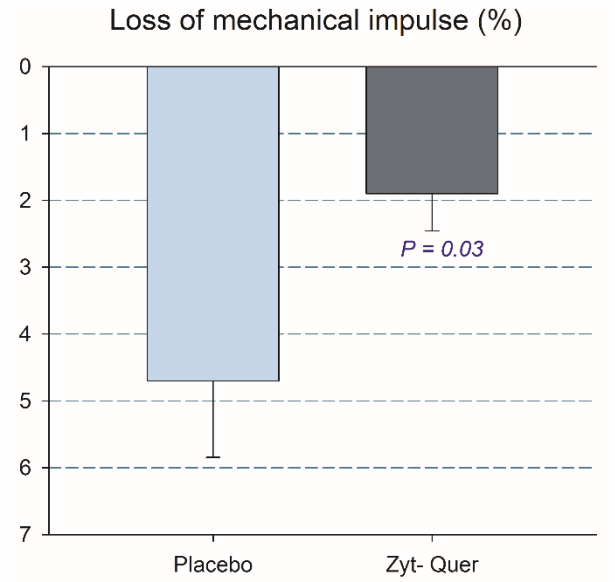

Figure 3. Mechanical impulse lost 24 h after a 10 km race followed by 100 drop jumps. The error bars represent the standard error of the mean. Placebo, $N = 23$; polyphenols, $N = 25$.

3.3. Biomarkers of Muscle Damage

Myoglobin serum concentration was increased 2-fold 24 h after the exercise protocol (from 24.4 ± 21.2 to 48.3 ± 42.9 ng mL^{-1}, $p < 0.001$). The ingestion of polyphenols attenuated the increase of myoglobin in men, but not in women (time by treatment by sex interaction; $p = 0.045$) (Figure 4A). The concentration of hs-CRP in serum was increased 8-fold 24 h after the exercise protocol (0.2 ± 0.2 to 1.9 ± 1.5 mg L^{-1}, $p < 0.001$), without a significant effect of polyphenol ingestion (time by treatment interaction, $p = 0.53$; time by treatment by sex interaction, $p = 0.19$) (Figure 4B). CK serum enzymatic activity was increased 3.5-fold 24 h after the exercise protocol (306 ± 623 to 1081 ± 1285 mg L^{-1}, $p < 0.001$), without a significant effect of polyphenol ingestion (time by treatment interaction, $p = 0.95$; time by treatment by sex interaction, $p = 0.26$) (Figure 4C). In men, supplementation with polyphenols attenuated the increase of ALT 24 h after the exercise protocol (from 19.3 ± 14.3 to 24.8 ± 15.7 and from

23.3 ± 13.6 to 24.7 ± 16.1 U L^{-1}, placebo and polyphenol treatment, respectively, $p < 0.001$; time by treatment interaction, $p = 0.01$) (Figure 4D).

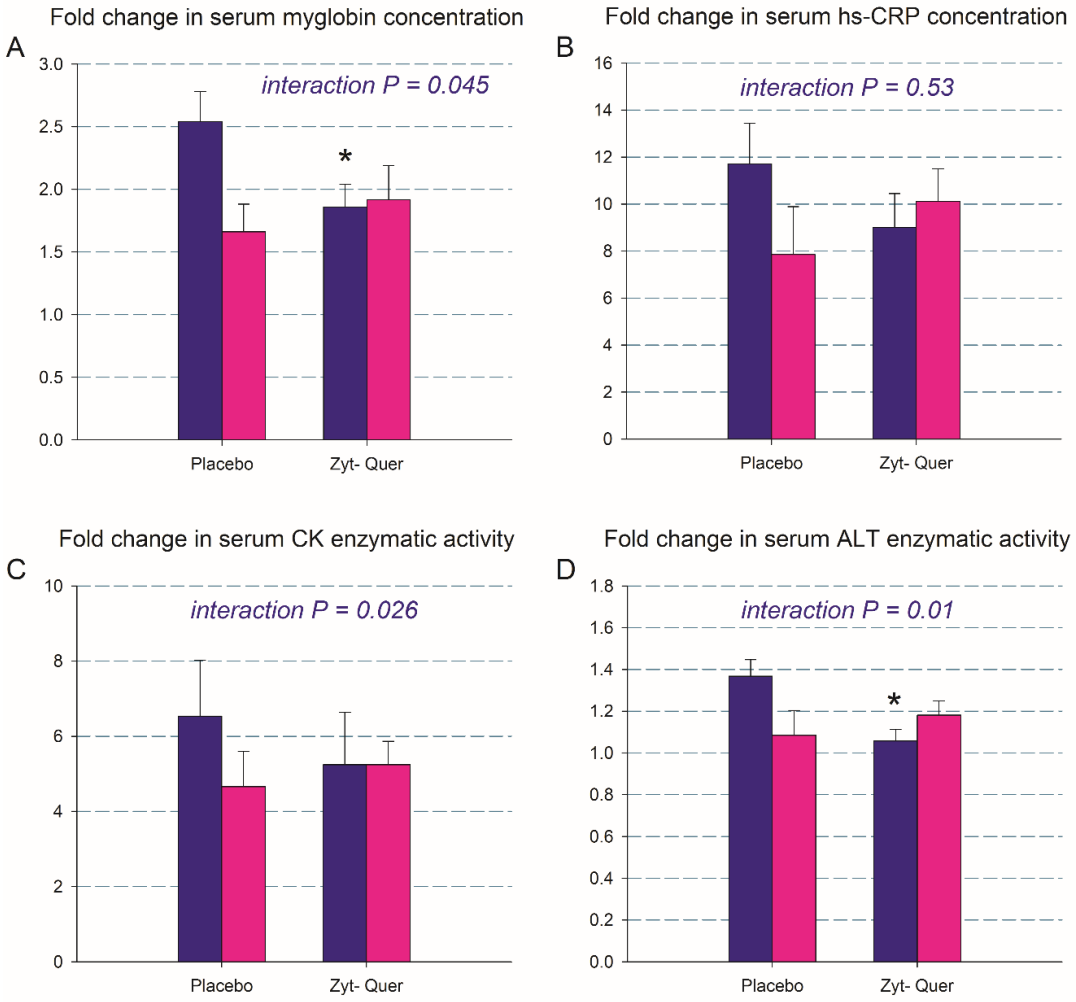

Figure 4. Changes in biomarkers of muscle damage 24 h after a 10 km running competition followed by 100 drop jumps. * $p < 0.05$ compared with placebo. Interaction refers to time by treatment by sex (men: purple; women: pink). The error bars represent the standard error of the mean. Placebo, $N = 22$; polyphenols, $N = 24$.

3.4. Efficiency of Concealment

Sixteen of the 23 subjects taking placebo correctly guessed they were on placebo, while only 7 of the 24 subjects on polyphenols guessed that they were taking polyphenols, at the end of the 10 km competition.

4. Discussion

This study has shown that a single dose of 140 mg Zynamite® combined with a similar amount of quercetin, taken one hour before exercise eliciting muscle damage, followed by three additional doses every 8 h (420 mg/24 h of each polyphenol during the recovery period), attenuates the pain and the muscle damage elicited by the race and accelerates the recovery of muscle performance. No significant differences in running times were observed between the placebo and the treatment group.

The protocol here applied induced muscle damage as shown by the degree of pain reported, and the increase of circulating biomarkers of muscle damage and inflammation 24 h after the exercise protocol (myoglobin, CRP, CK and ALT). The increase observed here in CK, CRP and myoglobin is similar to that reported after long distance running [2,35]. Consequently, jumping performance was reduced 24 h after the race and drop jumps, which is one of the best indicators of muscle damage and reduction of muscle functional capacity [36].

Exercise-induced muscle damage is initially caused by mechanical disruption of the ultrastructure of muscle [37], which affects several sarcomere proteins [38]. This causes one-half sarcomere nonuniformity and overstretching of sarcomeres beyond filament overlap, reducing the number of myosin-actin cross-bridges and hence, causing a reduction in the capacity to produce force and an overload of the sarcolemma and T-tubule structures [39]. This is followed by the opening of stretch-activated Ca^{2+} channels, membrane ruptures, and excitation-contraction coupling dysfunction. The increase of sarcoplasmic Ca^{2+} may stimulate calpain proteases with loss of contractile proteins prolonging the loss of force [40–42]. An intrinsic characteristic of EIMD is the induction of a protective effect when a similar exercise is repeated within the following days after the first damaging exercise (repeated bout effect) [43]. In the present investigation, we took care to measure well the performance levels of our subjects, although using a shorter distance to minimize the repeated bout effect. Despite a potential protective effect from the 5 km run, our protocol elicited EIMD likely due to the longest distance of the main competition, and the execution of 100 drop jumps 30 min after the 10 km race.

The muscle pain is likely triggered by inflammation of the extracellular matrix [36], by neurotrophic factors released by the muscle fibers and satellite cells, as well as by invading polymorphonuclear cells in the following days [39]. This causes a mild inflammatory response in which reactive oxygen species are involved [14,15]. Likely, several mechanisms act conjointly to elicit pain, but the nature may differ in the immediate post-exercise phase from the subacute phase (48–72 h after the exercise bout, which was not assessed here). Despite the hypothesized role of ROS in EIMD [14,15], the administration of N-acetylcysteine (a thiol-base antioxidant) may be counterproductive, as it has been associated with lower recovery of strength eight days after the exercise [16]. The protective effects of polyphenols after prolonged administration may depend more of the stimulation of the endogenous antioxidant systems through Nrf2 and antioxidant response element pathway signaling [44–46], rather than on a direct free radical-scavenging effect [2]. In the case of the combination of Zynamite® with quercetin, the antioxidant/anti-inflammatory effect due to the inhibition of the ROS-producing enzymes XO and NADP oxidase may explain the effect observed even though only a single dose was administered before exercise.

In the present study, the administration of Zynamite® combined with quercetin attenuated the muscle pain reported at the end of the race. In agreement, a trend to lower pain during post-exercise ischemia was observed for this polyphenolic combination, although given at larger doses [17]. This analgesic effect could have been mediated by the free radical-scavenging properties of Zynamite® and quercetin since free radicals have been implicated in nociception [47]. In addition, adenosine accumulation due to XO inhibition by Zynamite® and quercetin may have partly attenuated nociception reducing the pain felt by the subjects that received the polyphenol mixture, as suggested by animal experiments with allopurinol [48]. In agreement with our results, analgesic effects counteracting exercise-induced muscle pain have been reported in previous studies with polyphenol supplementation during the days preceding the exercise [6].

In the current investigation, we employed a realistic model of muscle damage, by using normal athletic activities of the lower extremities, which are more resistant to muscle damage than the upper extremities [49,50]. Therefore, not surprisingly, the loss of jumping performance was moderate, but similar to that reported following eccentric leg exercise on isokinetic dynamometers [50]. Interestingly, our polyphenolic combination was associated with faster recovery of jumping performance. In agreement with our findings, a more rapid recovery of muscle performance after eccentric exercise has also been reported for other polyphenol-rich extracts, in a dose-dependent effect [6].

Although EIMD is similar between men and women, the inflammatory response is more marked in men [51,52]. This sex dimorphism has been attributed to the anti-inflammatory and antioxidant properties of estrogens [53,54]. In the women studied here, polyphenols accelerated the recovery of performance and muscle pain, regardless of the intake of oral contraceptives. Five women in the placebo group were taking oral contraceptives, which may have contributed to attenuate the release of biomarkers of muscle damage in the placebo group [54], masking a potentially similar effect by the polyphenols.

This study has some limitations. The experimental design employed in the present investigation impedes to ascribe the results reported here to additive or synergistic effects of the polyphenol combination. Besides, the sample size might have been small to show significant effects of this polyphenol combination on biomarkers of muscle damage in the female group and, therefore, we cannot rule out a type II error. Although the diet was not standardized and the polyphenol content of the usual diet was not determined, the randomized adscription to each group should have minimized any potential bias due to differences in the diet.

5. Conclusions

In summary, a single dose of 140 mg Zynamite® combined with a similar amount of quercetin, taken one hour before exercise eliciting muscle damage, followed by three additional doses every 8 h (420 mg/24 h of each polyphenol during the recovery period), attenuates the pain felt after the exercise, reduces the muscle damage caused by the exercise, and accelerates the recovery of muscle performance. These effects are likely due to the antioxidant and anti-inflammatory properties of the combination of Zynamite® with quercetin. No similar results have been reported previously for such a low dose and short supplementation time when quercetin is ingested alone.

Author Contributions: Conceptualization: J.A.L.C. and J.C.W.; methodology: J.A.L.C., J.C.W., R.A.-O. and L.L.-R.; formal analysis: M.M.-R., M.G.-R., V.G.-A., A.G.-S., S.P.-R., A.S. and J.A.L.C.; investigation: M.M.-R., M.G.-R., V.G.-A., A.G.-S., M.M.-C., S.M.-R., S.P.-R., R.A.-O., C.D., D.M.-A. and J.A.L.C.; resources: J.A.L.C., J.C.W., R.A.-O. and L.L.-R.; data curation: J.A.L.C., M.M.-R., M.G.-R., and V.G.-A.; writing—original draft preparation: J.A.L.C., and M.M.-R.; writing—review and editing, all co-authors; validation, J.A.L.C., M.M.-R., M.G.-R., and A.S.; visualization, M.M.-R., M.G.-R. and J.A.L.C.; supervision, J.A.L.C., M.M.-R., and M.G.-R.; project administration: J.A.L.C., M.G.-R., and M.M.-R.; funding acquisition: J.A.L.C. and J.C.W. All authors have read and agreed to the published version of the manuscript.

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VIII

CONCLUSIONS

CONCLUSIONS

Study 1

Supplementation with the combination of mangiferin and luteolin enhanced exercise sprint performance, likely by improving brain oxygenation and allowing a higher muscle extraction of oxygen.

These effects were observed following 48h and 15 days of supplementation without significant differences between the two doses tested.

Study 2

The two supplements containing a MLE rich in mangiferin enhanced performance during high intensity exercise. In women, improved brain oxygenation at rest and during exercise, and increased peak VO_2 during high-intensity exercise. Moreover, both polyphenolic combinations tended to reduce the pain evoked by the occlusions.

The MLE with tigernut extract and quercetin supplement had a remarkable effect increasing peak power output after ischemia-reperfusion.

Study 3

A single dose of Zynamite® combined with quercetin administered one hour before exercise improves muscular performance and O_2 extraction. Moreover, Zynamite® combined with quercetin facilitates mitochondrial O_2 consumption during ischemia, improving sprints performed after ischemia.

Adding sunflower phospholipids to the Zynamite®-quercetin mixture had no additional beneficial effects.

Study 4

A single dose of 140 mg Zynamite® combined with 140 mg quercetin, taken one hour before exercise-induced muscle damage, followed by three additional doses every 8 hours after the exercise, reduced the muscle damage caused by the exercise and accelerated the recovery of muscle performance. Nevertheless, this polyphenol combination in a single dose pre-race did not improve running performance during a 10 km competition.

The administration of Zynamite® combined with quercetin attenuated the muscle pain reported at the end of the race.

IX

FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

Despite the rapid increase in prevalence of obesity, a favourable aspect to be considered is that it is a preventable disease. A multisector approach with the promotion of a healthier lifestyle in terms of nutrition and physical activity is needed. Specifically, the use of dietary regimen rich in anti-inflammatory nutrients, like the polyphenols included in the present thesis, besides to adding an exercise routine, could be considered as a promising preventive and therapeutic model against obesity.

Exercise actively helps to reduce obesity. However, given the potential effects of polyphenols intake for the same purpose, it is necessary to study how both natural polyphenols and exercise interact to achieve reductions in body weight and other health-related outcomes. It has been shown that specific polyphenolic combinations increase performance in sprint exercise, as well as improve recovery after very fatiguing exercises and intense protocols. However, how these polyphenols modulate the responses to strength-type of exercise remains unknown. Furthermore, there is some controversy regarding the chronic intake of antioxidant vitamins such as vitamin C during training [123, 140], which has been reported to blunt some of the expected adaptations in the supplemented groups. However, not all antioxidants are polyphenols and the effects of chronic ingestion of polyphenolic supplements during training remains largely unknown. Moreover, new studies should determine the absorption and bioavailability of these polyphenols, due to the fact that these molecules may undergo considerable degree of modifications during digestion and absorption and that the modified forms may have different biological properties and potencies.

Current knowledge from cell cultures and animal models suggests that polyphenols, including quercetin, curcumin, and resveratrol, play beneficial

effects in obesity, potentially by alleviating intracellular oxidative stress, reducing chronic low-grade inflammation, inhibiting adipogenesis and lipogenesis, and suppressing the differentiation of preadipocytes to mature adipocytes [207]. More studies are needed to better understand how these polyphenols act in obese patients, as well as to investigate whether mangiferin and luteolin would behave in a similar way, by the same or other mechanisms. In fact, a great number of xanthone compounds, from natural or synthetic origin have been studied, with promising inhibitory effects against one of the main targets of diabetes treatment, the enzyme α-glucosidase. The α-glucosidase plays an important role in carbohydrate metabolism and its inhibition help to maintain glucose homeostasis. Specifically, it has been shown that natural mangiferin (from different sources such as *Celastraceae, Asparagaceae, Thymelaeaceae*) poses inhibitory effects against α-glucosidase [208]. This opens a new line of research: testing whether mangiferin obtained from *Mangifera indica* L. (*Anacardiaceae*) exerts inhibitory effects against α-glucosidase. And, if so, to perform experiments with the polyphenolic formulations and exercise models already presented, in obese patients.

Browning of adipose tissue and thermogenesis are processes stimulated by endogenous factors such as thyroid hormones and catecholamines, or external factors such as cold exposure or dietary phytochemicals [209]. Although human studies to date are limited, dietary phytochemicals, specifically polyphenols, could induce browning and thermogenesis [209]. Thus, future research should determine which mechanisms let these compounds favour the stimulation of white adipose tissue cells browning, promoting at the same time thermogenesis. This may be used as complementary treatment for obesity and its comorbidities, where the polyphenol combinations used in the present thesis could be tested.

In skeletal muscle tissue, future work is needed to elucidate the molecular mechanisms by which polyphenols act, to examine whether mangiferin and

luteolin modulate RONS-induced signalling or how it prevents oxidative stress specifically in skeletal muscle. It is known that polyphenols (mainly curcumin, resveratrol and cocoa) health-related mechanisms rely on the modulation of mitochondrial biogenesis and the stimulation of enzymes or transcription factors involved in the cellular stress responses, as well as a nutritional deficiency (NRF2, PGC1α, forkhead box(FoxO3, AMPK, Sirt1), that regulate gene expression of key antioxidant proteins (SOD, Catalase, Glutathione system, etc.). Cell culture and animal studies have also shown that polyphenols modulate inflammatory processes (Nf-KB, COX, LOX, etc.) and the immune system response (Th1/Th2 balance) [210]. To date, it has been shown that mangiferin exerts protective effects on skeletal muscle structure and function in obese rats, preserving skeletal muscle mass as well as fibre type composition [211]. These effects were associated with the inhibition of inflammatory signalling pathways, but it is necessary to establish how these mechanistic links work, particularly in human models.

Another large area of future research is the relationship of these polyphenols with gut microbiota, their absorption and how these polyphenols may interact to affect muscle performance and weight loss. Up to 95% of ingested dietary polyphenols reaching the colon are transformed by the gut microbiota (GM) [212]. GM is in balance with the host under normal conditions, but this balance is affected in several diseases [213]. The pattern of the GM associated with obesity is not homogeneous among the population, in addition to exhibiting a lesser diversity and changes in some bacterial populations [214]. The impact of polyphenols on gut health and the modes of action could be through modulation of intestinal barrier function, innate and adaptive immune response, signalling pathways, as well as the ability to modify gut microbiota composition. An adequate and healthy composition of gut microflora can form a physical barrier against infections, whereas disturbance in the balance of gut ecology (dysbiosis) causes higher susceptibility to pathogens. A large body of literature has

discussed the linkage between dysbiosis and disease development, including obesity, inflammatory bowel disease (IBD) and cancer [215-217]. Therefore, a clear understanding of these mechanisms is required, including the interactions between polyphenols and gut microbiota. This would be crucial to gain insight on the implications on gut health [218].

Currently, there is shortage of data on the role of microbiota on polyphenol absorption, distribution, metabolism and elimination, and their impact on athletic performance. Moreover, in many cases, the application of nutritional genomics to sports performance is extrapolated from data of single genetic polymorphisms analysed in reference to other conditions, or in specific diseases, so it is necessary to confirm the relevance of these data in athletes.

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XII

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Por último, pero no menos importante, a la población de **Canarias** en general y, particularmente a la de Gran Canaria, por su acogida, por la belleza natural de su tierra, por los infinitos recovecos descubiertos y por la gente linda que en ella he conocido. Porque **Las Canteras y El Confital** han supuesto para mí un oasis de paz extraordinaria, un soplo de aire fresco y una renovación de energía diaria que me ha permitido crecer y, seguir con fuerza durante este período de 8 años. Indudablemente agradecida a esta isla por presentarme a la mejor persona que he conocido, a **Sergio Herrero**. Gracias por ser el impulso y equilibrio necesarios, por ser la felicidad personificada, por ayudarme a relativizar la vida como si de un niño se tratara, y por seguir disfrutando de nuestro presente y próximo futuro. Siempre equipo. No me olvido tampoco de mi familia adoptiva **Herrero-Menéndez** y sus **allegados**, que me han cuidado, como si de mi familia se tratara, en las innumerables ocasiones y circunstancias en las que he estado en Madrid.

Esta tesis doctoral va dedicada a todos vosotros.

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APPENDIX

CERTIFICATE OF
ACHIEVEMENT

EUROPEAN COLLEGE OF SPORT SCIENCE

2021 Young Investigators Award

The 3rd prize (€1000) in the conventional poster presentation is awarded to:

Miriam Gelabert Rebato

for your presentation at the

26th ECSS Virtual Congress entitled:

"Sex differences in metaboreflex activation and functional reserve during exercise to exhaustion revealed by post-exercise ischaemia and repeated supramaximal exercise"

Professor Flemming Dela
Chair - Scientific Board, ECSS

Professor Jørn Wulff Helge
President, ECSS

EUROPEAN COLLEGE of SPORT SCIENCE
ECSS

ECSS
Young Investigators Award

Gobierno de Canarias
Consejería de Economía, Conocimiento y Empleo
Agencia Canaria de Investigación, Innovación y Sociedad de la Información

Unión Europea

Modelo 18. CERTIFICADO DEL CENTRO RECEPTOR TRAS LA ESTANCIA BREVE/CERTIFICATE OF STAY IN A FOREING INSTITUTION

1. Solicitante/ Applicant:
Nombre y apellidos/ Name: Miriam Gelabert Rebato
D.N.I./ National identity Card: 40374823W
Centro de aplicación de la beca/ Home Institución: Universidad de Las Palmas de Gran Canaria. Instituto de Investigaciones Biomédicas y Sanitarias.
2. Centro en el que se ha realizado la estancia/ Host institution:
Nombre/ Name: 1. Copenhaguen University & 2.Institute of Sports Medicin (ISMC) Bispebjerg Hospital
Dirección/ Adress: 1. Nørregade 10, 1165 København, Dinamarca. 2. Bispebjerg Bakke 23, 2400 København, Dinamarca
Localidad/ Country: Copenhaguen, Denmark
3. Investigador responsable en el centro de la estancia/ Responsable person in the Host
Institución/ Institution: Institute of Sports Medicin (ISMC) Bispebjerg Hospital
Nombre/ Name: Michael Kjær
Cargo/ Post: Professor, Chief Physician
CERTIFICO: que el doctorando arriba mencionado ha realizado una estancia en este centro en las siguientes fechas: desde: 12 / 05 / 2021 hasta 12 / 09 / 2021 THIS IS TO CERTIFY: that the above mentioned person has performed a stay in this Institution in the following dates: From: 12 / 05 / 2021 To: 12 / 09 / 2021 Lugar y fecha / City and date: Copenhaguen, 12 / 09 / 2021
Firma y Sello/ Signature & Stamp MICHAEL KJAER INSTITUTE OF SPORTS MEDICINE COPENHAGEN Bispebjerg Hospital - Building 8, 1st floor Nielsine Nielsens Vej 11 DK-2400 Copenhagen NV Denmark

Canarias avanza con Europa

Fondo Social Europeo