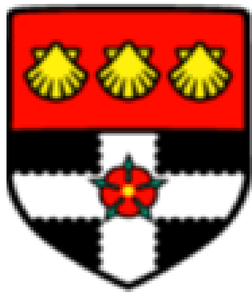

University of Reading

'An investigation of health benefits associated with consuming olive leaf extract as part of a healthy lifestyle'

A thesis submitted towards a Doctorate of Philosophy

Department of Food and Nutritional Sciences

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Sponsoring company

Declaration of original authorship:

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

………………………………..

Bandhita Saibandith

Abstract

A polyphenol rich extract derived from the leaves of olive trees (Olea europaea) may be used as a nutritional supplement. This olive leaf extract (OLE) has a high concentration of the secoiridoid, oleuropein and its derivative, hydroxytyrosol (HT). Recent randomised control trials demonstrate hypotensive and anti-hyperglycaemic activity for OLE in human volunteers; the mechanisms for these effects are still uncertain. The high consumption of olive polyphenols may, in part, explain the noted protection against chronic disease associated with the Mediterranean diet. The classical Mediterranean lifestyle as observed by Ancel Keys, involved not only a healthy diet, but also a high level of physically activity. Research investigating the synergistic benefits of including olive polyphenols as part of an active lifestyle is limited.

The aims of this thesis were; first, to explore plausible mechanisms that might explain previously observed anti-glycaemic and hypotensive responses to OLE in human volunteers; and second, to investigate the synergistic hypotensive effects of consuming olive leaf extract alongside increasing physical activity in individuals with slightly elevated blood pressure. Our hypotheses were that olive polyphenols would inhibit enzymes involved in glucose and fat digestion in the small intestine, and that these polyphenols inhibit glucose uptake by the gut epithelia resulting in improved blood sugar control. In relation to their anti-hypertensive effects we hypothesised that they would enhance NO production and suppress angiotensin enzymes. Finally, we hypothesised that there might be a synergistic effect of consuming the OLE and increasing physical activity in terms of reducing blood pressure.

OLE may influence the digestion of macronutrients. We show it to inhibit pancreatic α-amylase and lipase in a dose dependent manner; IC50 OLE; 3.23 ± 0.33 mM and 1.83 ± 0.03 mM, respectively. Further, we suggest that the rate of sugar absorption in the small intestine may be influenced by the presence of OLE, this is based on our observation of a reduction in the mRNA expression for genes encoding intestinal glucose transporters (SGLT1, GLUT2) in Caco-2 cells which was coupled to a reduction in glucose uptake measured in vitro.

To explain the anti-hypertensive effects of OLE, we first studied NO production in HUVEC cells. At concentrations of OLE equivalent to 100 μM oleuropein, NO production is enhanced compared to that observed in untreated controls ($p < 0.05$). However, we deem these concentrations super-physiological, and more work is needed to establish the potency of olive leaf phenolic metabolites on this cell system. The inhibitory effects of OLE and its principal polyphenols were assessed against renin and against the angiotensin converting enzyme (ACE). OLE inhibited renin and ACE activity with an IC50 value of 63.08 ± 2.90 μM and 60.86 ± 5.68 μM, respectively, again the concentrations required to reduce the activity of these enzymes in order to explain the reductions in blood pressure observed in intervention study are unlikely to be achieved in vivo.

Following the in vitro studies, we conducted a double-blind, four-arm parallel trial in 63 pre-hypertensive overweight adults aged 25-70y who were assigned to one of four treatment arms for 12 weeks: i) a placebo control group- with a daily capsule; ii) an increasing physical activity and a placebo capsule group; iii) an olive leaf extract arm (equivalent to 132 mg of oleuropein per day; iv) a physical activity and olive leaf extract arm. The primary outcome measure for the study was the change in 24-h ambulatory blood pressure (ABP). Secondary outcome measures were arterial stiffness index, plasma HbA1C levels, fasted glucose and insulin, serum lipid profiles and body composition. 24-h SBP (-5.80 (±SD 7.63), $p = 0.011$) and day time SBP (-5.07 (±SD 7.16), $p = 0.025$) were significantly lower following OLE intake. The PA intervention alone resulted in lower 24-h SBP (-3.69(±SD 9.39), $p = 0.031$) and daytime SBP (-4.19 (±SD 10.27), $p = 0.041$). Consuming the OLE alongside being increasing physical active reduced 24-h SBP (-3.88 (±SD 6.65), $p = 0.027$) relative to the baseline. The magnitude of SBP change observed in this thesis would suggest that regular OLE intake as part of healthy lifestyle may be associated with a 9-14 % reduction in CHD risk and a 20-22 % reduction in risk of stroke. There were however, no effects on stiffness index, body composition, lipids, HbA1c, glucose and insulin in all groups after 12 weeks ($p > 0.05$).

In this thesis, we provide mechanistic explanations for the findings of previous intervention studies. Understanding the biological mechanisms is a key component of the Bradford-Hill criteria when it comes to proving efficacy. Publications arising from this thesis may therefore provide further evidence for the assessment of health claims by

olive producers in the European market. In addition, we believe that following this thesis, there is now abundance of data proving that, olive phenolics when consumed chronically, at appropriate doses, reduce blood pressure in pre-hypertensive consumers. However, we still do not have a fundamental grasp of the mechanisms involved or an understanding of the relative potency of the individual phenolics in this crude extract. Nevertheless, the findings presented herein have commercial and public health significance. Consuming the OLE delivers the beneficial polyphenols at high doses and minus the need to ingest large amounts of energy dense olive oil. OLE consumption, in the absence of increasing physical activity, led to a more optimal blood pressure profile, however the benefits of increasing physical activity are wide and we would continue to recommend it to OLE consumers.

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I would like to thank Dr Kim Jackson for all the support and care that she has given me during my PhD years and who helped guide me in lab work, especially when times were looking very dark for me, you helped me get through it.

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List of abbreviations

°C	The degree Celsius
µCi	microcurie
ACE	Angiotensin-converting enzyme
ANOVA	Analysis of variance
BMI	Body mass index
BP	Blood pressure
Caco-2 cells	Human epithelial colorectal adenocarcinoma cells
CD	Chow Diet
CNIDs	Chronic, non-infectious diseases
cm^2	centimetre squared
CO_2	Carbon Dioxide
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DMEM	Dulbecco's modified Eagle's minimum essential medium
DMSO	Dimethyl sulfoxide
DNS	3, 5-dinitrosalicylic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediamine tetraacetic acid
EVOO	Extra virgin olive oil
FBS	Foetal Bovine Serum
FFA	Free fatty acid
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT2	Glucose transporter 2
HDL-C	High-density lipoprotein
HFD	High fat diet

Hr	Hour
HT	Hydroxytyrosol
HUVECs	Human umbilical vein endothelial cells
IC_{50}	Concentration estimated to give 50% inhibition
kg	kilogram
LDL-C	Low-density lipoprotein
MBq	Mega Becquerel
METs	Metabolic syndrome
mg	milligram
min	minute
mL	Millilitre
mM	mili Molar
nm	Nanometre
NO	Nitric oxide
OL	Oleuropein
OLD	OLE-supplemented diet
OLE	Olive leaf extract
PA	Physical activity
PBO	Placebo
PCR	Polymerase chain reaction
pNP laurate	p-nitrophenyl laurate
RAS	Renin-angiotensin system
rpm	Revolutions per minute
s	second
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of mean
SGLT1	Sodium dependent glucose transporter 1

U	Units
UK	United Kingdom
UV	Ultraviolet
v/v	volume to volume
w/v	weight per volume
WHO	World Health Organization
µL	microliter
µM	micro Molar

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Chapter 1 General background

1.1 The burden of chronic, non-infectious diseases

The four chronic, non-infectious diseases (CNIDs) of the greatest burden to public health are cardiovascular diseases, cancers, diabetes and chronic lung diseases [1]. CNIDs are seen as the leading causes of death and disease worldwide accounting for 38 million, or 68% of global deaths each year. Furthermore, CNIDs are projected to account for more than 75% of deaths worldwide by 2030 [2]. In the UK, CNIDs are estimated to account for 89% of total deaths. **Figure 1** shows causes of death in the UK; Cardiovascular diseases account for most CNIDs deaths (31%), followed by cancer (29%), respiratory disease (8%), and diabetes (1%) [3]. The total cost of CVD to the UK economy is estimated as £30 billion, 60% due to health-care costs [4], and diabetes costs the NHS around £10 billion each year [5].

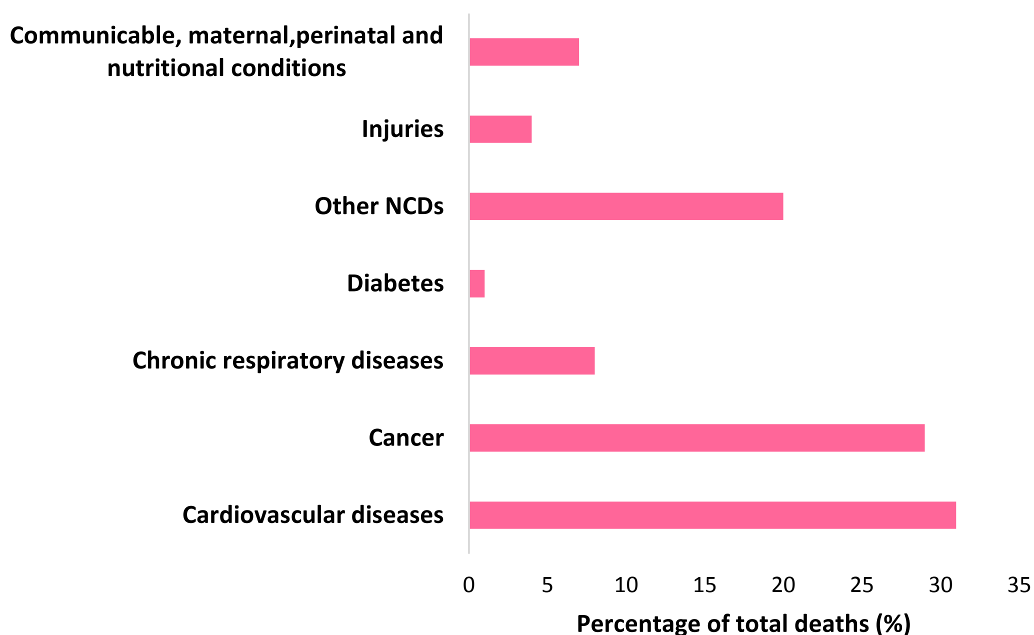

Figure 1 Proportional mortality (% of total deaths, all ages, both sexes) in UK [3].

1.2 Risk factors of chronic, non-infectious diseases

Intermediate biological risk factors in the pathogenesis of CNIDs include hypertension, hyperglycaemia, hyperlipidaemia, overweight and obesity, and these may be modifiable

and linked directly to lifestyle factors, including smoking, alcohol abuse, unhealthy diets and physical inactivity [6].

1.2.1 Metabolic risk factors

Metabolic risk factors include central obesity, dyslipidaemia (raised triglycerides and lowered high-density lipoprotein cholesterol), raised blood pressure, and raised fasting glucose [7]. People exhibiting a cluster of these abnormalities may be defined as satisfying the criteria for the metabolic syndrome (MetS). Diagnostic criteria for the metabolic syndrome have been establish by the International Diabetes Federation (IDF) in 2006 (**Table 1**). The IDF estimates that one-quarter of the world's adult population has the MetS [8]. The MetS confers a 5-fold increase in the risk of type 2 diabetes mellitus (T2DM) and 2-fold the risk of developing CVD over the next 5 to 10 years [9].

According to the IDF definition, for a person to be defined as having the metabolic syndrome they must have:
Central obesity (defined as waist circumference ≥ 94cm for Europe men and ≥ 80cm for Europe women, with ethnicity specific values for other groups)
plus, any two of the following four factors:

raised TG level	≥ 150 mg/dL (1.7 mmol/L), **or specific treatment for this lipid abnormality**
reduced HDL cholesterol	< 40 mg/dL (1.03 mmol/L) in males and < 50 mg/dL (1.29 mmol/L) in females, **or specific treatment for this lipid abnormality**
raised BP	systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg, **or treatment of previously diagnosed hypertension**
raised fasting plasma glucose	≥ 100 mg/dL (5.6 mmol/L), **or previously diagnosed type 2 diabetes**. If above 5.6 mmol/L or 100 mg/dL, OGTT is strongly recommended but is not necessary to define presence of the syndrome.

TG, triglycerides; HDL, High-density lipoproteins; BP, blood pressure

Table 1 The International Diabetes Federation (IDF) definition of the metabolic syndrome [8].

Obesity is a condition in which abnormal or excessive fat accumulation in adipose tissue impairs health. It increases the risk of all-cause mortality by approximately 50% [10]. In the UK, 62.9% of adults are overweight or obese [11]. An increased risk of CNIDs is associated with obesity. **Table 2** shows the classification developed by a National Heart, Lung, and Blood Institute task force, along with the associated disease risk with increasing BMI. A 10-kg higher body weight is associated with a 3.0 mm Hg higher systolic and a 2.3 mmHg higher diastolic blood pressure[12]. For an 8- to 10.9-kg increase in body weight, the relative risk of diabetes was 2.7 among women [13]. The global increase in the prevalence of obesity is projected to add 6–8.5 million incidence cases of type 2 diabetes and 5.7–7.3 million cases of CVD, in the UK the projected cost to the economy is £1.9–2 billion per year by 2030 [14].

	BMI (kg/m^2)	*Disease Risk Relative to Normal Weight and Waist Circumference*	
		Men ≤ 102 cm; Women ≤ 88 cm	Men ≥ 102 cm; Women ≥ 88 cm
Underweight	<18.5	***	***
Normal	18.5–24.9	***	***
Overweight	25.0–29.9	Increased	High
Obesity, class			
I	30.0–34.9	High	Very high
II	35.0–39.9	Very high	Very high
III (extreme obesity)	≥40	Extremely high	Extremely high

Table 2 Classification of overweight and obesity by BMI, waist circumference and associated disease risk for type 2 diabetes, hypertension, and CVD [15].

Blood pressure is defined as the pressure within the arteries as the heart forces blood to circulate around the body. In clinical practice, blood pressure is measured and expressed

as a single value of the systolic (SBP) and diastolic pressures (DBP). SBP is the maximum blood pressure during contraction of the ventricles; DBP is the minimum pressure recorded just prior to the next contraction [16]. **Table 3** provides a classification of BP for adults aged 18 years and older in reference to the Seventh Joint National Committee (JNC 7), with hypertension, defined as a persistent raised blood pressure of 140/90 mmHg. Major pathophysiologic mechanisms of hypertension include activation of the sympathetic nervous system and renin–angiotensin–aldosterone system [17]. Changing patterns of BP occur with increasing age [18]. Nearly 30 per cent of adults in the UK have high blood pressure [19]. Public Health England (PHE) reveal that diseases caused by high blood pressure are estimated to cost the NHS over £2 billion every year. In addition, prolonged exposure to elevated blood pressure is associated with an increased risk for cardiovascular mortality (e.g. stroke, heart failure), and renal failure events. Longitudinal data from the Framingham Heart Study have specified that BP values of 130–139/85–89 mmHg (prehypertension) are associated with a more than two-fold increase in relative risk of CVD as compared with those with normal BP levels [20]. Moreover, NICE hypertension guidelines indicate that BP control can be achieved by both pharmacological and lifestyle interventions. Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers are useful antihypertensive treatments. A blood pressure reduction of 10 mmHg systolic or 5 mmHg diastolic can reduce the risk of CHD events by 22% the incidence of stroke by 41% [21].

BP classification	*SBP mmHg*	*DBP mmHg*
Normal	<120	and <80
Prehypertension	120–139	or 80–89
Stage 1 Hypertension	140–159	or 90–99
Stage 2 Hypertension	≥160	or ≥100

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure

Table 3 Classification of blood pressure for adults [17].

The presence of both the metabolic syndrome and impaired fasting glucose was associated with approximately a 9-fold (7.47–10.45) increased risk of new onset diabetes [22]. Besides, a cohort study of nondiabetic individuals, showed that insulin resistance is related with a 2.8-fold increased risk of first ischemic stroke [23]. In addition, impairment in glucose metabolism leads to physiological imbalance with the onset of the hyperglycaemia. Epidemiological studies show that postprandial glucose, together with related hyperinsulinemia and lipidaemia is implicated in the aetiology of chronic metabolic diseases such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) [24]. Therefore, maintenance of glucose homeostasis is of utmost importance to human physiology. **Figure 2** gives an overview of the putative relationships between postprandial glycaemia and CNIDs.

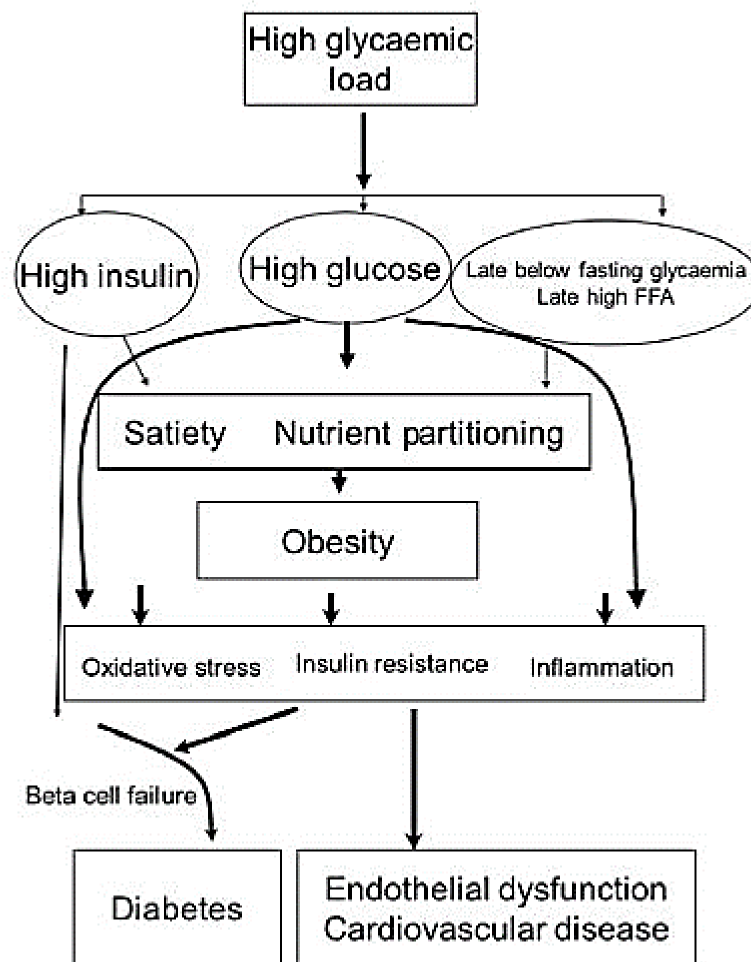

Figure 2 Putative relationships between postprandial glycaemia and risk factors for obesity, diabetes and CVD [25].

Dyslipidaemia is characterised by an elevation or attenuation of the plasma concentration of lipoproteins including triglycerides, chylomicron remnants, and of free fatty acids. The subsequent increased transport of FFAs into non-adipose tissue, such as liver and skeletal muscle, contributes to the formation of reactive lipid moieties such as diacylglycerol and fatty acyl-CoA accumulation, resulting in cellular dysfunction or cell death, a phenomenon known as lipotoxicity, as major mediators of insulin resistance, diabetes and cardiovascular disease [26, 27]. Inhibiting pancreatic lipase is an interesting approach to reducing fat absorption by the intestine in man and suppressing cholesterol, triglyceride and FFA level in the plasma [28]. The results of a meta-analysis of 10 large cohort studies showed that for each 0.6 mmol/L reduction in serum cholesterol levels in the risk of CHD decreased by 27 percent [29].

1.2.2 Lifestyle risk factors

Unhealthy diets, physical inactivity, smoking and the harmful use of alcohol, increase the risk of dying from CNIDs [30, 31]. The WHO estimates that approximately 1.7 million (2.8%) deaths worldwide are attributable to low fruit and vegetable consumption, and that this accounts for about 20% of CVD worldwide. Only a quarter of UK adults and one in five children consume the recommended minimum five portions of fruit and vegetables per day. About 3.2 million deaths annually can be attributed to insufficient physical activity. People who are insufficiently physically active have a 20–30% increased risk of all-cause mortality compared to those who adhere to the physical activity guidelines. Nearly two out of five adults in the UK do not achieve recommended levels of physical activity. Smoking, including exposure to second hand smoke, is responsible for 6.3% of the global burden of disease [32]. Exposure to tobacco smoke is associated with a 4-fold increased risk of development of metabolic syndrome that most likely contribute to smoking-induced CVD [33]. In 2015, there were 8,758 deaths which were related to the consumption of alcohol in the UK [34]. A prospective cohort study has reported an association between alcohol drinking and the metabolic syndrome [35].

1.3 Prevention of chronic, non-infectious diseases

Lifestyle modification regarding a healthy diet and physical activity is the preferred strategy for preventing chronic disease development. Evidence suggests that combining a healthy diet, and increasing physical activity level will have synergistic benefits to health.

1.3.1 Dietary polyphenols and the prevention of CNIDs

Increased consumption of plant-based foods such as fruit and vegetables, and plant-derived beverages has been recommended as a key component of a healthy diet for the prevention of CNIDs [36]. A meta-analysis of prospective cohort studies concluded that a higher consumption of plant-based foods is associated with a lower risk of all-cause mortality, particularly cardiovascular mortality [37]. For example, adequate intake was estimated to lead to a 20% to 30% reduction in risk of CVD [38]. and a 2-fold reduction in cancer risk [37]. These benefits may be, in part, attributed to the presence of natural antioxidants in these foods, such as ascorbic acid, tocopherols, carotenoids and phenolic compounds (polyphenols) [39]. Interestingly, among these compounds, polyphenols have demonstrated highest *in vitro* antioxidant capacity [40].

Polyphenols are secondary metabolites of plants that generally involved in defence against ultraviolet radiation or aggression by pathogens of plants [41]. They have been classified by their source of origin, biological function, and chemical structure. With over 8,000 structural variants, they denote many substances with aromatic ring(s) bearing one or more hydroxyl moieties. They are broadly categorized as the phenolic acids, the flavonoids, the stilbenes, the coumarins, and the tannins. Flavonoids are the most common and widely distributed group of plant phenolic compounds. Fruit and beverages such as berries, apples, grape, dark chocolate, olive products, tea, and red wine constitute the main sources of polyphenols [42]. The amount and type of polyphenols consumed is largely dependent on the dietary patterns of individuals and populations. It has previously been estimated that total polyphenol intake in western populations is approximately 1 g/day [43].

Polyphenols have been shown to have potent antioxidant/anti-inflammatory activities in *in vitro* and *in vivo* studies, suggesting that they could contribute to the health protective properties of plant foods [44]. Epidemiological evidence supports beneficial effects of polyphenol-rich diets on glucose homeostasis. Polyphenols may attenuate postprandial

glycaemic through different mechanisms, including the inhibition of carbohydrate digestion, glucose absorption in the gut or of its uptake by peripheral tissue [45]. In addition, observational studies have indicated an inverse association between the consumption of polyphenol-rich foods e.g. red wine, grape juice, tea, dark chocolate and olive products and the prevalence of hypertension [41]. The antihypertensive effect of polyphenol-rich foods for protection and improving endothelial function with vascular relaxation may occur via increasing NO bioavailability and inhibiting renin angiotensin system enzymes [46]. The polyphenol-rich extracts from foods such as apple, berries, red wine and green tea may also inhibit the lipase enzyme resulting in lowering lipid profiles and improving weight control [47].

The olive tree is valuable source of bioactive phenolics [48]. Olive oil, is believed to be a crucial constituent underpinning the health benefits of the Mediterranean diet; for example, supplementation with extra virgin olive oil improves modifiable cardiovascular risk factors in hypertensive volunteers [49]. Furthermore, there is evidence that a waste product of olive oil production, the olive leaf has a high phenolic content, 30-40 times greater than may be found in extra virgin olive oil [50]. Olive leaves (*Olea europaea* L.) have been used as folk remedy for centuries in Mediterranean and Middle Eastern countries. The olive leaf extract (OLE) has long been recognized as containing antioxidants, such as oleuropein, and hydroxytyrosol. Oleuropein is the most abundant phenolic in olive leaves, and has been used in a number of modern therapies [51], for instance, it may increase lipid metabolism in the obese, protect against oxidation of phospholipids in the arterial wall, an early event in cardiovascular disease [52], and regulate enzymes in cancer patients [53]. Other bioactive compounds in OLE also such as hydroxytyrosol, luteolin and rutin also demonstrate antioxidant activity; hence, olive leaf extract has potent anti-oxidant capacity due to the synergy of all the active phenolic compounds. Consuming olive leaf extract reduces some markers of chronic disease risk (**Table 4**); however, the mechanisms for these effects is still not well recognized. In intervention studies, olive leaf extract has been shown to reduce risk factors for CVD [54, 55], and has both anti-inflammatory and anti-cancer activities [50, 56]. Further studies are needed to collect data examining the effects of olive leaf extracts on health benefits in humans.

Table 4 Beneficial health effects of olive leaf extract on human studies

Target group	*Intervention*	*Duration*	*Result*	*References*
Healthy young adult male and female subjects (aged 18-25 y, n = 45)	Single-center, randomized, single-blinded, prospective pilot one capsule or 5-mL OLE liquid or placebo consisted of a 1:9.5:15 mix of vinegar, water, and glycerol three times per day	28 days	Not alter oxidative status compared with the control group.	[57]
The stage-1 hypertension patients (aged, 43-58 y,n=232)	Randomized, double blind, active-controlled clinical trial 4-week; single-blind placebo 8-week; double-blind treatment with: active control drug (Captopril) 12.5 mg or Olive leaf extract. 500 mg extract, twice daily	12 weeks	↓ SBP (11.5 mm Hg), DBP(4.8 mm Hg), LDL (3.9 mg/dL) and TAG (11.9 mg/dL) in OLE	[55]
Type II diabetes patients (adult, n=79)	Randomized, double-blind, placebo- controlled, clinical trial a tablet of olive leaf extract (500 mg) or matching placebo in a 1:1	14 weeks	↓ HbA1c (0.9%), fasting insulin levels (2.4 IU)	[58]
Consecutive cancer patients (aged 20.5± 9 n=25)	Placebo-controlled, randomized, double-blind, and cross-over randomly assigned to receive a mouth wash containing OLE (333 mg/ml), benzydamine hydrochloride (0.15 g/100ml), or placebo (normal saline) 3-4 time daily	14 days	↓TNF-α (49.6 pg/ml), IL-1b (96.5pg/ml)	[59]

Overweight Men (aged 46.4±65.5, n=46)	Randomized, double-blinded, placebo-controlled, crossover trial 12 weeks extract (OLE; 51.1 mg oleuropein, 9.7 mg hydroxytyrosol per day)or placebo, crossing over to other treatment after a 6-week washout	12 weeks	↑ improvement in insulin sensitivity (15%), pancreatic β-cell (28%), Interleukin-6 (0.18pg/ml), IGFBP-1 (0.26ng/ml), IGFBP-2 (14ng/ml)	[60]
Overweight Men (aged 18-65 y, n= 60)	Randomized, double-blinded, placebo-controlled, crossover trial 12 weeks extract (OLE; 51.1 mg oleuropein, 9.7 mg hydroxytyrosol per day) or placebo, crossing over to other treatment after a 6-week washout	12 weeks	↓ 24hr SBP(3.33 mmHg) ,Day SBP(3.95 mm Hg),24hr DBP (2.42 mm Hg),Day DBP (2.48 mm Hg),FTM 24hr SBP(4.76 mmHg),TAG(0.18m mol/L), TC(5.6mmol/L),LDL(0.20 mmol/L) ↑ HDL (0.05 mmol/L)	[61]

In addition, advocating the consumption of a supplement for health benefits is a sub-optimal public health approach as supplement usage can be used to justify an otherwise unhealthy lifestyle such as physically inactive. Accordingly, evidencing the synergistic benefits of including a supplement as part of a healthy lifestyle is desirable. However, no data are yet available to investigate into health benefits associated with consuming OLE as part of a healthy lifestyle.

1.3.2 Physical activity and disease prevention

Increasing physical activity (PA) has the potential reduce all-cause mortality and improve life expectancy. Prospective cohort studies showed that a higher level of total physical activity is strongly associated with a lower risk of breast cancer, colon cancer, diabetes, ischemic heart disease, and ischemic stroke [62]. A classic study of CVD risk in London's double-decker bus conductors and drivers demonstrated that the more active conductors were less likely to suffer from CVD than the inactive drivers [63]. In addition, a large prospective study reported that moderate physical 12, physical fitness is a good predictor of death amongst those with chronic disease. **Figure 3** adapted from Myers et al. shows that subjects with a history of hypertension, or chronic obstructive pulmonary disease (COPD), or diabetes, or who smoking, or who had an elevated body mass index (BMI ≥ 30) or a high total cholesterol level (TC ≥ 5.70 mmol/L), could mitigate the relative risk of death from any cause with increasing exercise capacity [64].

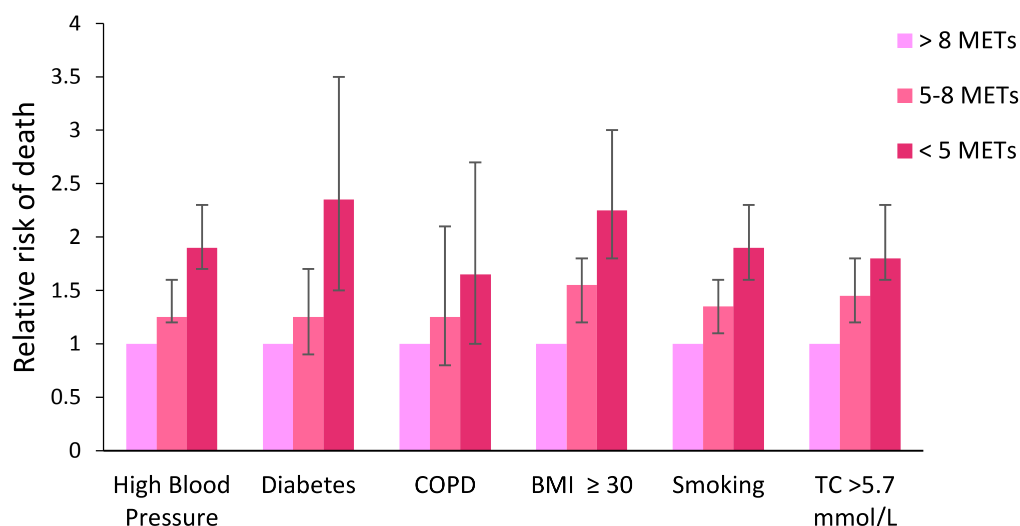

Figure 3 Relative risks of death from any cause among participants with various risk factors and exercise capacity (Less than 5 MET, 5 to 8 MET or more than 8 MET. Adapted from [64].

In 2011, the Chief Medical Officers of the UK revised the guidelines for physical activity to state that at least 150 minutes of moderate-intensity PA per week provides some health benefits for adults [65]. In a meta-analysis of 54 randomized controlled trials (2419 participants) with intervention and control groups which differed only in aerobic exercise showed that exercise was associated with a significant reduction in blood pressure of 3.8/2.6 mmHg [66]. Despite the widely reported benefits of PA, adults and children do not get enough PA [67].

1.4 Olive polyphenols and the metabolic syndrome: A systematic research review of dietary intervention trials

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Abbreviations: EVOO, Extra virgin olive oil**; HFD**, High fat diet**; HT**, Hydroxytyrosol**; MetS**, Metabolic syndrome; **OL**, Oleuropein**; OLE**, Olive leaf extract; **TC**, Total cholesterol

Keywords: Central obesity /Dyslipidaemia /Hyperglycaemia /Hypertension /Metabolic syndrome /Olive polyphenols

Abstract

Here, the effects of consuming polyphenol-rich olive products, including olive leaf and its crude extract, and extra virgin olive oil, on aspects of the metabolic syndrome are reviewed. We have sought to summarize the available scientific evidence from dietary intervention trials demonstrating a role for these phytochemicals in ameliorating aberrant glucose metabolism, high blood pressure and elevated blood lipids, and we discuss potential mechanisms underpinning these observations. Searches for relevant literature published in English were conducted via PubMed and Science Direct. Based on this literature there is convincing evidence to show that olive polyphenols, independently of olive lipids, reduce risk factors for metabolic syndrome, in particular improving blood sugar and blood pressure control. There is more limited evidence to suggest that the consumption of olive polyphenols or related products can reduce body weight, visceral fat or impede weight gain, and similarly there are some limited data suggesting improved lipid profiles. The consumption of olive polyphenols within the context of a healthy pattern of food intake may, in part, explain the reduced risk of metabolic disease associated with adherence to the Mediterranean diet.

1.4.1 Introduction

The metabolic syndrome (MetS) is characterised by a cluster of interrelated markers of ill health, including obesity, hyperglycaemia, dyslipidaemia and hypertension [7]; up to 25 percent of the world's adult population may satisfy the criteria for this syndrome [68]. Individuals with the MetS carry a five-fold greater risk of developing type 2 diabetes [69] and a two-fold increase in the risk of developing subsequent cardiovascular disease [24]. It is generally accepted that the MetS is related to modifiable behavioural risk factors, including unhealthy patterns of food consumption, physical inactivity, smoking, and alcohol abuse [30, 31].

Dietary guidelines consistently emphasise the importance of placing plant-based foods at the core of a healthy pattern of food consumption [70]. This predominance of plant foods is a defining feature of the Mediterranean dietary pattern, which is also characterised by a high consumption of olive products. Adherence to the Mediterranean diet is associated with a reduced risk of MetS [71]. There may be specific benefits arising from olive polyphenols within the context of the Mediterranean diet [50, 72-75]. The most well studied bioactive small molecules present in olive products are the catecholic compounds, oleuropein (OL) and hydroxytyrosol (HT). Both OL and HT have established bioactivity, with demonstrable experimental antioxidant [76], anti-inflammatory [77], and antimicrobial properties [78]. These compounds are thus credited with some of the health benefits observed in those consuming high quantities of olive foods [79, 80]. The current review will present the evidence illustrating the relationship between the chronic supplementation of polyphenol-rich olive products and MetS. It will also discuss the potential mechanisms linking olive polyphenol consumption to improvements in markers of health.

1.4.2 Phenolic content of olive products

The olive tree is a valuable source of polyphenols [48]; these are present in the plant as secondary metabolites to combat pests, bacterial infection and other stresses [41]. Olive oil is a product of the mechanical extraction of the olive fruit; compositionally it is an oleic acid (MUFA) rich product with a lesser (0.4-5%) non-triglyceride component [81]. Olive polyphenols are found in both the lipid and the water fractions of olive oil [82]. The total phenol content in olive oil varies between oils, according to crop, and harvest conditions

and according to processing, storage and preservation methods [83, 84]. In refined oils, phenolics may be present at concentrations of approximately 62±12 mg/kg, whereas in high quality extra virgin olive oils (EVOO) this concentration may be much higher [72]. The olive leaf has even higher concentrations of total phenolic compounds relative to the olive fruit and olive oil: 1350 mg/kg fresh leaf [85] vs. 232 ± 15 mg/kg of EVOO [82, 86]. A number of olive leaf extracts (OLE) are available to consumers and are marketed as food supplements or nutraceuticals containing concentrated amounts of phenolics (range 1057– 4831 mg/kg dry extract) [87]. The possibility of using phenolics extracted from olive leaves to enrich edible oils is also being explored [88, 89]. OL is the most abundant phenolic compound present in olive leaf, seed, pulp and peel of unripe olives [48, 51]. During fruit maturation, OL undergoes hydrolysis, yielding different products, including (2-(3, 4-dihydroxyphenyl) ethanol) (HT), which is the major phenolic compound in EVOO (14.42 mg/kg) [85, 90]. Other phenolics, namely rutin, caffeic acid, verbascoside and the flavone-7- glucosides of luteolin and apigenin, are also present in olive products, but in lower concentrations [50, 91]. The radical scavenging activity of some of these minor phenolics may also be very high [81], and it is feasible that the combination of phenolics, when consumed in a food or a crude extract, has a greater influence on the health of the consumer than would a purified individual compound.

1.4.3 Effect of olive polyphenols on the main features of metabolic syndrome

Manuscripts evaluated for the purposes of this review were identified using the following key search words: olive phenolics, olive leaf phenolics, olive oil phenolics, HT, OL. Abstracts were screened and papers were selected for this review where there was a dietary intervention involving the delivery of olive polyphenols, and where the endpoints were related to the metabolic syndrome, i.e. obesity, hyperglycaemia, hypertension, lipid peroxidation, hyperlipidaemia cardiovascular disease, diabetes mellitus. A summary of the identified studies is provided in **Table 5**.

Table 5 Summary of key human studies showing effectiveness of OLE and EVOO on MetS and associated risk factors

Source	***Intervention***	***Participants***	***Dose***	***Main finding***				***References***
				Central obesity	***Hyperglycaemia***	***Hypertension***	***Hyperlipidaemia***	
Olive leaf extract	*A randomised, double-blind, placebo-controlled, cross-over, acute intervention trial*	*18 male and female (healthy volunteers)*	*1600 mg OLE (51.12 mg OL and 9.67 mg HT)*	*na*	*na*	*↓DVP-SI*	*↓IL-8*	*[92]*
Olive leaf extract	*8-week, an open, controlled, parallel-group, co-twin study*	*40 monozygotic twins (pre-hypertensive subjects)*	*500 mg OLE/day (104 mg OL/day) or 1000 mg OLE/day (208 mg OL/day) (control group receiving no medication)*	*ns*	*ns*	*↓SBP ↓DBP*	*↓LDL*	*[93]*
Olive leaf extract	*28 days, a longitudinal, controlled, randomized, and double-blind intervention study*	*39 male and female (hyperlipidaemia subjects)*	*1,200 mg OLE/day (control group receiving placebo)*	*na*	*na*	*na*	*↓TC ↓LDL ↓oxLDL ↓TC/HDL ratio ↓TAG*	*[94]*
Olive leaf extract	*8-week, A double-blind, randomized, parallel and active-controlled clinical study*	*148 male and female (stage-1 hypertensive subjects)*	*1000 mg OLE/day (control group receiving Captopril 12.5 mg)*	*na*	*na*	*↓SBP ↓DBP*	*↓TC ↓LDL ↓TAG*	*[55]*

Source	*Intervention*	*Participants*	*Dose*	*Main finding*				*References*
				Central obesity	***Hyperglycaemia***	***Hypertension***	***Hyperlipidaemia***	
Olive leaf extract	*14-week, A randomized, double-blind, placebo controlled, clinical trial*	*79 male and female (Type II diabetic subjects)*	*500 mg OLE/day (control group receiving placebo)*	*ns*	*↓HbA1C*	*ns*	*ns*	*[58]*
Olive leaf extract	*30-week, A randomized, double-blinded, placebo controlled, crossover trial*	*46 male (overweight subjects)*	*OLE/day (51.1 mg OL and 9.7 mg HT)*	*ns*	*↑insulin sensitivity*	*ns*	*ns*	*[91]*
Olive leaf extract	*16-week, A randomized, double-blinded, placebo controlled, crossover trial*	*61 male (overweight subjects)*	*20 ml OLE/day (136.2 mg OL/day)*	*ns*	*ns*	*↓24h SBP ↓24h DBP ↓Daytime SBP ↓Daytime DBP*	*↓TC ↓LDL ↓TAG*	*[61]*
Olive oil (unrefined)	*3 months, A randomized, two-period, crossover design*	*24 male (peripheral vascular disease subjects)*	*Replacement of culinary oils with extra virgin olive oil (control group replacing with refined olive)*	*ns*	*na*	*na*	*↓ox-LDL*	*[95]*

Source	**Intervention**	**Participants**	**Dose**	**Main finding**				**References**
				Central obesity	**Hyperglycaemia**	**Hypertension**	**Hyperlipidaemia**	
Olive oil (unrefined)	*6 months, A double-blind, randomized, crossover study*	*23 male and female (hypertensive patients)*	*30-40 g of oil per day (control group receiving sunflower oil)*	*ns*	*ns*	*↓SBP ↓DBP*	*ns*	*[96]*
Olive oil (unrefined)	*30 days, A randomized crossover trial*	*25 male and female (healthy subjects)*	*10 g of extra virgin olive oil (control group receiving corn oil)*	*na*	*↓ blood glucose ↑ insulin*	*na*	*↓ox-LDL*	*[97]*
Olive oil (unrefined	*3 months, A randomized, single-blinded and placebo-controlled trial*	*41 male and female (overweight or obese subjects)*	*Replacement of culinary oils with extra virgin olive oil (control group replacing with 10 % corn oil and 90 % soybean oil)*	*ns*	*ns*	*↓SBP*	*↑HDL*	*[98]*
Olive oil (unrefined)	*1 year A randomized, placebo-controlled trial*	*351 male and female (type 2 diabetes or ≥3 CVD risk factors*	*MeDiet + extra-virgin olive oil (control group receiving MeDiet + nuts (walnuts, almonds, and hazelnuts), or a control low-fat diet.*	*↓BMI ↓WC ↓body fat distribution*	*na*	*na*	*na*	*[99]*

Source	***Intervention***	***Participants***	***Dose***	***Main finding***				***References***
				Central obesity	***Hyperglycaemia***	***Hypertension***	***Hyperlipidaemia***	
Olive oil (unrefined)	*5-year, A parallel-group, multicenter, randomized clinical trial*	*7447 male and female (type 2 diabetes or ≥ 3 CVD risk factors)*	*MeDiet + 50 mL extra-virgin olive oil (control group receiving MeDiet + 30 g nuts (walnuts, almonds, and hazelnuts), or a control low-fat diet.*	*↓central adiposity*	*na*	*na*	*na*	*[100]*
Olive oil (unrefined)	*3-week, A double-blind, crossover, randomized, controlled clinical trial*	*30 male and female (healthy subjects)*	*25 mL of virgin olive oil over three meals per day (control group receiving 25 mL of refined oilve oil per day)*	*ns*	*ns*	*na*	*↑HDL ↓ox-LDL*	*[101]*
Olive oil (unrefined)	*3-week, A randomized, double-blinded, placebo controlled*	*40 male (hypertensive subjects)*	*50 mL of virgin olive oil per day (control group receiving 50 mL of refined oilve oil per day)*	*ns*	*ns*	*↓SBP*	*↓oxLDL ↓Lipoperoxides*	*[102]*
Olive oil (unrefined)	*5-week A randomized, two-period, crossover design*	*200 male (healthy volunteers)*	*25 mL of virgin olive oil per day (control group receiving 25 mL of refined oilve oil per day)*	*ns*	*ns*	*ns*	*↑HDL ↓TAG ↓ox-LDL*	*[103]*

Source	***Intervention***	***Participants***	***Dose***	***Main finding***				***References***
				Central obesity	***Hyperglycaemia***	***Hypertension***	***Hyperlipidaemia***	
Olive oil (unrefined)	*3 years, a randomized dietary trial*	*187 male and female (metabolic syndrome subjects)*	*MeDiet + extra-virgin olive oil* *(control group receiving MeDiet + nuts (walnuts, almonds, and hazelnuts), or a control low-fat diet.*	↓*WC*	*na*	*na*	*na*	*[104]*
Olive oil (unrefined)	*4 months,* *A double-blind, randomized, crossover dietary intervention trial*	*24 female* *(mind hypertensive subjects)*	*30 mg/day of polyphenols from olive oil* *(control group receiving polyphenol-free olive oil)*	*na*	*ns*	↓*SBP* ↓*DBP*	↓*ox-LDL*	*[105]*

↓↑, *significant augmentation or diminution; ns, not significant; na, not assayed or not report*

Central obesity

A progressive increase in total adiposity is associated with insulin resistance and all the other components of MetS, including increased blood pressure, glucose and lipid concentrations [106]. Consumption of foods rich in polyphenols including green tea catechins, resveratrol and curcumin are weakly associated with anti-obesogenic effects [107]. Further, the Mediterranean dietary pattern (MedDiet) itself is associated with a lower risk of obesity despite the relatively high consumption of energy rich olive oils [108, 109]. In a follow-up of a large Spanish cohort (The SUN project) high intakes of olive oil were not associated with increased risk of subsequent weight gain (Table 1.) [110]. At least three long-term human dietary intervention studies with the Mediterranean diet plus a high intake of olive oil have demonstrated a reduction in body mass [99, 100, 104]. The Mediterranean diet has a comparatively lower energy density than the standard western diet; however, studies in animal models fed polyphenol-rich olive extracts suggest a further contributing role for olive phenolics in body weight management. In one study, mice were randomly divided into groups that received either a chow diet, a high-fat diet (HFD), or a 0.15% OLE-supplemented diet for 8 weeks. The OLE-fed mice showed significantly reduced body weight gain, and had lower visceral fat-pad mass, compared to the HFD-fed mice [111]. Elsewhere mice fed a 0.03% OL-supplemented HFD for 10 weeks showed reduced body weight gain (−55%) compared to control [112]. Furthermore, HT supplementation 10 mg/kg/day prevented HFD-induced obesity in mice at 17-weeks [113].The addition of rutin [114], luteolin [115], caffeic acid [116] and apigenin [117] to the diet of experimental animals have all been shown independently to ameliorate weight gain, liver, and adipose tissue mass. Unfortunately, human dietary intervention studies with polyphenol-rich OLE or with isolated olive phenolics have typically been relatively short in duration, and show no demonstrable reduction in body-weight reported.

Despite the paucity of human data showing a direct effect on either weight loss, or the attenuation of weight gain, potential mechanisms through which olive polyphenols might influence body composition have been suggested. In experimental models olive phenolics inhibit lipid and carbohydrate digestion in the gut [118], thereby suppressing macronutrient absorption and uptake [58]. In addition, *in vitro* and *in vivo* models have suggested that olive polyphenols can inhibit pre-adipocyte differentiation, suppress

lipogenesis, induce lipolysis, and promote adiponectin secretion [119-121], possibly mediated through the suppression of adipogenic gene expression (PPARγ, C/EBPα, CD36, FAS, and leptin) at the mRNA and protein levels [111, 115, 117, 122-124].

Hyperglycaemia

Impaired carbohydrate metabolism and insulin resistance are defining features of MetS [24]. Polyphenol-rich foods, including tea, cocoa, cinnamon, grapes, and berries, are reported to modulate carbohydrate metabolism, and attenuate hyperglycaemia and insulin resistance [45, 125]. Dietary supplementation with olive polyphenols has been shown to exert an anti-hyperglycaemic response in animal models [118, 126, 127]. For example, consumption of a phenolic-rich olive extract significantly decreased serum glucose in alloxan-induced diabetic rats after a 4 week intervention with a dose of extract equivalent to 8 mg/kg OL and 16 mg/kg HT [128]. Similarly, diabetic rats consuming 0.5 mg/kg OLE for 30 days showed improved blood glucose, and increased plasma insulin [129]. These observations are mirrored in a study in obese diabetic mice, which also showed improved glucose control when fed an OL-enriched diet independently of any change in body weight [130]. Further, in a rabbit model of diabetes, the consumption of extracted OL (at 20 mg/kg body weight) reduced blood glucose after 16 weeks compared to controls [131].

More importantly these anti-hyperglycaemic effects are also observed in human dietary intervention studies in volunteers with pre-existing elevated blood sugar [58, 60, 97, 132]. For example, de Bock et al demonstrated that supplementation with olive leaf extract for 12 weeks (51.1 mg OL, 9.7 HT per day) was associated with a 15% improvement in insulin sensitivity in overweight middle-aged men (n=46) [60]. An intervention study in recent-onset type-2 diabetic patients consuming 500 mg of OLE once daily led to improved long-term sugar control as evident by a reduction in glycated haemoglobin (HbA1c) after 14 weeks [58].

The minor phenolics may also contribute to the observed anti-hyperglyceamic response. Experimental supplementation with luteolin [133] or apigenin [134] have all be shown to significantly decrease insulin resistance in diet-induced obese mice. Additionally, a dietary intervention with 500 mg daily of supplementary rutin reduced fasting glucose levels by

over 10 % in diabetic patients at 30 and 60 days compared to baseline; this was possibly coupled to a concurrent reduction in body weight [135].

Potential mechanisms underpinning the anti-hyperglycaemic activity of dietary polyphenols have been proposed, *in vitro* studies show potential inhibition of amylase and α-glucosidase in the gut resulting in a suppressed glycaemic response to foods [136-138]. Other studies have suggested that polyphenols exert a direct suppression of the proteins involved in intestinal transport of dietary carbohydrate, this has previously been reviewed [45]. Incidentally, green tea phenolics have been credited with directly stimulating insulin-mediated glucose uptake in myocytes [139], this has not, as yet, been demonstrated for the olive phenolics and certainly not for their circulatory plasma metabolites.

Hypertension

Adherence to the Mediterranean diet is inversely associated with both systolic and diastolic blood pressure [49]. Dietary interventions with extra virgin olive oil show marked improvements in blood pressure in hypertensive and pre-hypertensive volunteers. For example, in a crossover study in which medicated hypertensive volunteers in the south of Italy were asked to replace their fats with either EVOO or sunflower oil, The EVOO led to a reduction in blood pressure (BP) and for some a decreased reliance on anti-hypertensive medication [96]. Similarly, in a dietary intervention in overweight and obese US cohort, BP was markedly lowered when the source of fat was replaced with EVOO [98]. Moreno-Luna et al. compared the effect of interventions with polyphenol-rich olive oil and high-oleic sunflower oil (polyphenol-free) in hypertensive women; the olive oil rich-diet significantly reduced both systolic blood pressure (SBP) and diastolic blood pressure (DBP) [105]. Further, Fito et al. reported a decrease in SBP after high-phenolic olive oil consumption but not with a low-phenolic refined olive oil, when consumed by hypertensive stable coronary heart disease patients [102]. These studies collectively suggest that the hypotensive effects of the EVOO may be due to the phenolic compounds and that they are not a consequence of the fat composition of the oils. However, Kaseb et al., induced a 10.95 mm reduction in blood pressure from baseline in hyperlipidaemic Iranian volunteers, asked to consume 20 ml of refined olive oil for six weeks. Refined oils

are low in polyphenols which would suggest quite significant anti-hypertensive benefits from the lipid fraction of the oil [140].

Human dietary intervention studies with supplemental lipid-free OLE strongly support anti-hypertensive effects for the polyphenols. For example, a study with 40 borderline hypertensive pairs of monozygotic twins discordantly assigned to consume either 500 mg (equivalent to 104 mg OL) or 1000 mg (equivalent to 208 mg OL) of OLE daily for 8 weeks was performed by Perrijaquet-Moccetti et al. They observed a dose-dependent drop in SBP, with a mean reduction of 6 mmHg on the 500 mg dose and 13 mmHg on the 1000 mg dose [93]. Elsewhere in a double blind, randomized, parallel and active-controlled clinical study, stage-1 hypertensive patients (n = 46) were given either 1000 mg (equivalent to 199 mg OL) aqueous OLE daily or captopril for 8 weeks. They observed reductions in SBP and DBP from baseline by -11.5 ± 8.5 and -4.8 ± 5.5 mmHg, respectively for those in the OLE group and by -13.7 ± 7.6 and -6.4 ± 5.2 mmHg, respectively for those in the captopril group [55]. Further, Lockyer et al. performed a randomized placebo-controlled trial with OLE using ambulatory blood pressure as the primary endpoint. In this study 60 volunteers consumed either olive leaf extract, containing 136 mg OL and 6 mg HT, or a control for six weeks prior to crossover. They observed a reduction in 24h SBP/DBP of about 3 mmHg and following the intervention [61].

These observations are also mirrored in work performed in animal models of hypertension. OLE (15% w/w OL) when fed to hypertensive rats at 30 mg/kg body weight for 5 weeks significantly reduced SBP (−21.6 ± 5.5 mmHg) [141]. Khayyal et al considered the dose response to an OLE (containing 18-26% w/w equivalent of OL) in L-NAME induced hypertensive rats. With daily oral administration of 25, 50 or 100 mg/kg of OLE, they observed a dose-dependent prevention of induced blood pressure with the 100 mg/kg dose completely preventing any rise in blood pressure after 8 weeks [142].

The mechanisms of action for olive phenolics in the lowering of blood pressure are uncertain. Lockyer et al. observed a decrease in vascular stiffness relatively soon after the consumption of OLE in an acute human dietary intervention study [92]. This suggests that the phenolics influence production of nitric oxide, thereby improving vascular function in the short term, and with sustained consumption resulting in an improvement in blood pressure over the longer term. Indeed, *in vitro*, OL has been shown to increase NO production in LPS-stimulated mouse macrophages [143] plausibly through the modulation

of enzymes such as NADPH oxidase and nitric oxide synthase [144]. Further, OL and HT may act synergistically with other phenolics such as verbascoside to exert ACE inhibitory [145] and calcium channel blocking activities [146]. However, the phenolics themselves survive digestion relatively poorly, small quantities are present in the circulation as conjugated metabolites [147] and the specifics effects of these metabolites on the blood pressure control systems have not been well studied. More mechanistic studies are needed to fully elucidate the mechanisms involved.

Dyslipidaemia

The major components of dyslipidaemia associated with the metabolic syndrome are raised flux of free fatty acids, increased fasting and postprandial triglyceride-rich lipoproteins (TRLs), decreased high-density lipoprotein (HDL), and increased small, dense low-density lipoprotein (LDL) particles [148]. Exaggerated postprandial lipaemia links MetS to the progression of atherosclerosis [132]. Olive oil with its high MUFA content is shown to reduce TC and LDL-C levels when substituted into the diet in place of saturated fats [149]. The additional beneficial effects of the phenolics in EVOO have also been studied [72]. In a large multi-centre crossover trial in healthy men (n=200), Estruch et al. demonstrated the dose dependent improvements in plasma HDL status in response to increasing polyphenol concentrations in olive oils [150]. Several human chronic dietary intervention studies with aqueous olive leaf extracts have also demonstrated favourable plasma lipid responses. Perrinjaquet-Moccetti et al., Sasulit et al., Lockyer et al. and Fonollá J et al. all observed significant reductions in total cholesterol, LDL-cholesterol and triglyceride levels in human volunteers [61, 93, 94, 151]. In contrast, De bock et al. did not observe improvements in lipid profiles after OLE supplementation [60]. In each of these studies plasma lipids were measured as secondary endpoints; and therefore, more focussed human studies are needed to fully elucidate potential benefit of the phenolics independently of the oil in relation to dyslipidaemia.

In rodent models, chronic OLE supplementation led to a reduction in total cholesterol, LDL-C and triglycerides [111, 152-154]. Further Ghosian Moghaddam et al. observed an improvement in HDL-C in diabetic wistar rats fed powdered olive leaf mixed into chow at 6.25 % w/w [155] and Jemai et al. observed protection against dyslipidaemia in cholesterol fed rats for both HT and an OL-rich olive leaf extract [128, 156].

The evidence for anti-hyperlipidaemic effects of olive phenolics is not as strong as it is for anti-hypertensive and anti-diabetic effects, nevertheless potential mechanisms action has been postulated. In the small intestine, phenolics may inhibit pancreatic lipases, thus delaying post-prandial lipaemia [157], further better glucose and insulin control would reduce the accumulation of lipids in the liver, as observed in a cholesterol fed rat model [156], and potentially offset de-novo lipogenic pathways.

Lipid peroxidation

Oxidation of LDL is a free radical driven process which is believed to stimulate macrophage clearance of lipoproteins, and subsequently, to induce foam cell formation and inflammatory responses [158]. Elevated oxidized LDL is associated with increased incidence of metabolic syndrome and coronary heart disease [159].

There are consistent data on the effects of extra virgin olive oil on markers of lipid peroxidation. The European Food Safety Authority now accepts health claims concerning the effectiveness of the ingestion of olive oil with high concentration of HT and its derivatives (5 mg/d) at suppressing lipid peroxidation [97, 102, 105, 150, 160]. It has been assumed that *in vitro* the conferred protection against oxidative stress is mediated by HT and its derivatives [161], however this hypothesis has never been confirmed in humans [162]. Further, in an eight week long chronic intervention trial with 45 mg per day pure hydroxytyrosol, Lopez-Huertas et al., observed no significant reduction in oxidised LDL., they did however report elevated serum Vitamin C in cases versus controls, suggesting the sparing of vitamin C for antioxidant function with HT consumption [163].

Only one human dietary intervention study with a lipid free olive leaf extract demonstrated reduced LDL oxidation, with a 13% reduction in oxidized LDL reported in hypercholesteraemic subjects is reported in the literature and this was only presented in conference abstract form [94]. Protection against LDL oxidation for lipid free olive extracts is observed in animal models. Wistar rats fed a cholesterol-rich diet in combination with polyphenol-rich olive leaf extracts for 16 weeks showed reduced LDL oxidation, in combination with an upregulation of antioxidant enzymes [156]. Elsewhere mice fed 15% olive oil for 6 weeks had demonstrably reduced lipid peroxidation compared to controls [164]. Rabbits fed with 10% (w/w) extra virgin olive oil plus 7 mg/kg of OL were protected against LDL oxidation [165] and Wistar Rats fed a high energy diet

showed a reduction in markers of oxidative stress when given a 20 µg daily oral gavage with HT [123].

In vitro, olive polyphenols inhibit copper sulphate-induced oxidation of LDL [166], and counteract both metal- and radical-dependent LDL oxidation [167]. The direct anti-oxidant actions of polyphenols delivered through routine diet may however be somewhat less in vivo given their relatively low bioavailability and rapid clearance; Giordano et al. demonstrate a down regulation of the oxidative stress pathways in mouse adipocytes post HT consumption [168]. The influence of olive polyphenolics on the Nrf2 pathway in the combatting of oxidative stress has recently been reviewed by Piroddi et al., whilst not yet proven in man; this may be a viable mechanistic explanation for the antioxidant responses to olive polyphenol consumption [169].

1.4.4 Discussion

In this review, we have summarised the findings of dietary intervention studies with olive oil or with lipid free olive phenolic extracts, these studies have explored aspects of the MetS **(Figure 4.)**

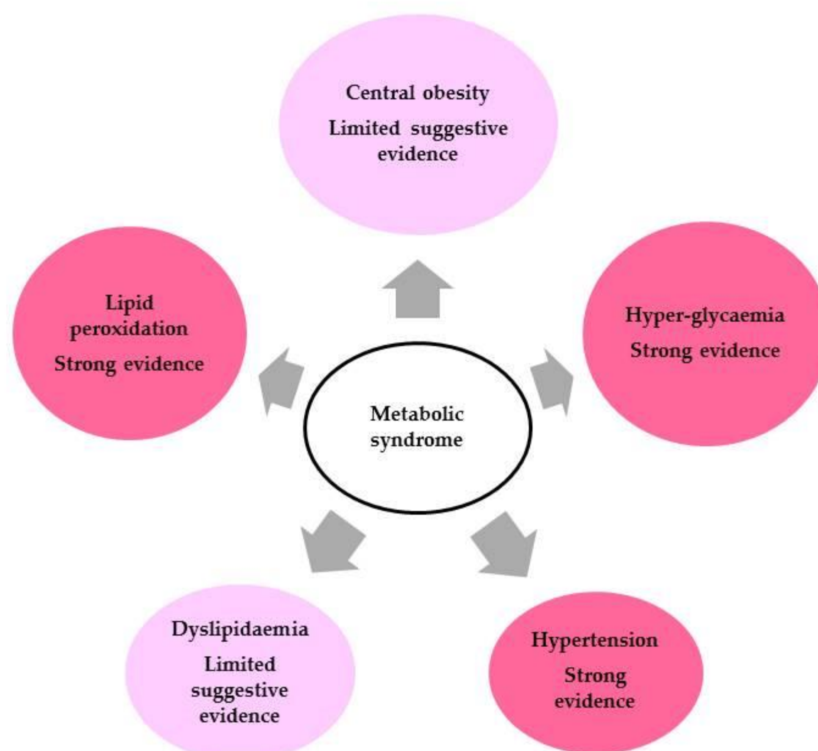

Figure 4 Influence of olive polyphenols on risk factors for the metabolic syndrome

The evidence for benefits directly attributable to olive phenolics coming from human dietary intervention studies is strongest for the observed protection against the oxidation of lipoproteins, protection against hypertension and protection against hyperglycaemia. There is limited, but suggestive evidence for effects in dyslipidaemia and the inhibition of weight gain.

In an obese age, the consumption of phenolic-rich, lipid-free valorised olive leaf products may be a preferential approach to ingesting these beneficial compounds. Intervention studies with EVOO consumed in the Mediterranean diet at quantities of up to 50g per day delivers a 5-20x lower dose of the phenolics than can be delivered in an olive leaf extract supplement. Directly replacing traditional spreads with EVOO in the UK diet (10g per day) would beneficially increase MUFA consumption, but would not substantially change total polyphenol intake. The PREDIMED intervention trial clearly demonstrates efficacy of a Mediterranean pattern of food consumption over a low fat diet, but does not show a reduction in mortality with an EVOO enriched Mediterranean over that observed for the same diet supplemented with nuts instead of EVOO [170].

A current weakness of the available evidence is the paucity of data on mechanisms of action, approximately 60% of ingested olive oil phenols are absorbed in man, mainly in the small intestine, and peaking, as derivatives, in ng ml concentrations, in plasma 1-2 hr post consumption [171]. During digestion and with metabolism oleuropein is hydrolysed, and are later recovered in urine, primarily as glucuronides of hydroxytyrosol and tyrosol [172] [147]. In vitro, mechanistic studies have typically evaluated the effects of the un-metabolized major phenolics at supra-physiological doses in cell systems which are often poorly validated against the *in vivo* system. Genomic and transcriptomic studies in animal models point to the activation of pathways involved in inflammation, oxidative stress and macronutrient metabolism as potential mechanisms of action [173], however further molecular nutrition research is still needed in order to complete our understanding of the interactions between the bioactive components of olive polyphenols. Further dietary intervention studies in which the primary outcome measure is dyslipidaemia or adiposity are also warranted.

1.5 Thesis structure and General hypotheses

1.5.1 Thesis structure

Our starting hypothesis for the PhD was that, consuming olive leaf extract (OLE) as part of a healthy lifestyle would confer health benefits. In the chapter 2 we describe the methodologies used in our experimental works including *in vitro* and human studies. Then in the chapter 3 and 4, we present our *in vitro* studies offering mechanistic support previous observation that polyphenols present in OLE might exerts anti-hyperglycaemia and anti–hypertensive. In the chapter 5 we evidence the synergistic benefits of consuming plant (and specifically olive) phenolics alongside being more physically active in individuals with elevated blood pressure. Finally, in the last chapter (chapter 6) we conclude with a general discussion of the study limitations, future perspective and impact of the studies.

1.5.2 Hypothesis and Objective of this thesis

Hypothesis to be tested in this study:

- Olive polyphenols inhibit enzymes involved in glucose and fat digestion in the small intestine, and inhibit glucose uptake by the gut epithelia.
- Olive phenolics may enhance NO production and/or inhibit angiotensin enzymes resulting in improved blood pressure control.
- Chronic OLE consumption lowers blood pressure in pre-hypertensive volunteers.
- There is synergistic hypotensive effect of consuming olive leaf extract combined with increasing physical activity in individuals with slightly elevated blood pressure.

Objectives of the proposed research

- To investigate the effects of OLE and its phenolic compounds on the inhibition of pancreatic α amylase and lipase activity.
- To examine the effects of OLE on the expression of intestinal glucose transporters in *in vitro* models.
- To assess the effects of OLE on the uptake of glucose by intestinal epithelial cell lines *in vitro*.
- To investigate the effects of OLE on NO production in endothelial cells.

- To explore the effects of OLE and its phenolic compounds on the inhibition of renin and ACE enzymes.
- To determine the effects of olive leaf extract consumption by human volunteers, over a 12-week period, for markers of blood pressure, lipid profiles and blood sugar
- To investigate the synergistic hypotensive effects of consuming olive leaf extract alongside increasing physical activity in individuals with slightly elevated blood pressure

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Chapter 2 General methods

2.1 Phenolic-rich olive leaf extract

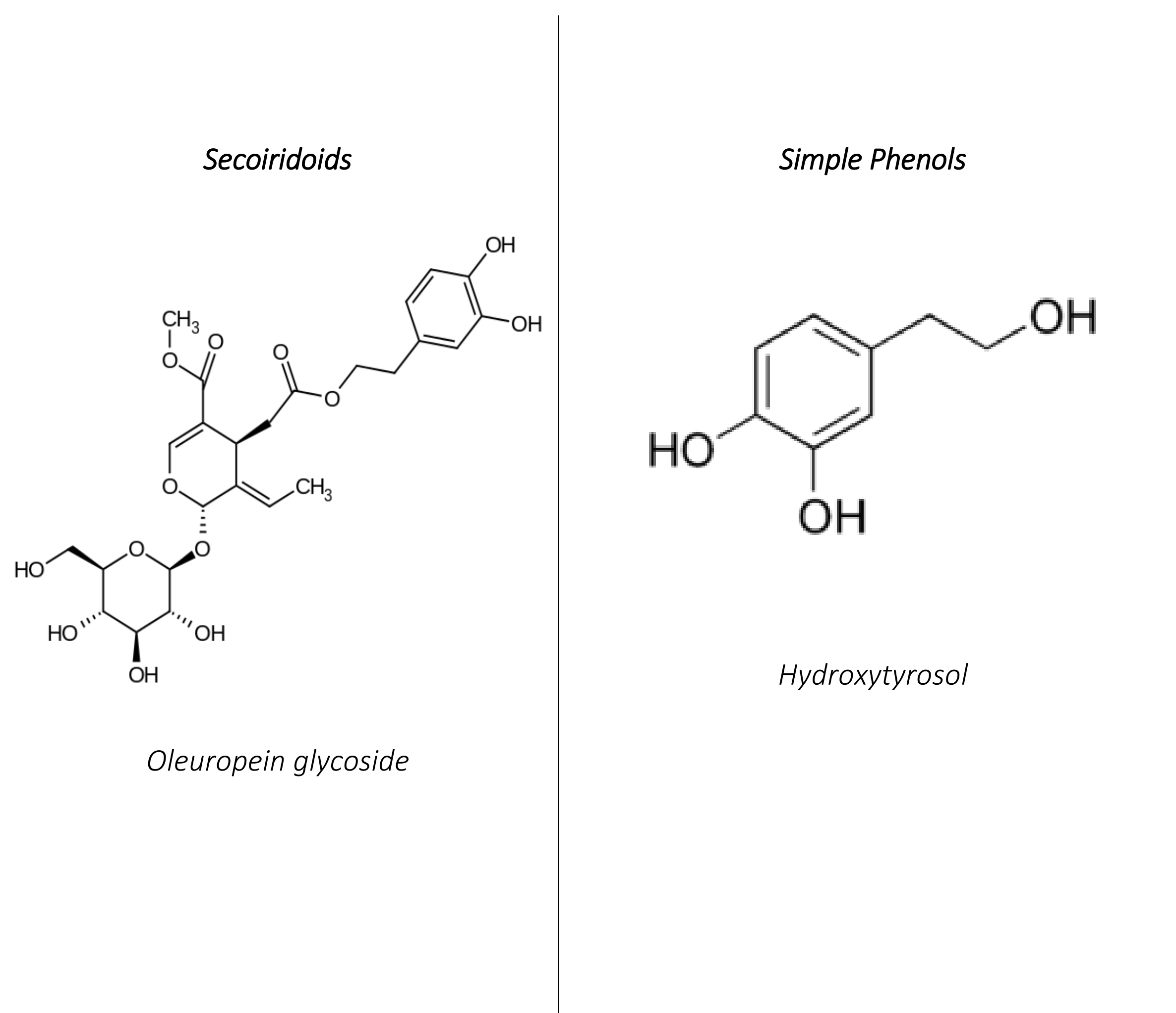
The most common species of olive tree (Olea europaea) is a source of the olive fruit and olive leaf from which phenolics can be extracted [1]. Traditional methods of recovering polyphenol rich fractions from olive leaf include, maceration and Soxhlet extraction. Soxhlet extraction increases the yield of recovered phenolics [2]; however the maceration method is the easier approach, is separates soluble phenolic compounds by diffusion, briefly a large cloth sack containing milled fresh leaves is placed in boiling vats of water, then the solvent is evaporated and a concentrated extract solution is left behind as the product [3]. OLE products are now available for human consumption either as liquid or capsules. Comvita® produce the OLE product used in the experimental work presented in this thesis, via the maceration extraction protocol. Comvita® OLE capsules are produced by mixing the dried extract with a safflower oil carrier. For the liquid extract product, the crude extract is mixed with glycerol and water for stability (**Figure 5**).

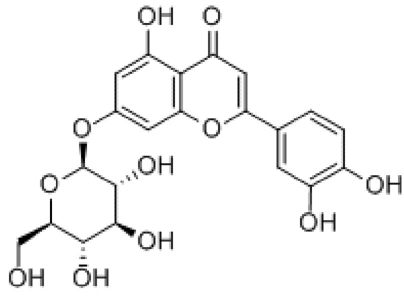
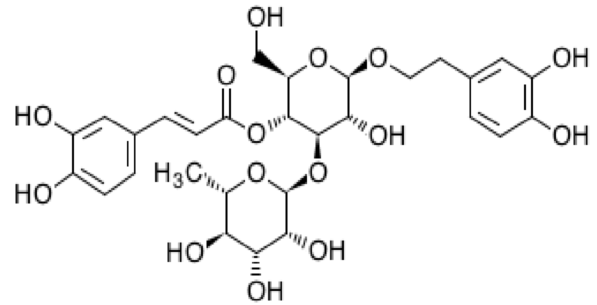
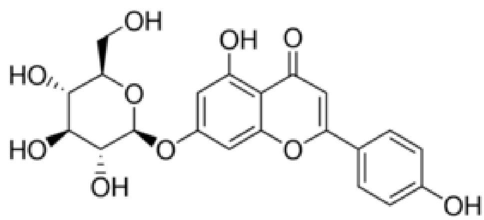
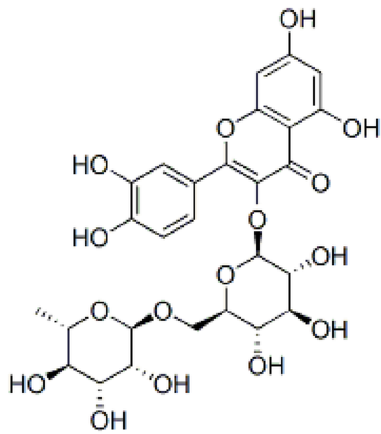
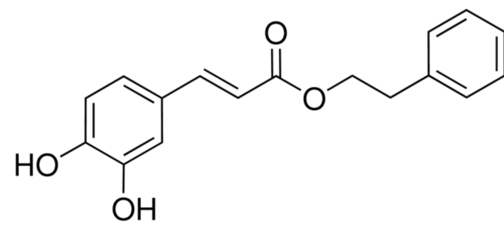
Figure 5 The range of commercial OLE products (Comvita, New Zealand), which are marketed for general health-promoting effects.

Phenolic compounds present in olive leaf extract are shown in **Figure 6**. They may be divided into different groups with regard to major molecular characteristics: secoiridoids (oleuropein); flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7- glucoside, luteolin, and diosmetin); flavonols (rutin); flavan-3-ols (catechin), and substituted phenols (hydroxytyrosol, tyrosol, vanillin, vanillic acid, and caffeic acid) [4]. The oleuropein

content of OLE is influenced by several factors; the harvest seasonal, industrial processing techniques such as drying conditions, extraction techniques, storage and preservation methods [5,6]. In the same olive-leaf cultivars, dried olive leaf has a higher concentration of oleuropein than the fresh leaf; On a dry matter basis the oleuropein concentration of the olive leaf may reach up to 140 mg/g on a dry matter basis in young olives and 60-90 mg/g of dry matter in the leaves [7]. Olive leaf dried at room temperature provides higher oleuropein content than those dried at 50˚C [8].

Figure 6 Chemical structure of the most abundant phenolic compounds in OLE.



Flavonoids	**Cinnamic acid derivatives**
 *Luteolin-7-glucoside*	 *Verbascoside*
 *Apigenin-7-glucoside*  *Rutin*	**Hydroxycinnamic acids**  *Caffeic acid*

2.2 The fate of dietary olive phenolics

Studies have been performed looking at the absorption of phenolics after consumption of olive oil consumption [9, 10]. Vissers et al. suggest that ~60% of ingested olive oil phenols are absorbed in man, mainly in the small intestine 1-2 hrs post consumption. after absorption, oleuropein may be hydrolysed, and as is recovered in urine as hydroxytyrosol and tyrosol [9]. Hydroxytyrosol may enter intestinal cells via passive diffusion [11]. Oleuropein as a glycoside is larger and more polar, entry into the enterocytes may therefore be mediated via the sodium-dependent glucose transporter (SGLT1) although its large size renders it poorly absorbed [12]. OLE contains a predominance of oleuropein glycoside, versus the oleuropein aglycone abundant in olive oil [13], therefore the bioavailability and metabolism might be difference the two. However, the bioavailability of OLE polyphenols is still unclear, and highly dependent on a number of factors, including preparation (capsule/liquid) [14]. Oral administration of a single dose (100 mg/kg) of oleuropein in rats allowed the unmodified oleuropein in plasma reaching a peak value of 200 ng/ml within 2 hrs [15]. In a human intervention trial, de Bock et al., studied the fate of oleuropein and hydroxytyrosol ingested as OLE over 24 hr using an LC-ESI-MS/MS based analysis of plasma and urine samples. Peak oleuropein concentrations in plasma were greater following ingestion of liquid OLE as opposed to capsule preparations (0.47 versus 2.74 ng/mL; p = 0.004). Conjugated metabolites of hydroxytyrosol (sulfated and glucuronidated) were the primary oleuropein metabolites recovered in plasma and urine [14].

2.3 Potential mechanisms explaining the anti-glycaemic activity of polyphenol rich olive leaf extract

The inhibition of the digestive enzyme pancreatic α amylase and lipase and/or the down regulation or inactivation of well as glucotransporter (GLUT2 and SGLT1) in the gut are potential therapeutic mechanisms to improve glycaemic control [16, 17].

2.3.1 Inhibition of α- amylase activity

α–amylases (α -1,4-glucan-4- glucanohydrolases) of both salivary and pancreatic origins are key enzymes in the digestion of dietary carbohydrate [18]. They catalyse the hydrolysis of internal α-(1,4) glycosidic linkages in starch releasing maltose and other oligosaccharides (**Figure 7**).

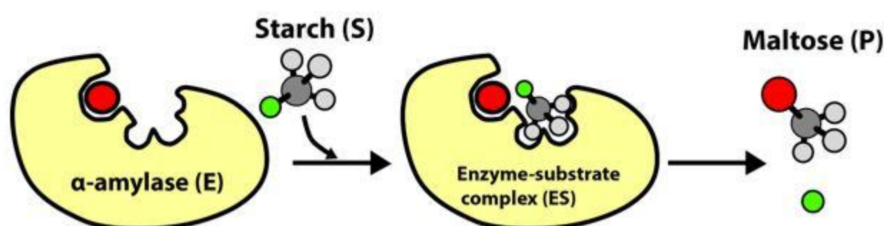


Figure 7 The hydrolysis of starch by α–amylases.

Methods for determination of carbohydrate activity are typically based on the analysis of reducing sugars (RSs) formed as a result of the enzymatic hydrolysis of the glycosidic bond. The 3, 5- dinitrosalicylic acid (DNS) assay described by Miller is the most widespread assay used, and has also been recommended by the IUPAC commission on biotechnology for measuring standard cellulase activities against filter paper and carboxymethylcellulose (CMC) [19]. In addition, the DNS assay is simple, inexpensive and sensitive, and a given solution requires only one standard curve for each sugar [20]. After enzymatic activity, a carbonyl group (C=O) of maltose participates in an oxidation-reduction reaction with DNS (maltose is oxidised into maltonic acid and 3,5-dinitrosalicylic acid is reduced into 3-amino, 5-nitrosalicylic acid) that produces a change in colour

(yellow to orange or red with maximum absorption at 540 nm, depending the intensity of maltose produced) (See **Figure 8** below) [21, 22]. The colour produced is permanent, therefore it is unnecessary to pay special attention to the control of the conditions [20]. Due to the stability of enzymes, the optimum pH value for pancreatic amylase is 6.7-7 [23].

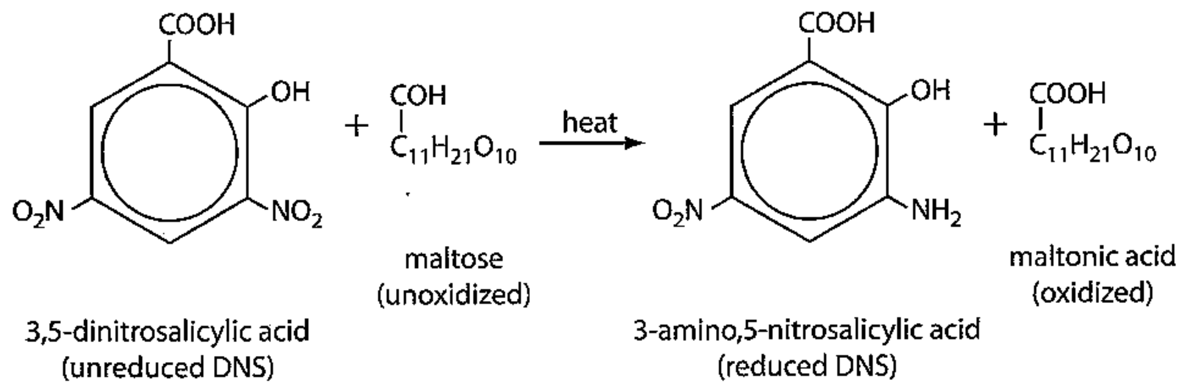


Figure 8 Oxidation-reduction between maltose and DNS.

A standard curve of absorbance at 540 nm against a known concentration of maltose was constructed to establish the dose response of the DNS method. The coefficient of determination = 0.979, and the inter-assay coefficient of variation was 8.69% (n=3)) (**Figure 9**).

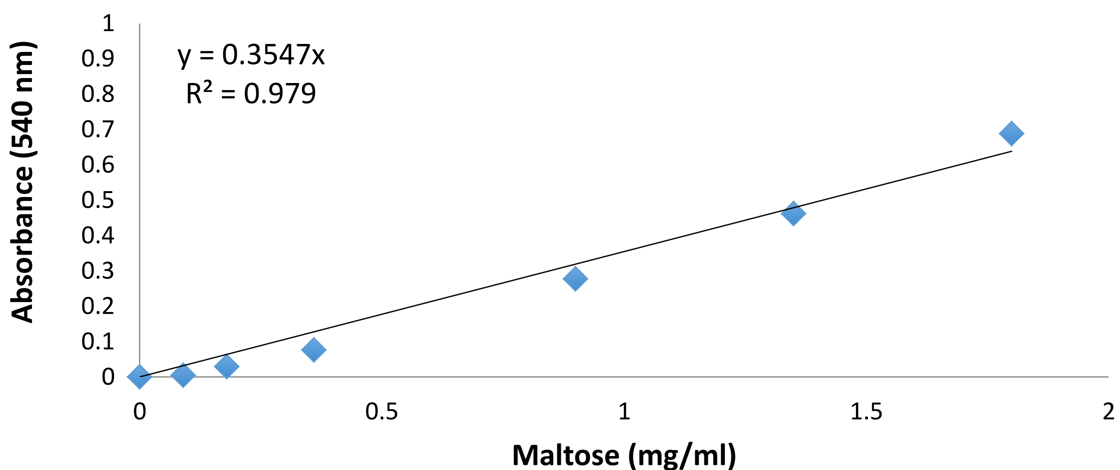
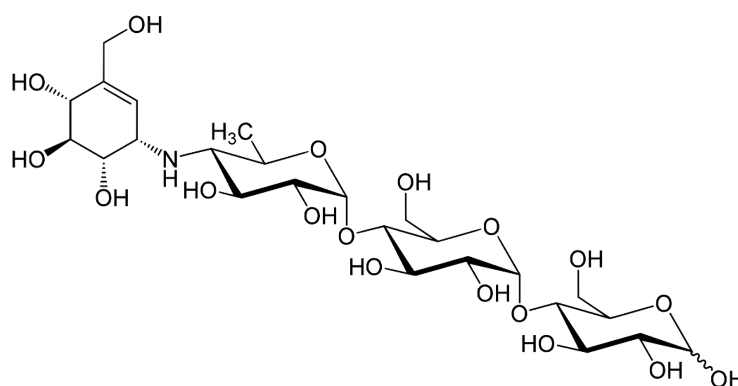


Figure 9 Maltose standard curve for a -amylase inhibitory assay.

Acarbose is a pharmacological inhibitor of amylase that has been used for the treatment of diabetes mellitus; although adverse gastrointestinal effects, such as diarrhoea and abdominal discomfort limit its utility [24]. Acarbose isolated from *Actinoplanesis* is a pseudotetrasaccharide with a conduritol ring at the non-reducing terminus (**Figure 10a**)[25, 26]. It competitively inhibits α–amylase in the brush-border of the small intestine (**Figure 10b**)[27, 28]; therefore, it was used as the positive control in our study.

(a)



(b)

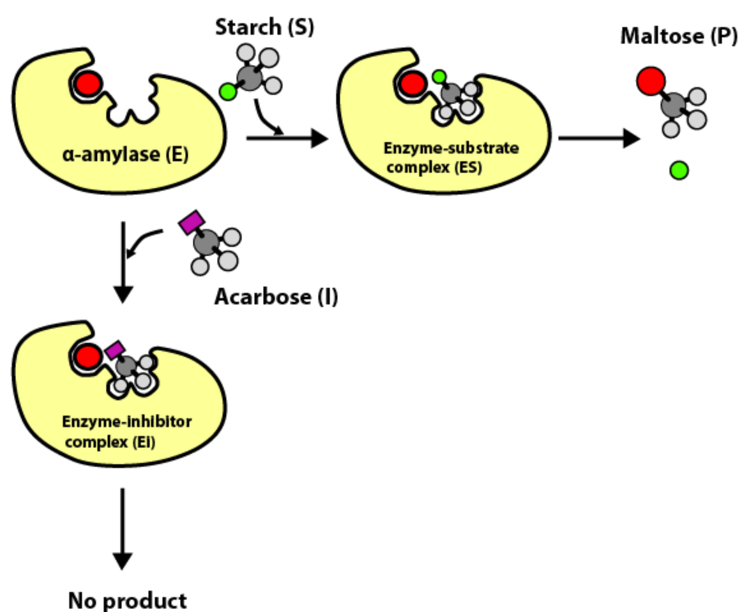
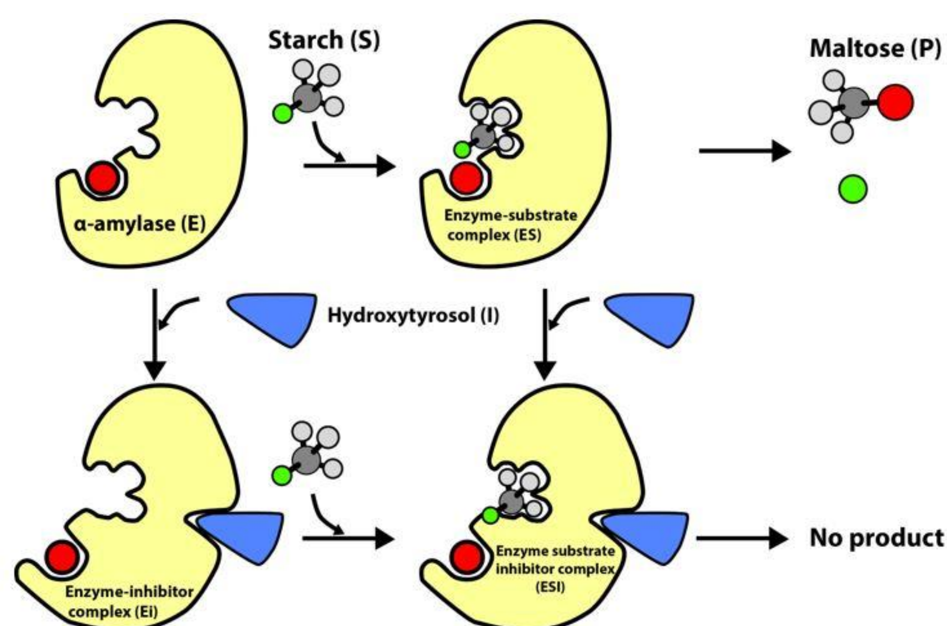


Figure 10 (a) Chemical structures of acarbose and (b) Mechanism of pancreatic α–amylase inhibition by acarbose.

The limitations of the DNS method are due to practical difficulties that occur during the require boiling or heating steps for colour development [29], and more pressingly that DNS reagent strongly interferes with the OH groups of polyphenols such as EGCG, which partially reduces the potential inhibition of α–amylase [30]. The Lineweaver-Burk plot is used to determine the binding potential of an enzyme, determined the initial velocity of the hydrolysis reactions catalysed by α–glucosidase, and found that hydroxytyrosol binds with free enzyme or with the enzyme-substrate complex (non-competitive) (**Figure 11a**) whereas, oleuropein only binds to the enzyme-substrate complex (uncompetitive inhibition) (**Figure 11b**) [31].

(a)



(b)

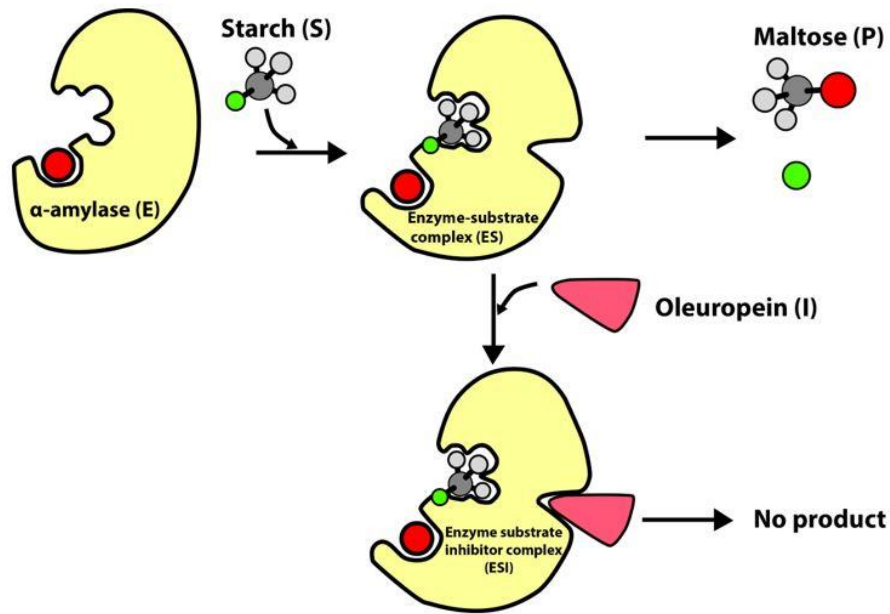


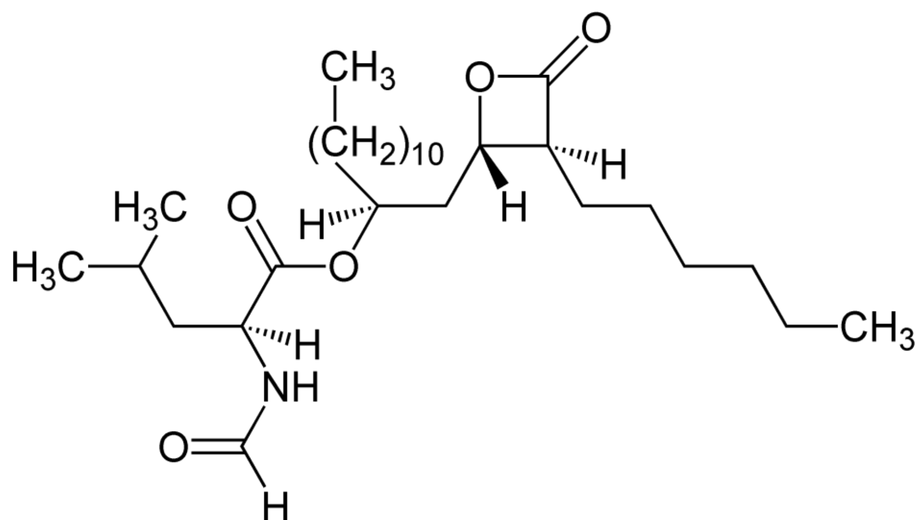
Figure 11 (a) Mechanism of pancreatic α– amylase inhibition by Hydroxytyrosol and (b) Oleuropein.

2.3.2 Lipase inhibition assay

Hyperglycaemia may be precipitated by the availability of lipid for muscle which can induce insulin resistance, regulating the rate of lipolysis may therefore also be beneficial [32]. Excess free fatty acid competes with glucose for uptake by peripheral tissues and influences endogenous glucose production [33]. Ingested fats are metabolized and absorbed in the intestine [34]. Human pancreatic lipase is an enzyme secreted from the pancreas, with responsibility for hydrolysing the triglycerides (fats) into three fatty acid and glycerol [35]. To investigate lipase inhibitory activity, the esters of long chain fatty acids, such as p-nitrophenyl laurate, were used as a substrate in our experiment [36-38]. The liberation of p-nitrophenol from through hydrolysis a simple and cheap colorimetric method of detecting lipase activity [39]. The limitation of this assay is that the reaction can only be performed at moderately alkaline pH which may not be suitable for some lipases [40]. Orlistat is an anti-obesity drug approved and authorized in Europe, which inhibits gastric and pancreatic lipases; thus, it was used as positive control in our study [41, 42]. It is a hydrogenated derivative of lipstatin, produced by the bacterium

Streptomyces toxytriciniis (**Figure 12a**)[34]. Orlistat is a competitive inhibitor of pancreatic lipase which binds covalently to the serine residue of the active site of gastric and pancreatic lipases (**Figure 12b**) [43].

(a)



(b)

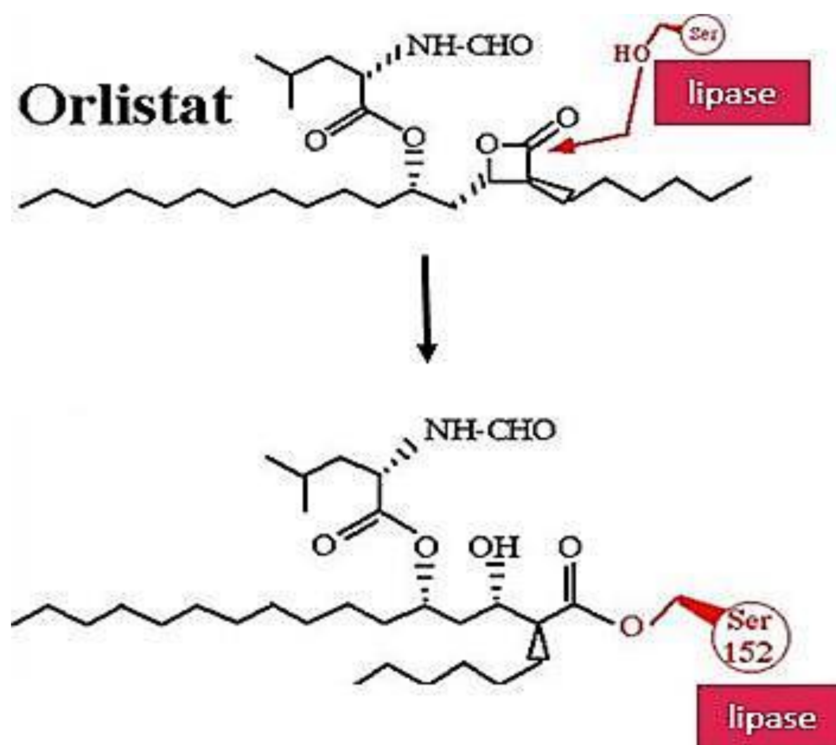


Figure 12 (a) Chemical structure of orlistat [34]and (b) Mechanism of pancreatic lipase inhibition by orlistat [43].

A standard curve of absorbance at 540 nm was established for known concentrations of p-nitrophenol (PNP) to establish the dose response to the lipase inhibition assay. The coefficient of determination was 0.998, and the inter-assay coefficient of variation was 4.95% (n=3)) (**Figure 13**).

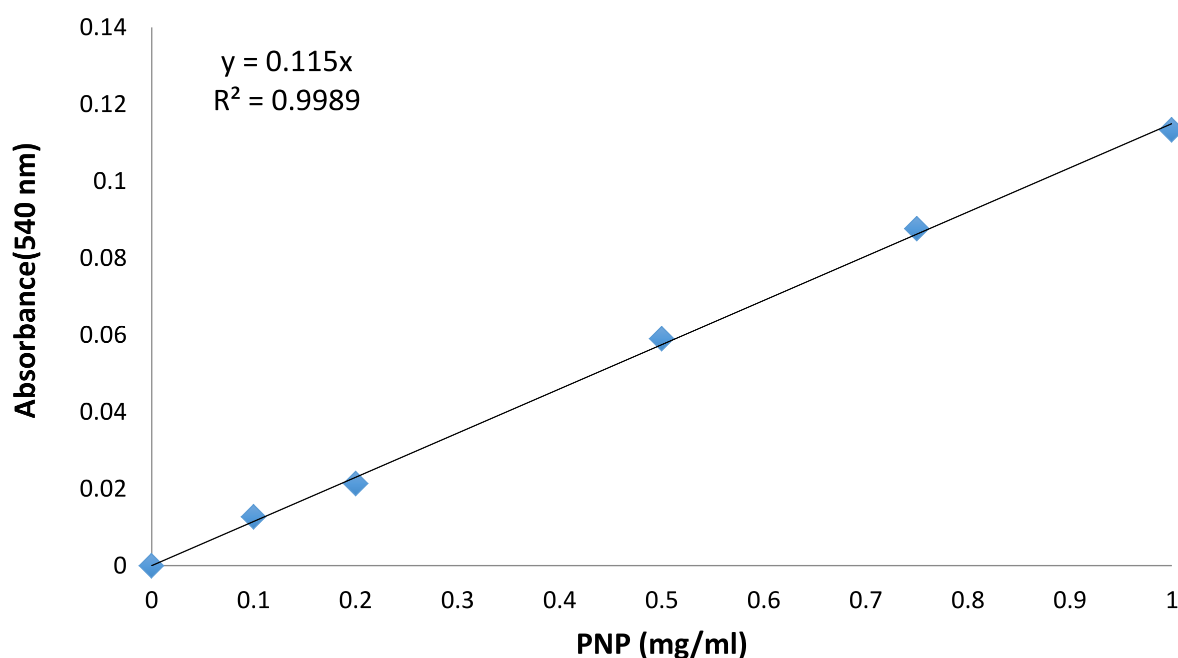


Figure 13 P-nitrophenol (PNP) standard curves for pancreatic lipase inhibitory assay. The standard curve was also generated each time the assay is performed.

2.3.3 Intestinal glucose transporter expression

Glucose is the main product of carbohydrate digestion; it is very hydrophilic and cannot cross biological membranes unaided [44]. In the intestine (see **Figure 14** below), glucose molecules are absorbed into enterocytes by active transport via the sodium-dependent glucose transporter SGLT1 and released into the circulation by facilitated sodium-independent transport via the glucose transporter GLUT2; conversely, suppression of both transporters is strongly related to lowering glucose absorption[44, 45].

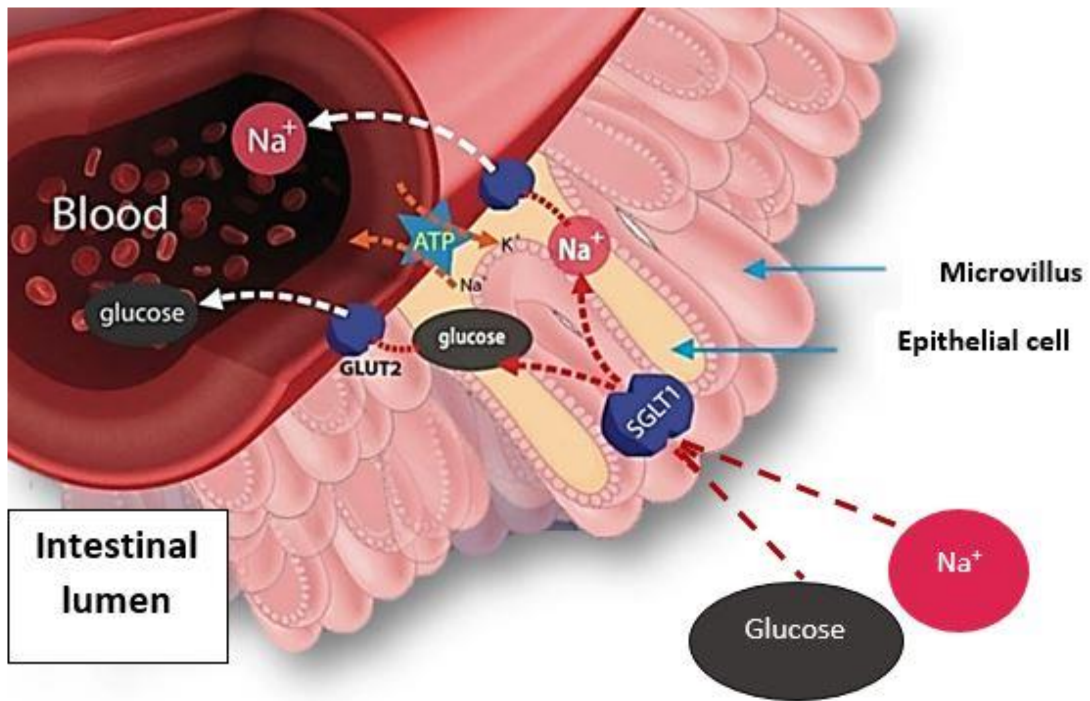


Figure 14 Absorption of glucose molecules by the enterocyte [44].

In our study, we have tested the effects of OLE on the sugar transporters GLUT2 and SGLT1 in intestinal Caco-2 cells, which express high levels of both transporters [44]. The human intestinal Caco-2 cell line represents an excellent *in vitro* model system for the study of drug and nutrient transport and metabolism [46]. These cells are originally obtained from a human colon adenocarcinoma; in culture, it exhibits a well-differentiated brush border on the apical surface and tight junctions [47] which is akin to the small-intestinal enterocyte phenotype [48]. Initially suitable exposures OLE and phenolics were established by assaying their effect on cell viability in a cytotoxicity assay. DAPI or 4', 6-diamidino-2-phenylindole is a blue fluorescent nucleic acid stain, which was used to quantify both live and fixed caco-2 cells [49]. Further to determine relative gene expression, quantitative, or real time, PCR (qPCR) is widely used [50]. PCR or the Polymerase Chain Reaction is a technique for amplifying DNA, real-time PCR has become a well-established procedure for quantifying levels of gene expression in real-time. SYBR Green is a fluorescent DNA binding dye, which specifically binds double-stranded DNA by intercalating between base pairs, and fluoresces only when bound to DNA. During the PCR cycle, an increase in reporter fluorescent signal at the end of the extension step is directly proportional to the amount of PCR product produced [50]. However, the

weakness of intercalating dyes is that they are non-specific, so a combination of primers and master mix in the reaction is required to produce a single gene-specific amplicon.

2.3.4 Glucose uptake assay

The determination of apical uptake refers to the amount of glucose inside the cell, and the amount of glucose in the basolateral side, representing the amount that passes through the apical and basolateral side which could be an indicator of how polyphenols might obstruct this process [44]. Glucose uptake is typically performed by using radiolabelled glucose. The rate of uptake of radioactively tagged glucose in differentiated caco-2 cells help to analyse the glucose uptake activity and the effect of plant extract on the glucose uptake activity. Treated cells with plant extracts elicit the translocation of glucose transporters to the cell surface allowing radiolabelled glucose enters the cell alongside with the normal glucose. The amount of radioactivity in labelled samples is directly measured are detected by using liquid scintillation counter. In scintillation counting, after radioactive samples combined with a liquid scintillation cocktail, the decay of radionuclide produces an ionizing particle. These particles are converted into light photons which can be detected by a liquid scintillation counter [51].

The glucose uptake assay was initially developed and validated by Manzano et al. when running it we used phlorizin and phloretin as positive controls and ran an untreated negative control. The inter assay coefficient of variation for the controls was 1.61% and 1.72%, respectively (n=3).Therefore, cells were cultured in 3 flasks in biologically independent manner (n=3).

2.4 Investigating the mechanisms underpinning the anti-hypertensive activity of olive leaf extract

2.4.1 Determination of Endothelial Nitric Oxide Production

In the vasculature, an imbalance of Nitric oxide (NO) and Angiotensin II (Ang II) has been implicated in the pathophysiology of hypertension [52]. Increasing the production of NO and modulating Ang II results in vasodilatation [53]. In endothelial cells, NO is synthesized by endothelial nitric oxide synthase (eNOS) via the conversion of L-arginine to L-citrulline in the presence of O_2 and NADPH (**Figure 15**) [54].

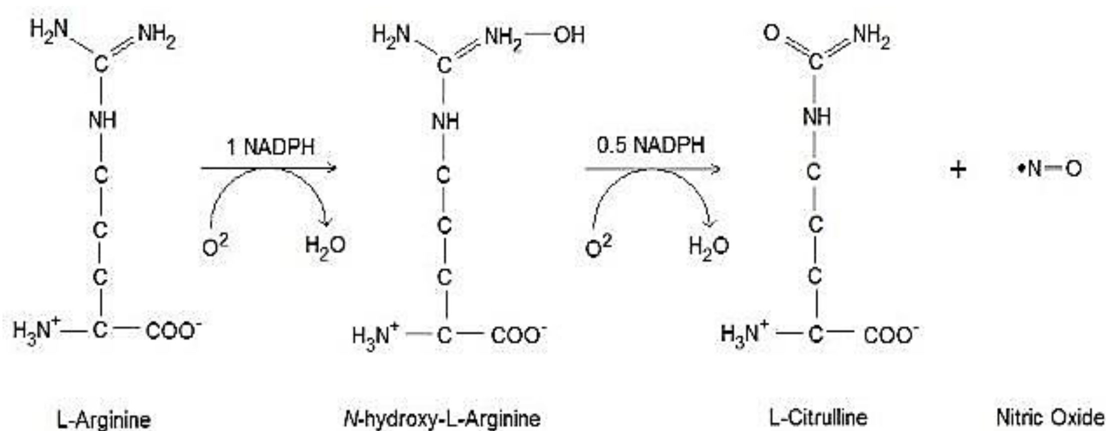


Figure 15 The biosynthesis of nitric oxide from L-arginine [54].

Human umbilical vein endothelial cells (HUVECs) isolated from the umbilical cord are typically used as a model system for the study of the regulation of endothelial cell function [55]. It provides a simpler alternative to screen the vasoactive potency of compounds [56]. However, NO production of HUVEC becomes limited with a high number of passages [57]. In our study, production of NO by HUVEC was measured by the Griess reaction, which is the indirect determination of NO through the spectrophotometric measurement of its stable decomposition products (NO_3^- and NO_2^-) [58]. Since the Griess reaction is specific for nitrite, enzymatic reduction of nitrate to nitrite is required prior to react with the Griess reaction [59]. NO_2^- reacts with a diazotizing reagent, such as sulfanilamide (SA) ($HO_3SC_6H_4NH_2$), under acidic conditions to form a transient diazonium salt ($HO_3SC_6H_4–N\equiv N^+$), then reacted with a coupling reagent, N-naphthyl-

ethylenediamine (NED) ($C_{10}H_7NH_2$), to form water-soluble azo dye ($HO_3SC_6H_4$–N═N–$C_{10}H_6NH_2$); a red-violet coloured, (λmax ≈ 540 nm) (**Figure 16**) [58].

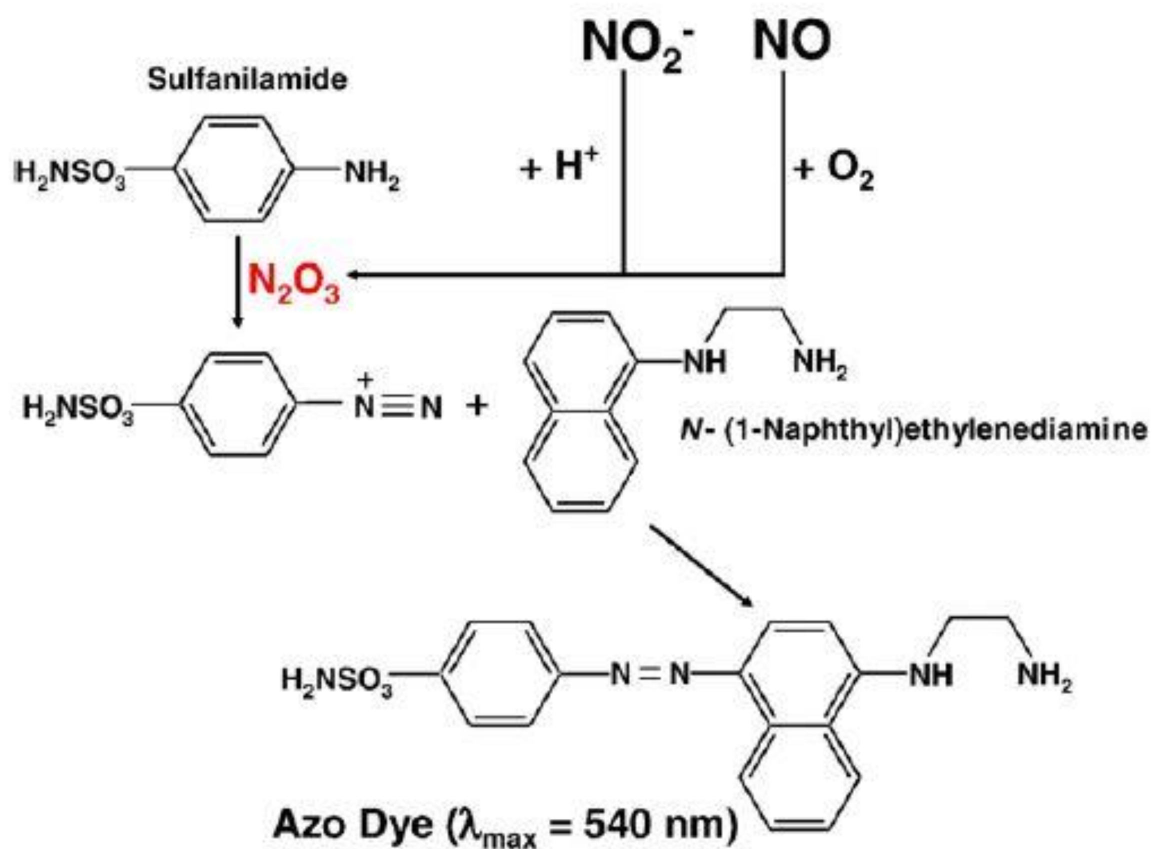
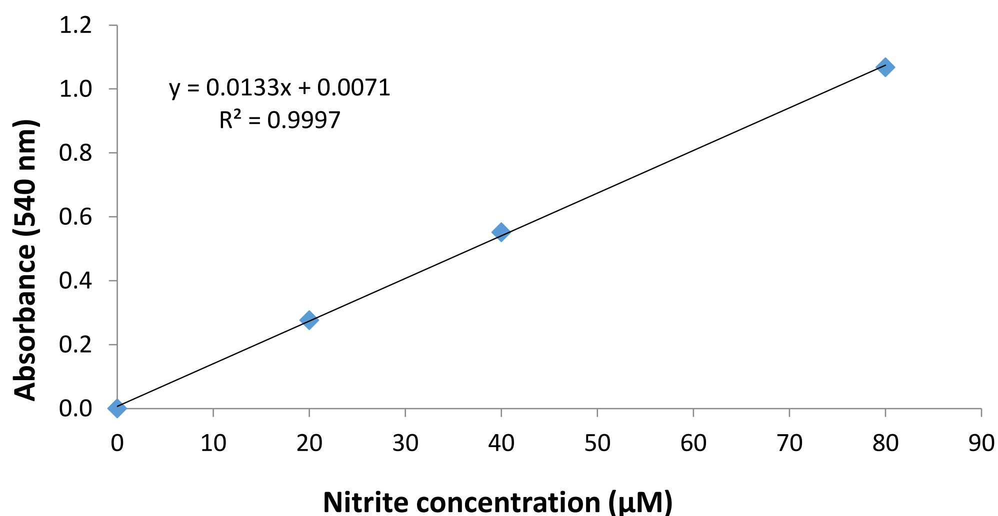


Figure 16 The overall reaction of Griess Reaction Assay [58].

Standard Nitrite and Nitrate + Nitrite curve was constructed to establish the linearity of the Griess assay, coefficients of determination were 0.999 and 0.998 respectively. The inter-assay coefficients of variation = 1.53% and 1.38% respectively, (n=3)). (**Figure 17**).

(a)



(b)

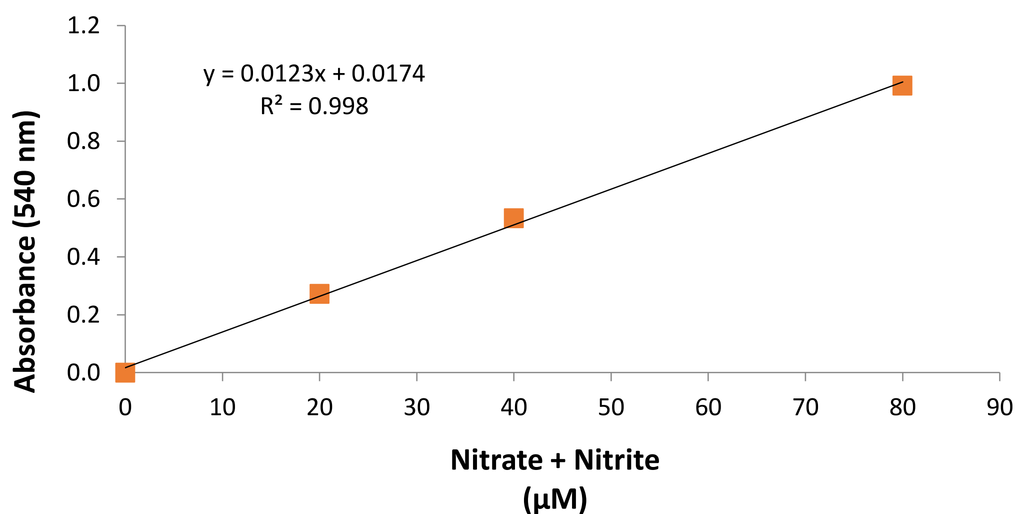


Figure 17 (a) Nitrite standard curve (b) Nitrate + Nitrite standard curve. The standard curve was generated each time the assay is performed.

2.4.2 Inhibition of the renin-angiotensin system (RAS) enzyme activities

Angiotensin II (Ang II) is a multifunctional peptide hormone of the renin-angiotensin system (RAS) [60]. Renin, released from renal juxtaglomerular cells, and angiotensin I-converting enzyme (ACE), produced by endothelial lung cells, are two principal enzymes involved in RAS [61]. Renin cleaves the N-terminus of angiotensinogen to yield angiotensin I, which further hydrolysed by ACE to release a potent vasoconstrictor (angiotensin II) [62]. Angiotensin II acts directly on blood vessel cells stimulating vasoconstriction and also triggers the adrenal gland to release aldosterone, which acts on the kidneys to stimulate reabsorption of salt and water (**Figure 18**) [63]. Inhibition of both renin and the ACE enzyme would be therefore an attractive strategy for the control of hypertension [64].

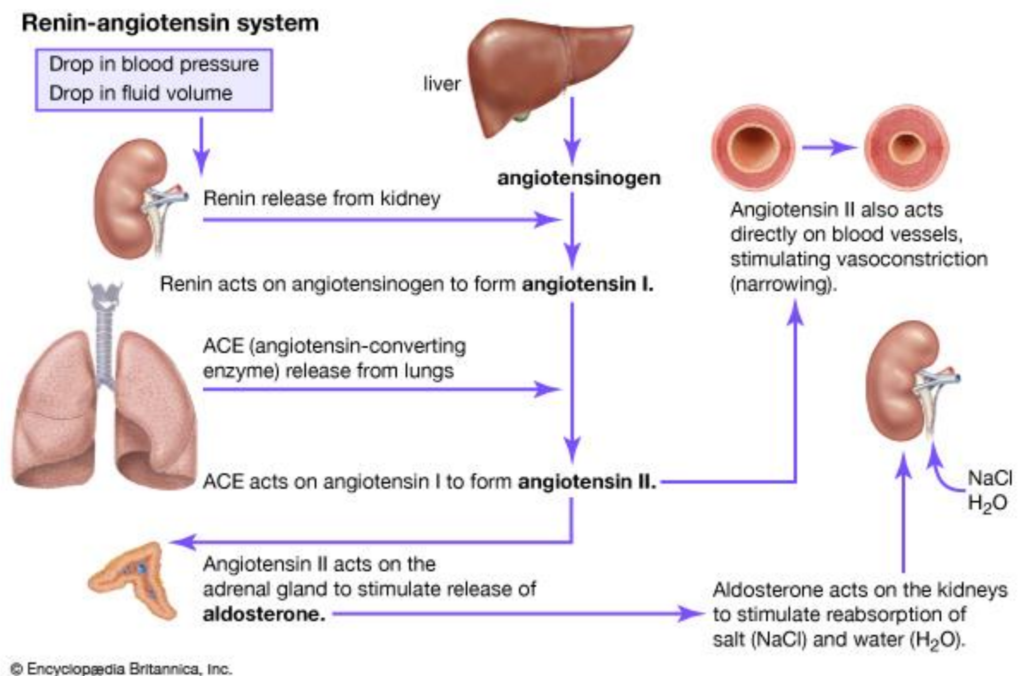


Figure 18 Renin-angiotensin system [64] **.**

2.4.3 Renin inhibition assay

In our renin inhibition assay, the renin synthetic peptide substrate; Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(DABCYL)-Arg, was used to incorporate the renin cleavage site that occurs in the N-terminal peptide of human angiotensinogen. Renin cleavages a (fluorophore-EDANS) to brightly fluorescent (peptide-EDANS), which can be easily determined using fluorescence plate reader, with an excitation of 335-345 nm, and an emission wavelength of 465-510 nm [65]. Aliskiren, the orally effective renin inhibitor, was used as positive control, it is used clinically to treat patients with hypertension [66]. Aliskiren is a highly selective, tight-binding inhibitor of human renin, and can be seen to occupy the S3 to S2’subsites, thereby blocking the catalytic function of the enzyme (**Figure 19**) [67].

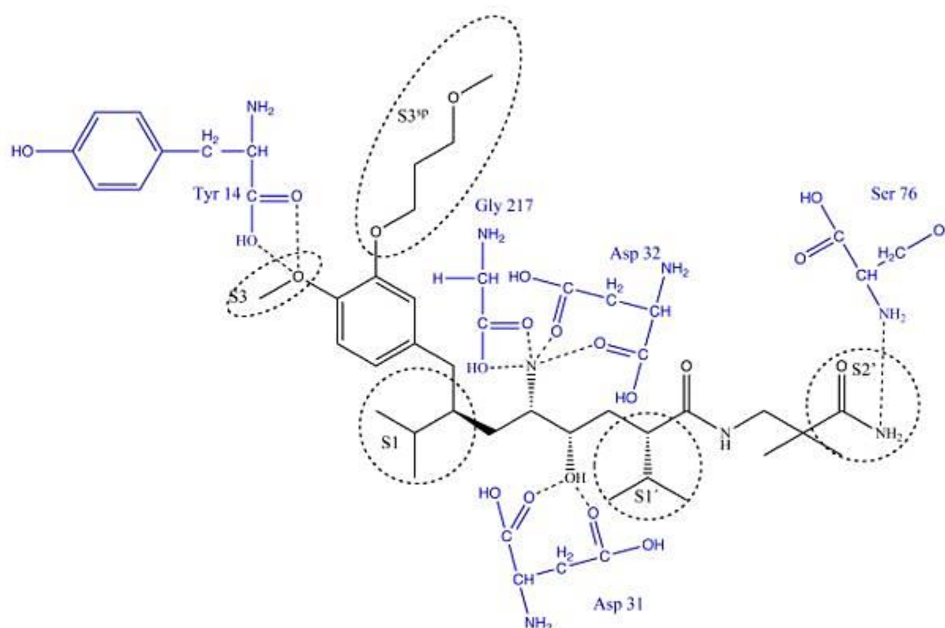


Figure 19 General view of aliskiren in the binding complex with human renin [67].

A commercial kit was used to perform this assay. It has been validated by the Cayman Chemical Company. Intra-assay coefficient of variation = 2.9% (n=75). Inter-assay coefficient of variation = 7.3% (n=5).

2.4.4 ACE inhibition assay

ACE inhibition has been widely used as a therapeutic approach to improve endothelium-dependent vasorelaxation [68]. A Spectrophotometric assay for measuring the inhibition activity of OLE on ACE was used. N-(3-(2-furyl)acryloyl)-phe-gly-gly (FAPGG) performs one of the highest activities with ACE [69]; therefore it was selected as substrate. During the assay the internally quenched fluorescent synthetic substrate, FAPGG, is hydrolysed by ACE leading to the production of amino acid (furanacryloyl-L-phenylalanine, FAP) and dipeptide (glycylglycine, GG) [70]. The final absorbance of FAP is only measured, since GG could not absorb at 340 nm [71]. A potent synthetic ACE inhibitor such as captopril was use as positive control. Captopril inhibits competitively the activity of ACE, a sulfhydryl (SH) group of captopril chelates the zinc ion contained in the active site of the ACE enzyme (**Figure 20**) [72].

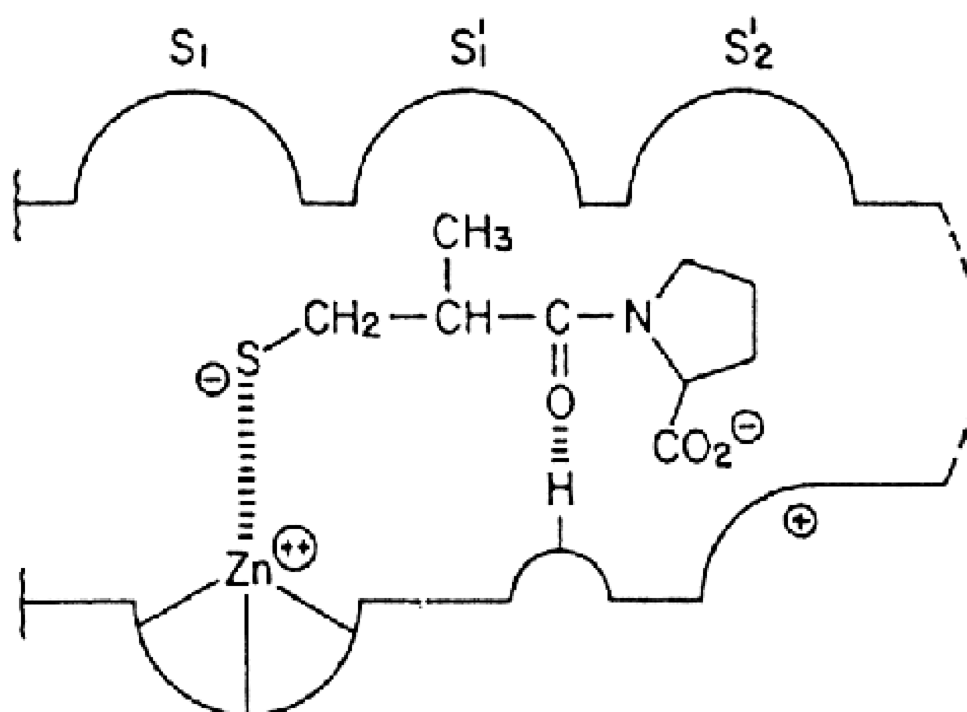


Figure 20 A representation of captopril bound to the active centre [72].

A standard curve of absorbance at 340 nm versus a known concentration of [N-(3-[2-furyl]acryloyl)-l-phenylalanine] (FAP)+[glycylglycine] (GG) was constructed to establish the dose response for this assay. The coefficient of determination was 0.993 and inter-assay coefficient of variation = 3.84%, (n=3)). (**Figure 21**).

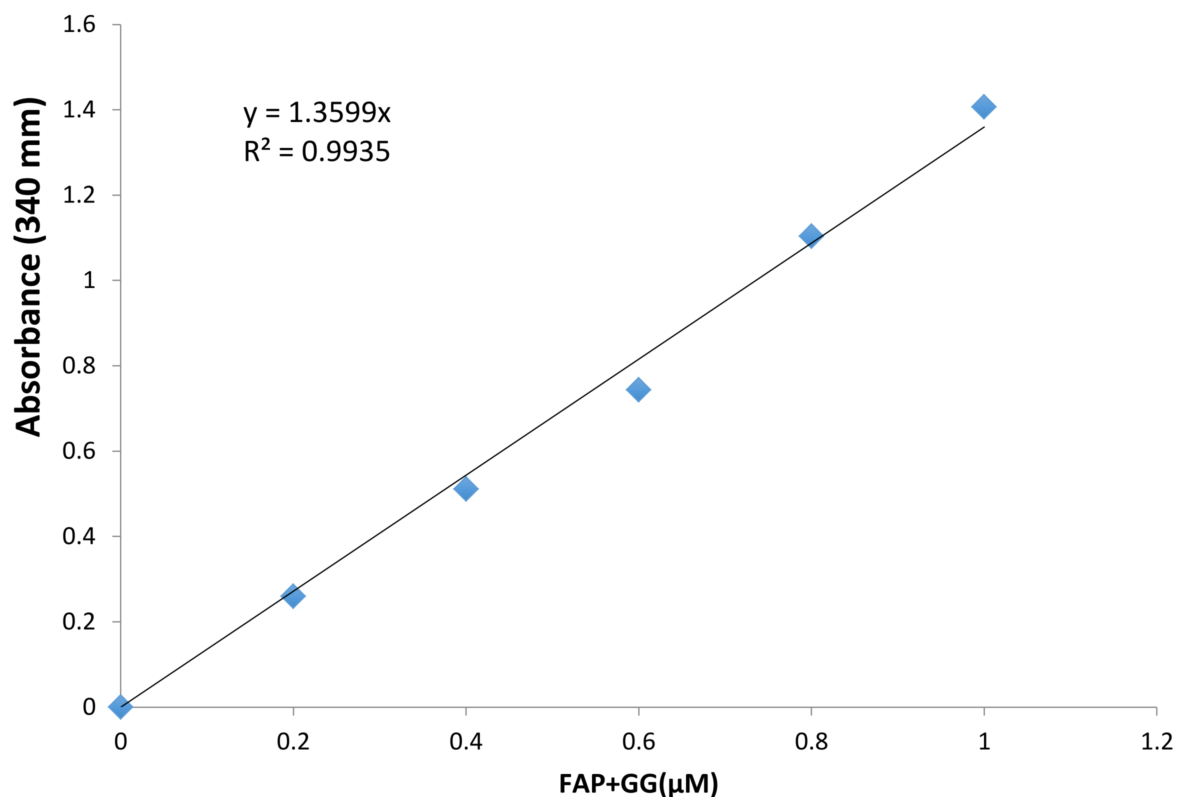


Figure 21 Standard curve of [N-(3-[2-furyl]acryloyl)-l-phenylalanine] (FAP)+[glycylglycine] (GG) versus absorbance at 340 nm.

2.5 The synergistic anti-hypertensive effects of consuming olive leaf extract alongside increasing physical activity

2.5.1 The ambulatory blood pressure measurement

Ambulatory blood pressure monitoring (ABPM) is a non-invasive blood pressure measuring technique that provides a profile of blood pressure behaviour over a period of time-usually over a 24 hr period and identifies of individuals with a white coat response [73]. ABPM uses an oscillometric method to detect blood pressure, the cuff is inflated until the pressure occludes flow within the brachial artery in the arm, when the pressure in the cuff is greater than the blood pressure inside the artery, blood starts to flow initiating fluctuations in the arterial wall, the pressure in the cuff when blood first passes through the cuffed region of the artery is an estimate of systolic pressure, and the pressure in the cuff when blood first starts to flow continuously is an estimate of diastolic pressure [74]. A large variety of ambulatory blood pressure measurement devices are now available on the market, the A&D Medical Ambulatory Blood Pressure Monitoring System (Model TM-2430, Scan Med, A&D Medical, UK) is an automated oscillometric upper-arm ABPM device, which has been evaluated for measurement accuracy using the 1990 and 1993 British Hypertension Society (BHS), European Society of Hypertension (ESH), and Association for Advancement of Medical Instrumentation (AAMI) standards (**Figure 22**).

Figure 22 The A&D Medical Ambulatory Blood Pressure Monitoring System (Model TM-2430, Scan Med, A&D Medical, UK).

This device has been verified accurate across the whole range of age and blood pressure level, and its performance does not vary according to sex [75]. To initiate ABPM, the TM-2430 recorder must be connected to the computer and be ready for operation via the multilingual Doctor Pro™ software. The software of the device issued to the volunteers will be programmed in accordance with British Hypertension Society guidelines to obtain measurements of BP and heart rate every 30 minutes between 07:00 and 21:59 hr, and every 60 minutes between 22:00 and 06:59 hr. The first two readings were excluded. To ensure accurate and appropriate data collection, subjects were asked to wear the monitors for a duration of 25 hrs.

The display of the device indicates the clock. The key to successful ABPM is educating the volunteer in the process of monitoring and instructions should be explained and printed on a diary card [73]. Volunteers were asked to maintain habitual physical activity levels on the day of the blood pressure recording. When the measurements are complete, data are downloaded; the quality of the recording can be checked. Number of measurements necessary for day time and night-time should be greater than 14 and 7 systolic and diastolic blood pressure measurements, respectively [76]. The most popular methods of analysing blood pressure values recorded during the 24 hrs cycle is to assess the time of awakening and sleeping from a diary card of activities, and to use a fixed time method in which the daytime period is set at 0900 to 2100 hrs and the night-time from 0100 to 0600 hrs; however, variations of the fixed time method that may possibly occur between the young and the old and in different cultures are to some extent excluded from the analysis [73].

2.5.2 Digital Volume Pulse (DVP)

A digital volume pulse (DVP), an accurate, simple, inexpensive and non-invasive photoplethysmography technique to analyse the peripheral pressure pulse waveform is suitable for use in large-scale trials [77]. Previous study has recently shown that DVP is ideal for pharmacological studies in which continuous monitoring of drug effects is required [78]. The DVP measures infrared light transmission at 940 nm through the finger pulp, and the amount of back scattered light is directly proportional to the wave-like movement of blood from the aortic root to the finger [77]. The DVP waveforms provide two indices from the contour analysis of the DVP: the stiffness index (SIDVP) which highly

correlated to arterial stiffness, and the reflection index (RIDVP) which relates with vascular tone [79]. For measurement of the SIDVP, all subjects rested in bed supine for 15 minutes in a quiet room prior vascular function measurements and the measurement was performed by a single trained investigator. A photoplethysmographic probe (Micro Medical, Kent, U.K.) is placed on the index finger of the right hand to obtain the DVP. Blood pressure and DVP waveforms will be recorded 3 times over 10 second periods with 5 minute intervals between measurements. The DVP waveform has two components. The first part of the waveform (systolic component) is formed as a result of pressure transmission along a direct path from the aortic root to the finger (a). The second part (diastolic component) is formed by pressure transmitted from the ventricle along the aorta to the lower body where it is reflected back along the aorta to the finger (b). The average peak-to-peak time (PPT), as the time interval between the first and the second peaks of a DVP, for each measurement will be calculated using the Pulse Trace PCA2 Software (Micro Medical, Kent, U.K.) (**Figure 23a**). SIDVP will be calculated by dividing the PPT value into height in metre and multiplying the result by 1000 to convert the result into m/sec. The reflection index (RIDVP) is calculated as the height of the diastolic peak of the DVP relative to that of the systolic peak expressed as a percentage of the total amplitude of the DVP waveform (**Figure 23b**) [80].

(a)

(b)

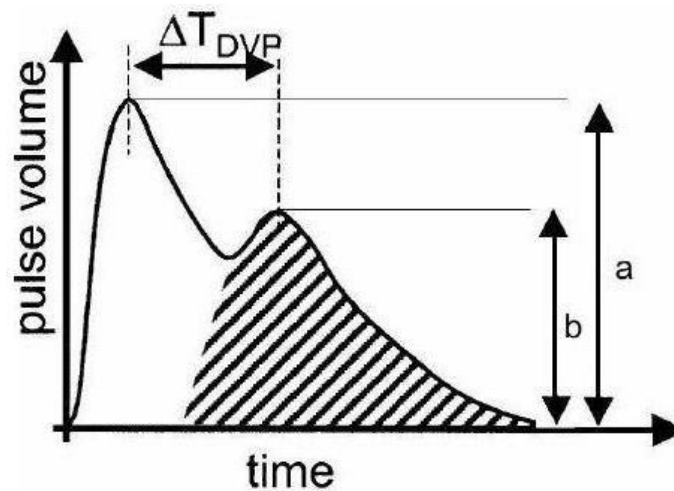


Figure 23 (a) PulseTrace PCA2 Software (Micro Medical, Kent, U.K.), and (b) The DVP waveform and its characteristic parameters as: time between the first and second tip (PPT), amplitude of the first tip identified by (a), amplitude of the second tip identified by (b).

2.5.3 Motivating physical activity

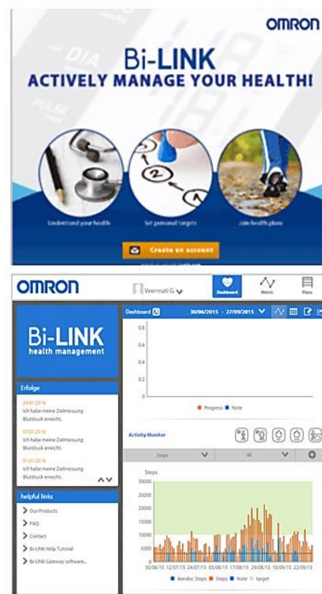
The World Health Organization (2011) defined physical activity as "any bodily movement produced by skeletal muscles resulting in energy expenditure, includes all forms of activity such as leisure time physical activity, transportation (e.g. walking or cycling), occupational (i.e. work), household chores, or structured exercise". Changing physical activity behaviour is a big challenge for public health. The four home countries' Chief Medical Officers recognised the importance of physical activity and launched "Start Active, Stay Active" campaign recommending that "adult should do at least 150 minutes of moderate-intensity physical activity or at least 75 minutes of vigorous aerobic activity every week and strength exercises on two or more days a week" [81]. Achieving the recommended levels of physical activity has been shown to reduce the risk of the development of a number of chronic diseases such as type II diabetes, cardiovascular disease and cancer, to improve bone function and also to enhance energy balance and weight control [82]. However, public health strategies now focus on regular moderate-intensity physical activity as part of an activity lifestyle rather than on structure programme [83]. The easiest technique for most people to increase their physical activity

is incorporating into daily routines, for example by using the stairs instead of lifts, walking or cycling instead of driving, and taking up more active hobbies such as gardening, painting. Physical activity interventions for adults have been predominantly focused on walking [84-86]. Walking is commonly associated and utilized in everyday activities, thus it is an excellent way for most inactive people to begin regular exercise [87]. Computed translations of free-living physical activity study mentioned that recommended moderate-to-vigorous physical activity are equivalent to 7,100 to 11,000 steps/day [88]. A review of physical activity promotion recommended that brisk walking has the greatest potential for increasing overall activity levels of a sedentary population and meeting current public health recommendations [89]. A package intervention consisting of self-monitoring, goal setting, and feedback has been achieved to increase physical activity in healthy adults [90]. Physical activity is a complex multi-dimensional construct; thus, accurate measurement of physical activity is required to monitor physical activity level and better define the dose response relationship between physical activity and health. Self-report and objective measures are available to assess free-living physical activity [91]. Self-report tools include questionnaires, diaries, logs and recalls, are the simplest and cheapest for collecting physical activity data from a large scale of people in a short time, but the least accurate including recall bias [92]. Objective methods such as motion sensors: pedometer and accelerometer are the best option of estimating energy expenditure (EE) [93]. They can provide more accurate information than self-report measures, but resources and expertise are required to collect and manage the data [94]. Pedometer is increasingly used in physical activity research and health promotion initiatives, and most sensitive to walking behaviours [95]. It is a convenient, inexpensive tool for estimating of physical activity volume by counting the numbers of steps taken and relating the count to guideline equivalents of physical activity [96]. Previous studies showed that the use of a pedometer is associated with significant increases in physical activity and significant decreases in blood pressure [97]. Although pedometers are extensively used as a physical activity–monitoring tool, they are unable to measure activity intensity [98]. Nowadays, advanced pedometer models such as Omron pedometer HJ-322U-E (Milton Keynes,UK) are available, and seem to provide valid and reliable step counting information in both lab-based and free-living settings [99]. Reviews by Tudor-Locke et al., have found that pedometer showed strong correlations (r = 0.86)

with data from accelerometers under free-living conditions [100]. Omron pedometer HJ-322U-E has a dual-axis acceleration sensor which counts steps when it is placed horizontally or vertically, and comes with a USB cable and software for installation on a personal computer. A researcher can set up a user account for each volunteer, connect the pedometer to the computer with the USB cable, and click a button to download the activity data. Moreover, it also provides an online database (Omron Bi-LINK) to keep track health and fitness data, and to set goals. The device stores 7 days of information including total step/day, distance, calories burned, aerobic steps on the display and stores 22 days in the memory. Aerobic steps are counted separately when walking more than 60 steps per minute and more than 10 minutes continuously which can be referred to brisk walking. This type of pedometer is unaffected by body mass index and can be worn to count an individual's steps at different positions on the body: at the hip, in the pocket or clipped to a bag [101]. However, it is important to be aware of the effect of stride length on each person; for example, those with shorter stride lengths will accrue a greater number of steps than those with a longer stride length. Thus, the correct average stride length is measured by divided the total length of ten steps. Omron pedometer HJ-322U-E were given to participants who were in physical activity group. Participants were instructed to wear pedometer either in their pocket or clipped with their belts for 12 weeks. Besides, they were instructed to use the pedometer during the day as motivational tools, and to upload their pedometer data every weekend via Omron Bi-LINK software where they could track their overall physical activity progress themselves (**Figure 24a and 24b**).

(a)

(b)



Activity Monitor

Date	Steps	Aerobic Steps	Aerobic Walking Time	Fat Burned	Calories	Distance
21/03/2016 00:00	5,915.00 steps	0.00 aerobic steps	0.00 min	-	110.00 kcal	4.10 km
22/03/2016 00:00	11,274.00 steps	3,000.00 aerobic steps	27.00 min	-	363.00 kcal	8.00 km
23/03/2016 00:00	7,829.00 steps	0.00 aerobic steps	0.00 min	-	151.00 kcal	5.50 km
24/03/2016 00:00	5,554.00 steps	0.00 aerobic steps	0.00 min	-	51.00 kcal	3.90 km
25/03/2016 00:00	11,614.00 steps	7,862.00 aerobic steps	79.00 min	-	444.00 kcal	8.20 km
26/03/2016 00:00	4,644.00 steps	0.00 aerobic steps	0.00 min	-	113.00 kcal	3.20 km
27/03/2016 00:00	10,465.00 steps	7,140.00 aerobic steps	69.00 min	-	449.00 kcal	7.40 km
28/03/2016 00:00	4,897.00 steps	1,916.00 aerobic steps	18.00 min	-	161.00 kcal	3.40 km
29/03/2016 00:00	8,014.00 steps	1,930.00 aerobic steps	18.00 min	-	231.00 kcal	5.60 km
30/03/2016 00:00	7,355.00 steps	0.00 aerobic steps	0.00 min	-	151.00 kcal	5.20 km
31/03/2016 00:00	5,637.00 steps	0.00 aerobic steps	0.00 min	-	81.00 kcal	4.00 km
01/04/2016 00:00	12,198.00 steps	0.00 aerobic steps	0.00 min	-	314.00 kcal	8.60 km
02/04/2016 00:00	1,700.00 steps	0.00 aerobic steps	0.00 min	-	26.00 kcal	1.20 km
03/04/2016 00:00	4,822.00 steps	0.00 aerobic steps	0.00 min	-	175.00 kcal	3.40 km
04/04/2016 00:00	7,888.00 steps	2,024.00 aerobic steps	17.00 min	-	252.00 kcal	5.60 km
05/04/2016 00:00	10,083.00 steps	2,823.00 aerobic steps	25.00 min	-	307.00 kcal	7.10 km

Figure 24 (a) Omron pedometer HJ-322U-E (Milton Keynes,UK), and (b) Omron Bi-LINK; an online database.

3 days of pedometer data is sufficient to estimate free-living adult pedometer-determined physical activity in a week [102], and pedometer data collection over a 7-day period is reliable estimate of monthly activity in adults [103]. The pedometer is most effective as a motivational tool when feedback is used in combination with individualised goal-setting and self-monitoring methods [94]. Goal-setting strategies have been used in pedometer-based interventions such as a generic fixed goal of 10,000 steps per day [104], a fixed increment steps per day over baseline [105, 106]. However, for inactive people, goals have to be individualized to feel confident of being achievable, an absolute step target may be de-motivating if it appears unachievable [107]. Strategies to improve compliance with increasing physical activity include the completion of an activity monitoring diary, reminder calls, SMS/email reminders, motivational hint sheets [93]. The use of daily activity records should be encouraged as a tool to increase self-efficacy and potentially long-term changes in behaviour, since the act of keeping a daily record needs conscious thought about activity levels and serves as a reminder to exercise [108]. A systematic review by Bravata et al., indicated that participants in pedometer-based

programmes who recorded their daily step count significantly increased their physical activity than those who were not required to record their data [97]. Regular follow-ups via mobile text message from healthcare professionals could help patients' self-monitoring of goal achievements [109]. Moreover, motivational physical activity interventions such as educational and motivational content [110] are modestly effective in changing physical activity behaviour among healthy adults [111]. The motivational messages including the benefits of exercise and some tips (e.g. walk up the stairs instead of taking the lift, get off the bus one stop earlier) and informed of the public health guidelines for physical activity are very important to increase awareness of behaviour [112, 113]. In our intervention trial participants received weekly motivational hint sheets through the post to encourage continued engagement in routine exercise on a regular basis over 12 weeks. Each sheet outlined some of the benefits of exercise and offered easy tips to be more active, and also provided diary for participants to record their total steps and other exercises in each day. (see **Figure 25** below).

University of Reading

Get

Active

OLE STUDY

Volunteer name: Week beginning:

OLE STUDY

Get active with us

[Week1]

Being physically active increases lifespan

British researchers in the mid twentieth century compared the risk of dying for bus conductors versus bus drivers, and for postmen and women to sorting office workers. In the US, similar studies were conducted comparing dock workers to desk based staff. These studies all strongly suggested that individuals with more physically active occupations had a lower risk of death.

Be more active

Many of us drive to work, we park close to our workplace and sit at our desks for much of the day. To increase your activity levels try walking to work, or park a five or a ten minute walk from the office. These extra steps all count towards improving your health and a short walk can be a great way to kick start your day.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
Monday		
Tuesday		
Wed		
Thursday		
Friday		
Saturday		
Sunday		

Figure 25 OLE-2 study weekly motivational hint sheets.

An accelerometer is more sophisticated than pedometer since it can measure the intensity of body movement each minute in one (uniaxial), two (biaxial) or three (triaxial) planes [117]. Thus, the physical activity data collected with the pedometer was validated against data collected using the accelerometer in our study. There are many commercially available accelerometer models. ActiGraph GT3X (Pensacola, FL, USA) activity monitors were used in our study, have been widely used as tools to measure activity including water-based activity in many research and clinical applications [118, 119]. They include both a micro-electro-mechanical system (MEMS) based accelerometer that is capable of measuring acceleration in three planes (vertical, front-to-back, and side-to-side) and an ambient light sensor. It is usually worn at the waist, and does not have a display screen to provide feedback to the user; therefore, it is intended for measurement of physical activity, not for motivating people to physical activity (**Figure 22**) [120].

Figure 26 ActiGraph GT3X (Pensacola, FL, USA) activity monitor.

Physical activities are regularly measured by determining the energy expenditure in kilocalories or by using the metabolic equivalent (MET) of the activity [121]. ActiGraph GT3X activity monitors are designed to monitor human activity and record energy expenditure (calories spent during normal activity, METs, everyday activity, and exercise). Device initialization, data processing and analysis were conducted using Actilife Data Analysis Software (Version 6.13.3). Raw data were collected from 7 am. on the first day of monitoring at a 30 Hz sample rate. At least 3–4 days of monitoring are needed to achieve 80% reliability in the variance of activity [122, 123]. It has been suggested that weekdays

as well as weekend days need to be included [124]. Therefore, participants were instructed to wear device directly above the right iliac crest during sleeping and waking hours (except for during water-based activities) for four days, including three weekdays and one weekend day. Participants also were required to completed a 4-d activity diary in order to data cleaning purposes. For analysis inclusion, participants were needed to have made counts on their activity monitor for ≥ 3-d (> 600 min/d of wear time) [125]. Non-wear-time was defined as ≥ 60 min of zero activity counts [119]. Data were summarized in 60-s epochs and mean energy expenditure from PA (EEPA) was calculated (kcal/d). Data were summarized in 60-s epochs [117] and cut-points were used to classify wear time as: sedentary behavior (< 100 counts/min), light/lifestyle PA (760 - 1951 counts/min), moderate PA (1952 - 5724 counts/min), vigorous PA (≥ 5725 counts/min) [126]. The Freedson equation is commonly used to calculate the cut-points for light, moderate, and vigorous activities, and it has been shown to have good agreement with time spent in different intensity categories [127].

However, to correctly interpret physical activity data, it is vital to account for the fact that the activity levels may also vary according to the season. Previous studies have indicated that adults in the UK displayed higher physical activity level in the summer in comparison to winter months [128, 129].

Endpoints	*Physical activity intervention*	*Olive leaf extract intervention*
✔*SPB, DBP*	overweight female (aged 40 -65 y, n=18) SBP (8 mmHg) and DBP (5 mmHg) [130] obese male (aged 32 -59 y, n= 31) SBP (13 mm Hg), DBP (7 mmHg) [86] Healthy, sedentary (aged 50-65 y, n=31) SBP (11.82 mm Hg), DBP (4 mmHg) [131] Mild hypertensive patients (aged 47± 1 y, n= 730) SBP (10.2 mm Hg), DBP (8.4 mmHg) [84] Type II diabetes patients (aged 30-74 y, n=39) SBP (8.4 mmHg), DBP (5.2 mmHg) [132]	The stage-1 hypertension patients (aged, 43-58 y, n=232) SBP (11.5 mm Hg), DBP (4.8 ± 5.5 mm Hg) [133] Overweight male (aged 18-65 y, n= 60) 24hr SBP (3.33 mmHg), Day SBP (3.95 mm Hg), 24hr DBP (2.42 mm Hg), Day DBP (2.48 mm Hg), FTM 24hr SBP (4.76 mm Hg) [134]
✔*HbA1c*	Type II diabetes patients (aged 40-70 y, n=40) HbA1c (0.75mmol/mol)[135]	Type II diabetes patients (adult, n=79) HbA1c (0.9%) [136]

Endpoints	**Physical activity intervention**	**Olive leaf extract intervention**
↓*TAG*	Healthy male (aged 37±9 y, n=44) TAG (23 mg/dl) [137]	The stage-1 hypertension patients (aged, 43-58 y, n=232) TAG (11.9 mg/dL) [133] Overweight male (aged 18-65 y, n= 60) TAG (0.18 mg/dL) [134]
↓*TNF-α*	Healthy male (aged 37±9 y, n=44) TNF-α (0.12pg/ml)[137]	Consecutive cancer patients (aged 20.5± 9 n=25) TNF-α (49.6 pg/mL) [138]

Table 6 Comparison of the health benefits of olive leaf extract versus physical activity in human studies

Table 6 illustrates the common endpoints of intervention studies of both physical activity and olive leaf; increasing physical activity level or consuming olive leaf extract reduce blood pressure; consequently, in this aspect of the study, blood pressure will be used as the primary endpoint with anticipated synergistic benefits for consuming olive leaf extract alongside achieving the recommended guidelines for physical activity. Secondary endpoints will include blood glucose and lipid profile.

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Chapter 3 Potential mechanisms explaining the anti-glycaemic activity of polyphenol rich olive leaf extract

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Abbreviations: FFA, free fatty acid; **GLUT2**, glucose transporter 2; **IC_{50}**, concentration estimated to give 50% inhibition; **OLE**, Olive leaf extract; **OL**, Oleuropein; HT, Hydroxytyrosol **SGLT1**, sodium dependent glucose transporter 1

Keywords: Amylase /Caco-2 cell/ Intestinal glucose transporter /Lipase/Oleuropein/Olive leaf extract

Abstract

Abnormal postprandial hyperglycaemia is a feature of insulin resistance and is considered a risk factor for metabolic and cardiovascular disease. A previous twelve-week randomized, double-blinded, placebo-controlled, crossover dietary intervention study with olive leaf extract led to improved insulin sensitivity in overweight men; the mechanisms for this effect remain uncertain. We hypothesis that olive polyphenols inhibit enzymes involved in glucose and fat digestion in the small intestine, and inhibit glucose uptake by the gut epithelia. The inhibitory action of OLE and its principal polyphenols, hydroxytyrosol and oleuropein, on the intestinal digestive enzymes α-amylase and lipase were assessed in vitro. Further, the level mRNA expression for genes encoding intestinal glucose transporters (SGLT1, GLUT2) was assessed in Caco-2 cells using real time PCR. Glucose uptake in Caco-2 cells was assessed using ^{14}C-labelled glucose. OLE inhibited pancreatic amylase activity with an IC_{50} value of 3.23 ± 0.33 mM and inhibited pancreatic lipase, IC_{50} 1.83 ± 0.03 mM. Cells treated with OLE show reduced glucose uptake and a decrease in the expression of both glucose transporters compared to untreated control. Our data suggest mechanisms that might explain previously observed anti-glycaemic effects of OLE and add weight to growing evidence that olive phenolics may be useful as supplements for the prevention of chronic diseases.

3.1 Introduction

With an ageing and increasingly overweight global population the WHO projects a surging prevalence of type 2 diabetes by 2030 to nearly 4.4% of the population, up from 2.8% in 2000 [106]. Elevated postprandial hyperglycaemia is evident prior to the onset of type 2 diabetes, and poor blood sugar and lipid control in the diabetic condition, are associated with complications, including liver toxicity, renal dysfunction, obesity and cardiovascular disease [107]. Intervention studies have demonstrated that blunting postprandial spikes in glucose and lipids decreases inflammation and improves endothelial function [108]. Thus, dietary, lifestyle or pharmacological approaches to attenuate these spikes may benefit those with impaired glucose tolerance.

Adherence to a Mediterranean dietary pattern reduces the risk of diabetes [109] and improves long term blood sugar control [110]. This dietary pattern is low in meat and rich in fruit and vegetables, and incorporates a modest contribution to energy from olive oil. Independently, supplementation with extra virgin olive oil improves modifiable cardiovascular risk factors in hypertensive volunteers [70] and reduces postprandial glucose and LDL cholesterol in healthy volunteers [22]

As a source of lipids, olive oil is an unremarkable blend of monounsaturated, polyunsaturated and saturated fatty acids, it is however rich in phenolic compounds, principally hydroxytyrosol and oleuropein, which may be of benefit to health [19]. Feeding studies in diabetic rodent models show that both oleuropein [58, 111] and hydroxytyrosol [41] are capable of exerting anti-hyperglycaemic effects. These compounds are present in very high quantities in the oil industry waste by product, olive leaf [9], and interest has therefore grown in the salvaging of these potentially beneficial compounds for use as dietary supplements [76, 112, 113]

There are data showing anti-glycaemic activity for OLE supplements in man: following a 12 week randomised crossover study in 46 overweight male volunteers, consumption of OLE resulted in a 15% improvement in insulin sensitivity compared with placebo [30]. These data are well supported by feeding studies with olive leaf extracts demonstrating anti-glycaemic activity in animal models [47]. A mechanistic explanation for these observations would add support to this evidence and further justify the potential use of

this extract to help control blood sugar, which may be of particular benefit to those at risk of diabetes.

Pharmacological approaches to managing postprandial hyperglycaemia and hypertriglyceridemia include targeting the digestive hydrolysing enzymes responsible for breaking down starches and triglycerides in the small intestine, and also targeting the action of the intestinal glucotransporters [53]. We hypothesised that the phenolics present in olive leaf extract might affect these same systems. Here we report findings from *in vitro* studies into the effects of olive leaf extract on digestive enzyme inhibition and on the expression and activity of glucose transporters in cultured intestinal epithelial cells.

3.2 Materials and Methods

3.2.1 Olive leaf extract

OLE liquid was provided by Comvita (Te Puke, New Zealand). This elixir contains *Olea europaea* (Olive) leaf extract (water, olive leaf solids) each 5 mL equivalent to 5 g fresh olive leaf standardized to 22 mg of oleuropein and with vegetable glycerine (stabiliser).

3.2.2 Chemical materials

Oleuropein, hydroxytyrosol, luteolin, rutin, acarbose, orlistat, phloretin, porcine pancreatic α-amylase enzyme (EC 3.2.1.1), Pancreatic lipase enzyme type II (Sigma product L3126), sodium phosphate buffer, bovine serum albumin, calcium chloride, potato starch, 3, 5-dinitrosalicylic acid (DNS), Triton X-100 and p-nitrophenyl laurate were purchased from Sigma-Aldrich (Poole, UK). Phlorizin dehydrate, fetal bovine serum, Dulbecco's modified Eagle's minimum essential medium (DMEM)(4.5 g/L Glucose with L-Glutamine), penicillin 100 U/mL, Trypsin-versene EDTA, glucose stock solution 100 g/L and Dulbecco's Phosphate Buffered Saline (DPBS) (without Ca & Mg) were purchased from Scientific Laboratory Supplies (Nottingham, UK) QIAGEN RNeasy Mini Kit, QuantiTech SYBR Green PCR kit, RT2 first strand kit and GAPDH, SGLT1, GLUT1 and GLUT2 primer were purchased from QIAGEN (Manchester, UK). Glucose, D-[1-14C]-, 50 µCi (1.85 MBq) was purchased from PerkinElmer (Beaconsfield, UK). Caco-2 cell line was purchased

from American Type Culture Collection (ATCC, Middlesex, UK). All other chemicals were obtained from the Department of Food and Nutritional science, University of Reading.

3.2.3 Inhibition of α- amylase activity

The amylase inhibition assay was slightly modified from that reported by Ahmed et al. Briefly, a maltose standard curve was prepared to determine the optimal concentration of starch solution and porcine pancreatic α-amylase enzyme. OLE, OL, HT and acarbose (as a positive control) were prepared in DMSO at concentrations ranging from 0 to 2.5 mg/mL. DMSO was used as negative control. Porcine pancreatic α-amylase (8U) was suspended in 50 mM sodium phosphate buffer (pH 6.9 at 20°C) containing 50 mM sodium chloride, 0.1% bovine serum albumin and 0.5 mM calcium chloride. Stock starch solution was prepared by dissolving 0.6% (w/v) soluble potato starch in 20 mM Sodium Phosphate Buffer with 6.7 mM Sodium Chloride (pH 6.9 at 20°C) and boiling the mixture for 15 min. A mixture of 0.5 mL of each of the samples and the enzyme solutions was incubated at 37°C for 30 min. Then 1 mL starch solution was added to the reaction mixture and incubated at 20°C for 3 min. Thereafter, 96 mM DNS solution (3, 5- dinitrosalicylic acid solution in sodium potassium tartarate tetrahydrate and 2N NaOH) was added to stop the reaction. For the blank, the DNS solution was added prior to addition of the starch solution. The mixture was then boiled for 10 min. After cooling to room temperature, the reaction mixture was diluted with 9 mL distilled water, and absorbance measured at 540 nm using UV spectrophotometer. Three independent replicates were performed.

3.2.4 Lipase inhibition assay

The lipase inhibition assay was conducted according to a previously described method [114]. The assay buffer was 100 mM Tris buffer (pH 8.2) and p-nitrophenyl laurate (pNP laurate) was used as the substrate. The substrate stock solution was prepared by suspending 0.8% (w/v) pNP laurate dissolved in 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100 and boiled for 1 min for dissolution, this was mixed then cooled to room temperature. Lipase from porcine pancreas type II was dissolved in ultra- pure water at 10 mg/mL and centrifuged at 4,500 rpm for 10 min to provide a supernatant for use in the assay. The negative control assay contained 400 µL assay buffer, 450 µL substrate solution and 150 µL lipase enzyme. OLE, phenolic compounds and orlistat (positive control) were dissolved in DMSO in 50 µL total volume. The buffer, enzyme and test

compounds were mixed with substrate and incubated at 37 °C for 2 hrs. Samples were then centrifuged at 4,500 rpm for 5 min and absorbance read at 400 nm in a UV spectrophotometer. All samples were assayed in triplicate and an inhibitor blank was prepared for each sample.

3.2.5 Cell cultures

Caco-2 cell cultures were prepared in 75-cm^2 flasks in biologically independent manner (n=3) with a seeding density of 3 x 10^4 to 5 x 10^4 viable cells/cm^2) and cultured at 37°C ± 1°C in an atmosphere of 5% CO_2- 95% air. The growth media was DMEM (4.5 g/L Glucose w/L-Glutamine), supplemented with 10% (v/v) heated-inactivated (45 min, 56°C) foetal bovine serum (FBS) + 1% (v/v) Penicillin-Streptomycin. The medium was changed every 2-3 days. Routine passage was carried out at 70-90% confluence with 0.25% Trypsin-EDTA solution and washing with Dulbecco's Phosphate Buffered Saline (DPBS). All experiments were carried out on cells between passage numbers 36 to 55.

3.2.6 Cytotoxicity assay

Caco-2 cells were cultivated in a 96-well culture plate, and incubated for 24 hr at 37°C. After 24 hr, treatments of OLE extract, OL, or HT were applied in DMEM at concentrations of 0.001-0.1 mM, or the equivalent of oleuropein in the OLE, were added to cell monolayers and these were incubated for a further 24 hrs. The medium was then removed from the wells. 100 µL of ice-cold methanol was added to each well and left for 5 min to incubate at room temperature. The methanol was then removed and the 96-well plate air dried in a hood for 15 min. 100 µL of DAPI (4',6-diamidino-2-phenylindole) prepared in DPBS (70 µL of DAPI stock solution (3 mM): 10.43 mL DPBS per plate), was add to each well and the plate then incubated in the dark for at least 30 min at 37°C. The percentage of viable cells relative to control was enumerated using a microplate reader (TECAN) with absorbance and emission at 340 nm and 465 nm, respectively.

3.2.7 Intestinal glucose transporter expression analysis

Caco-2 cells were grown for 19 days in a 6 well culture plate with the media changed every two days. The cells were then treated with 3 mL of OLE, OL and HT or negative control (normal cell media (DMEM)) for 24 hrs. The treated cells were washed three times with 2 mL DPBS, and then collected by scraping into a falcon tube (15 mL). Total mRNA was isolated from adherent cells by using the QIAGEN RNeasy Mini Kit, and cDNA was

synthesized by using RT2 first strand kit both according to manufacturer's instruction (QIAGEN; Manchester, UK). Purification of both mRNA and cDNA were measured by using a Nano drop (ND-1,000 V3.7.1). Following first strand cDNA synthesis the expression levels of GAPDH (house-keeping gene) and the respective glucose transporter mRNA were explored by real time RT-PCR 7300 system and the QuantiTech SYBR Green PCR kit. Primers were GLUT2; forward 5'-AGT TAG ATG AGG AAG TCA AAG CAA-3' and reverse 5'-TAG GCT GTC GGT AGC TGG-3' and SGLT1; forward 5'-TCT TCG ATT ACA TCC AGT CCA-3' and 5'-TCT CCT CTT CCT CAG TCA TC-3'. The PCR condition per cycle was 55°C for 0 min, 95°C for 15 min, 92°C for 30 secs, 55°C for 1 min and 72°C for 30 secs.

3.2.8 Glucose uptake assay

Caco-2 cells were seeded at a density of $1x10^5$ cells/well into Transwell inserts in 6 well plates (Corning® Transwell® poly polycarbonate membrane, pore size 0.4 mm, 24 mm diameter) and left to differentiate for 19 days with the media changed every two days. For the experiment, 1.5 mL of carrier media containing 0.001-0.1 mM were treated with 1.5 mL of samples or negative control (normal cell media (DMEM)) into apical side for 24 hrs prior to uptake studies. The media was discarded and cells were washed twice with 2 mL of HEPES buffer salt (HBSS, pH 7.5, 140 mM; NaCl, 5 mM; KCl, 1 mM; K_2HPO4, 1 mM; $CaCl_2$, 0.5 mM; $MgCl_2$) or HEPES buffer free salt solution (HBSS, pH 7.5,145 mM; KCl, 1 mM; K_2HPO4, 1 mM; $CaCl_2$, 0.5 mM; $MgCl_2$) applied to both upper and lower compartments. 1 mL HEPES buffer salt or free salt solution containing (D-[U-14C] Glucose) 0.5 µCi/mL and 1 mM total glucose was then added to the Apical side. Cells were then incubated at 37°C for 30 min. The media was removed and glucose uptake stopped by washing each membrane twice with ice-cold PBS. 1 mL of 0.1 M NaOH was then added to apical compartment to lyse the cells, and 300 µL of cell lysate was added to 5mL scintillation counting solution (Ultima Gold™) and analysed by scintillation counting using Wallac 1409 DSA Liquid Scintillation Counter.

3.2.9 Statistical analysis

The concentration of the extract required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by regression analysis. Determination of gene expression was

calculated using analysis of relative gene expression via the $2^{-\Delta \Delta CT}$ method [115], and data were normalised to the housekeeping gene (GAPDH). Data were expressed as the mean ± standard deviation (n= 3). Statistical analysis was performed by one-way analysis of variance using the software package SPSS (IBM SPSS Statistics 21). A p value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 α- Amylase inhibition assay

OLE and its phenolic compounds inhibited α-amylase in a dose-dependent manner (0.01-4mM oleuropein equivalent) (**Figure 27**).

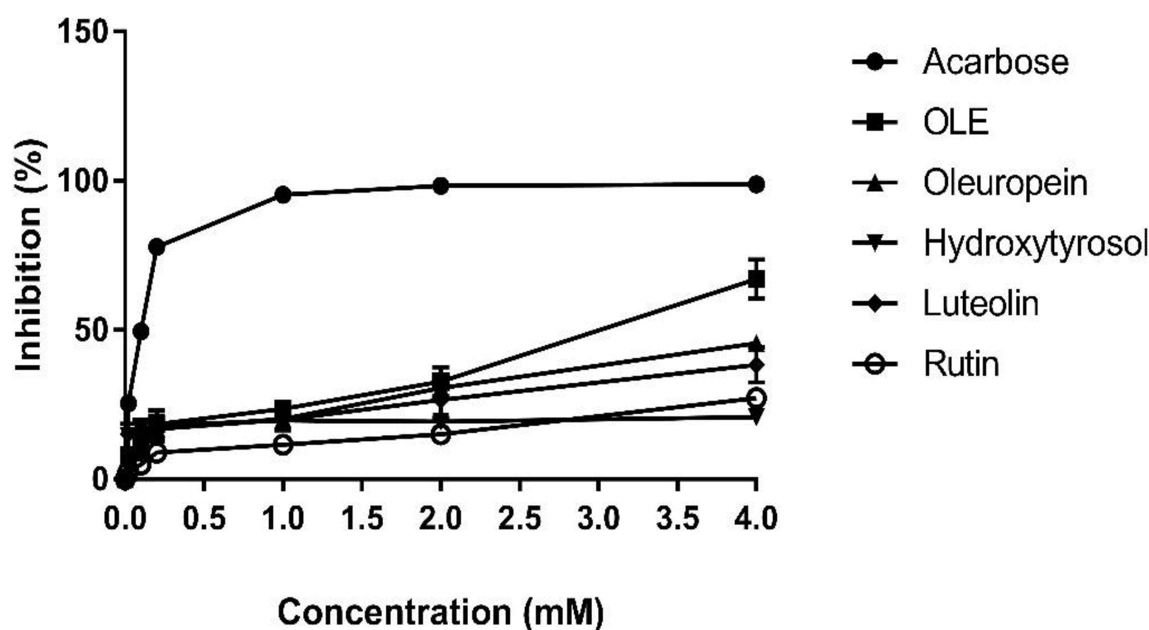


Figure 27 Percent inhibition of pancreatic α-amylase by OLE and individual olive phenolics, oleuropein, hydroxytyrosol, luteolin and rutin. Acarbose was used as a reference alpha amylase inhibitor; the results are expressed as means ± SD, (n=3).

The IC_{50} value for each was compared with the anti-diabetic drug, acarbose (**Table 7**). OLE had an IC_{50} value of 3.23 ± 0.33 mM which is greater than that of acarbose, 0.058 ± 0.001 mM (p = 0.014). Oleuropein had low IC_{50} value, 4.75 ± 0.27 mM, which is not significant

different from OLE. Luteolin, rutin and hydroxytyrosol all demonstrated inhibition of amylase but with higher IC_{50} values than OLE ($p < 0.01$).

Compounds	IC_{50} (mM) pancreatic α-amylase
Acarbose	0.058 ± 0.001^{b}
OLE	3.23 ± 0.33^{a}
Oleuropein	4.75 ± 0.27^{a}
Hydroxytyrosol	$62.23 \pm 3.99^{a,b}$
Luteolin	$12.16 \pm 2.55^{a,b}$
Rutin	$7.41 \pm 0.83^{a,b}$

Table 7 IC_{50} of pancreatic α-amylase by OLE and individual olive phenolics, OL, HT, luteolin and rutin. Acarbose was used as a reference alpha amylase inhibitor; the results are expressed as means ± SD, (n = 3). a: $p < 0.01$ as compared with acarbose. b: $p < 0.01$ as compared with OLE.

3.3.2 Lipase inhibition assay

OLE and pure polyphenols were tested for their ability to inhibit pancreatic lipase *in vitro*. There was a dose dependent reduction in lipase activity for all treatments. Therefore, the highest concentration of OLE (2 mM) tested showed a maximum inhibition of 31.20%. The percentage inhibition varied from 3.9-31.2% from the lowest concentration to the highest concentration of 2 mM. The IC_{50} value of this particular extract was compared with orlistat (**Figure 28**).

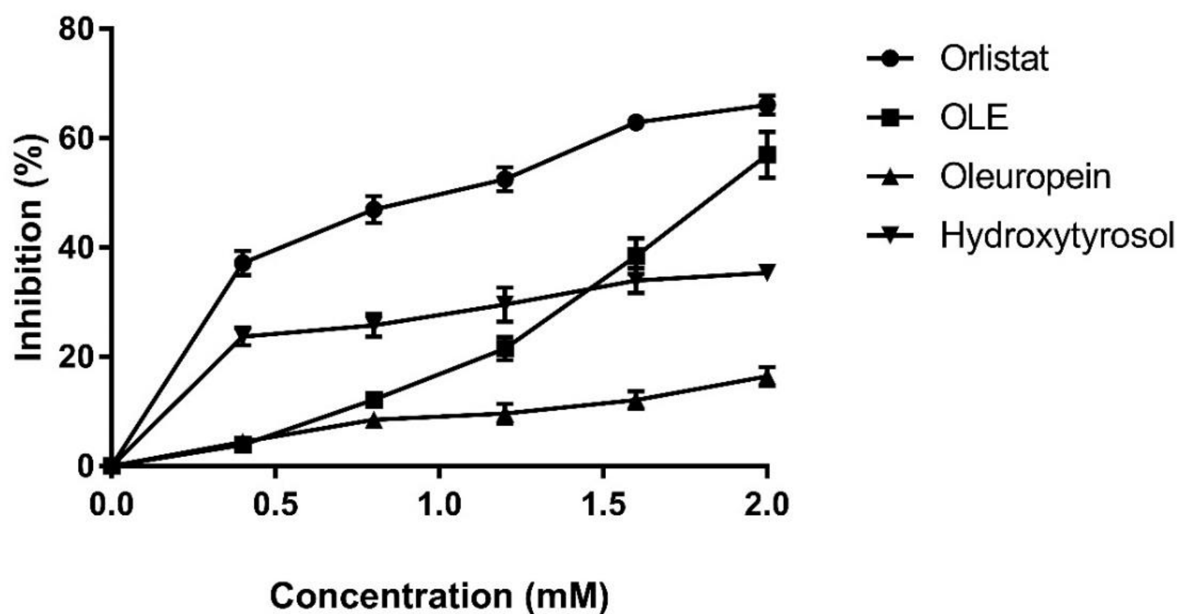


Figure 28 Percent inhibition of pancreatic lipase by OLE and individual olive phenolics, OL, HT. Orlistat was used as a reference alpha amylase inhibitor; the results are expressed as means ± SD, (n=3) (***p* < 0.01).

The IC $_{50}$ value for OLE (1.83 ± 0.03 mM) demonstrates less efficacy than shown for orlistat (1.20 ± 0.02 mM) (p = 0.006) (**Table 8**). Oleuropein and hydroxytyrosol also independently inhibited lipase activity but with higher IC_{50} values than OLE (p < 0.05 and p < 0.01, respectively).

Compounds	IC_{50} (mM) pancreatic lipase
Orlistat	1.20 ± 0.02 [c]
OLE	1.83 ± 0.03 [a]
Oleuropein	6.00 ± 0.36 [a,c]
Hydroxytyrosol	8.68± 0.48 [a,b]

Table 8 IC_{50} of pancreatic lipase by OLE and individual olive phenolics, OL and HT. Orlistat was used as a reference alpha amylase inhibitor; the results are expressed as means ± SD,

(n=3). a: $p < 0.01$ as compared with orlistat. b: $p < 0.05$ as compared with OLE. c: $p < 0.01$ as compared with OLE.

3.3.3 Intestinal glucose transporter expression

No significant toxicity was observed in the Caco2 cells exposed to the OLE at the tested concentrations. However, significant toxicity, as indicated by cell viabilities of less than 70% following exposure, was observed for HT concentrations greater than 0.05 mM and oleuropein concentrations greater than 0.10 mM) following incubation for 24 hrs. (**Figure 29**). As shown in **Figure 30**, OLE exerted a marked down regulation of SGLT1 and GLUT2 mRNA expression ($p < 0.05$). After 24 hr of treatment, the OLE inhibited SGLT1 and GLUT2 mRNA expression in a concentration–dependent manner (0.001-0.1 mM OL equivalent OLE). OL and HT also inhibited expression although at higher doses the inhibition of expression by OL and HTl was lower than OLE ($p < 0.01$), this may have been associated with toxicity.

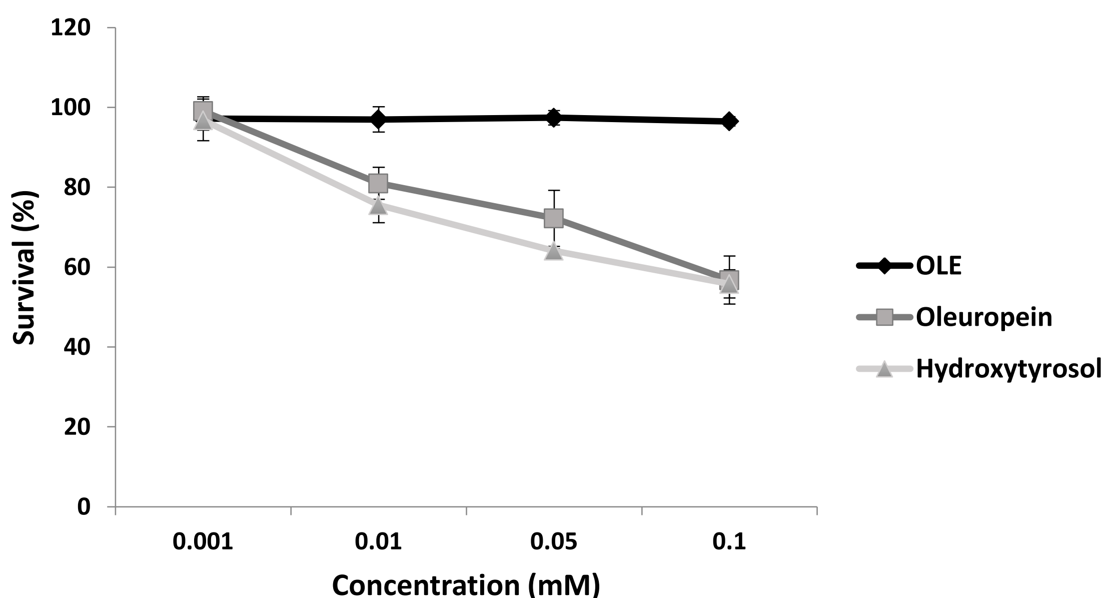


Figure 29 Viability of Caco-2 cells incubated for 24 hr with (0.001, 0.01, 0.05 and 0.1 mM) OLE, OL and HT dissolved in DMEM, as measured by the DAPI method. Each of the four concentrations was evaluated in triplicates. Error bars depict the primary results based on standard deviation.

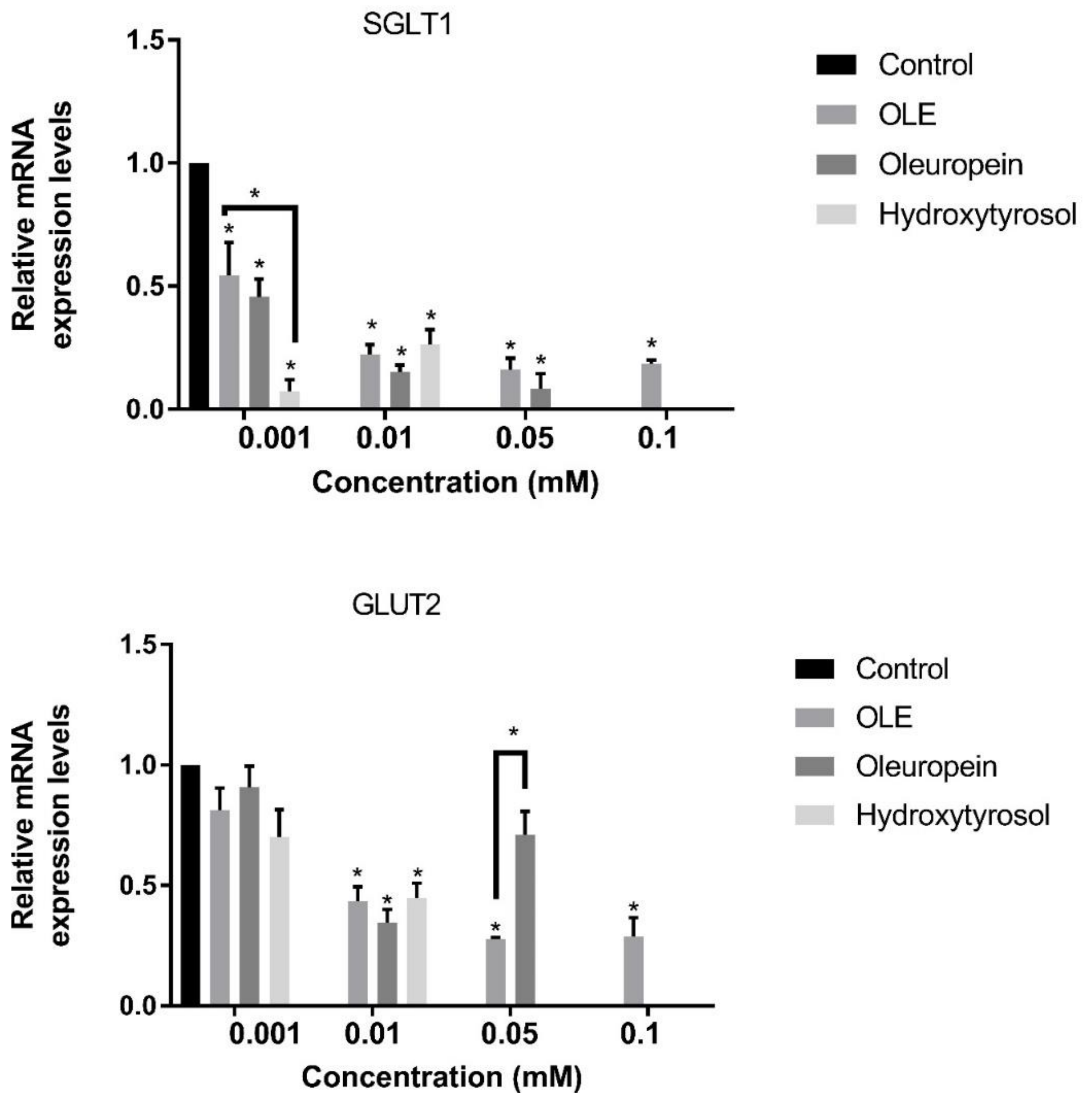
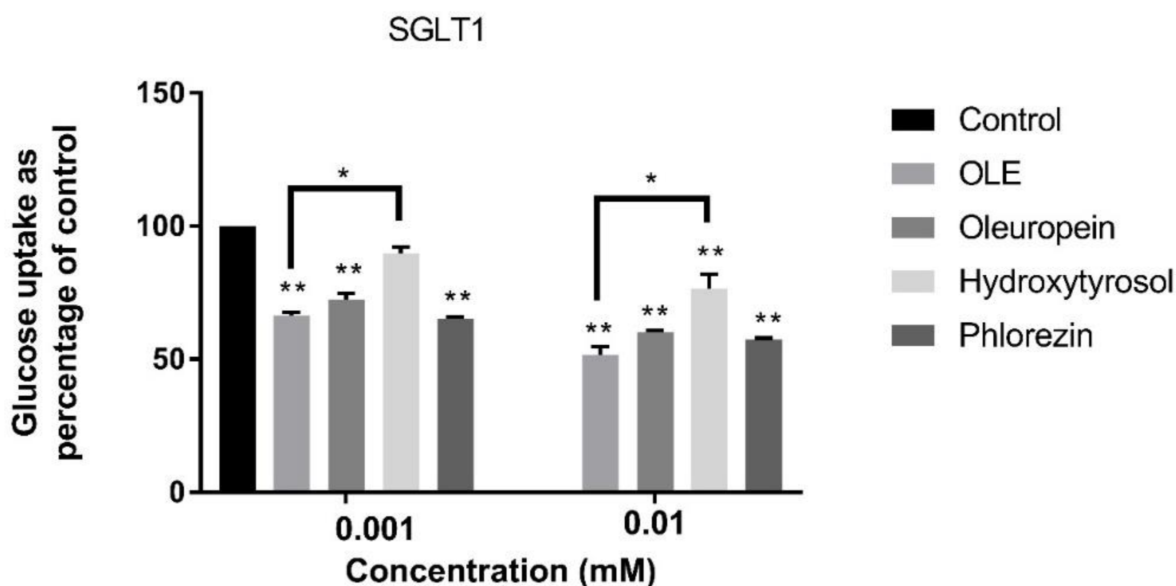


Figure 30 The effect of olive leaf extract and its phenolic compounds; OL and HT on SGLT1 and GLUT2 mRNA levels normalised to GAPDH as a house-keeping gene: real-time PCR analysis of the relative mRNA expression levels of SGLT1 and GLUT2 in differentiated Caco-2 cells treated with 0.001-0.1 mM OLE, 0.001-0.05 mM OL and 0.001-0.01 mM HT for 24 hrs. Normal cell media (DMEM) were used as a negative control. Results represent mean ± SD (n=3). * = p <0.01 compared with the expression in the untreated control group.

3.3.4 Glucose uptake assay

Glucose uptake was measured in sodium-containing buffer, to capture the transport action of both SGLT1 and GLUT 2 or sodium-free buffer to capture only facilitated glucose uptake via the GLUT2 transporter. Both processes were significantly decreased after Caco-2 cells were treated with OLE and its phenolic compounds for 24hrs. The OLE at a concentration equivalent to 0.01 mM) of oleuropein inhibited glucose uptake by 50 % in both experimental conditions. The phenolics tested exerted similar dose dependent reductions in glucose uptake in either the sodium-dependent (SGLT1) or sodium-free conditions (GLUT2) in a concentration–dependent manner (0.001-0.01 mM OL equivalent OLE) (**Figure 31**).



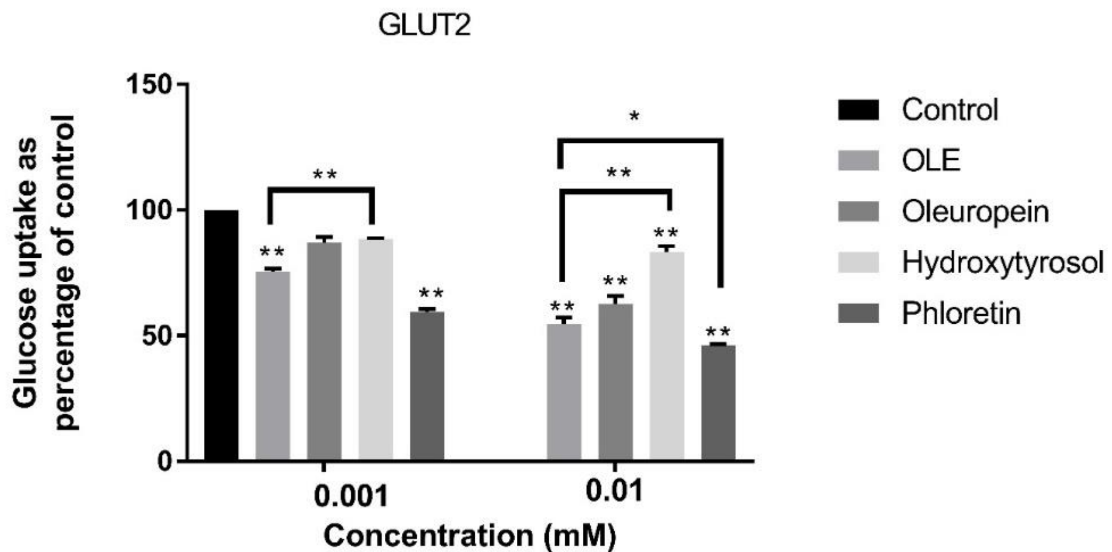


Figure 31 The effect of olive leaf extract and its phenolic compounds; OL and HT on glucose uptake in sodium (SGLT1) and free sodium condition (GLUT2). Caco-2 cells were treated with Caco-2 cells treated with 0.001-0.01 mM OLE, oleuropein and hydroxytyrosol for 24 hrs. Phlorizin and phloretin were used as positive control for SGLT1 and GLUT2 respectively. Normal cell media (DMEM) were used as a negative control. Results represent mean ± SD (n=3) (*p <0.05, **p < 0.01), compared with the respective untreated control group.

3.4 Discussion

Supplementation with OLE, containing the equivalent of 51.04 mg of OL, daily for 12 weeks, significantly improved insulin sensitivity in overweight middle-aged men [61]. Here we have set out to understand the mechanisms underpinning that observation. We hypothesised that the regulation of glucose and fat uptake in the gut might be involved, and we explored this hypothesis under *in vitro* conditions. In our *in vitro* studies, we have used concentrations of OLE extract which might be achievable in the upper gut through supplementation. De bock et al supplemented with a volume of OLE extract equivalent to 51.04 mg of OL, approximating a 1 litre dilution of the supplement capsule in the stomach and small intestine we can estimate concentrations in the small intestine of ~0.094 mM:

$$\left(\frac{51.04\, mg}{540.5\,\frac{mg}{mmol}\; x\; 1\; litre}\right) = 0.094\, mM$$

Besides, we used positives and negative controls which help to differentiate between invalid testing and product failure through observing control test. The positive control is used to assess test validity, while the negative control would infer that the treatment had no effect.

OLE inhibited α-amylase in a dose dependent manner (IC_{50} OLE; 3.23 ± 0.33 mM) although less efficiently than the positive control, acarbose (IC_{50} acarbose; 0.058 ± 0.001 mM). It also inhibited pancreatic lipase (IC_{50} 1.83 ± 0.03 mM), again less efficiently than the positive control orlistat (IC_{50} 01.20 ± 0.02 mM), but which may still be clinically relevant. In addition, olive leaf polyphenols OL, HTl, luteolin and rutin individually inhibit α–amylase and/or lipase with low IC_{50} values, hydroxytyrosol exerted a much weaker effect. Oleuropein, which is the principle polyphenol in OLE, may be exerting the greatest enzyme inhibitory effect in the OLE mixture. OLE and its phenolics also suppressed the expression of the genes for glucose transporters (SGLT1 and GLUT2) in cultured human intestinal epithelial cells (Caco-2 cells). Furthermore, cultured intestinal epithelial cells absorbed less glucose in the presence of OLE relative to control. These data demonstrate mechanisms which might explain observations made in human intervention study. Sales et al. evidenced that food components which delay the release of nutrients into the blood is desirable as this effect may be associated with improved blood sugar and appetite control, and may attenuate cardiovascular and metabolic disease risk [116].

The α amylase inhibitor acarbose has been used in the management of diabetes, however its use can lead to excessive bacterial fermentation of undigested starches in the colon with unwelcome side effects [117]. More subtle, food based approaches may therefore be preferable. A variety of polyphenolic rich plant extracts including those of pumpkin leaf, clove buds, rosella, chrysanthemum, mulberry, blue butterfly pea and *Rumex acetosella* roots, have previously been shown to inhibit α–amylase activity [118-120], possibly via non-specific competition for the enzyme's active site [121]. Sales et al. suggest that the hydroxyl groups on a polyphenol mediate the amylase inhibitory activity

[116]. The phenolic compounds found in OLE, principally OL and HT, are rich in hydroxyl groups, which may form hydrogen bonds with polar groups at the enzymes allosteric site. The chemical structure of polyphenols might also allow non-competitive inhibition. Further experimental work is needed to fully elucidate the interactions involved.

Hyperglycaemia is exacerbated by elevated plasma lipids with excess free fatty acids effectively competing with glucose for uptake in peripheral tissues and enhancing endogenous glucose production [122]. Orlistat, a weight-loss agent, inhibits pancreatic lipase to decrease systemic absorption of dietary fat, but with undesirable side effects [123]. Previous studies report that polyphenol-rich extracts from foods such as berries, red wine, green tea and some dietary phenolic compounds, such as cocoa procyanidins (IC_{50} = 0.034 mM), and tea (-)-epigallocatechin-3,5-digallate (IC_{50} = 0.00028 mM) can inhibit pancreatic lipase inhibitory activity *in vitro* [114, 124, 125]. We demonstrate here that OLE and its phenolic compounds also inhibit lipase, although not as efficiently as orlistat. Orlistat inhibits pancreatic lipases by forming a covalent bond with serine in the active site of the enzyme[123], no data are yet available to explain the inhibitory effect of the olive leaf phenolics.

GLUT2 and SGLT1 protein levels are upregulated about threefold in the intestinal tract of patients with type 2 diabetes [126], suppressing their expression decreases the rate of sugar transport across the gut barrier. Here, we have tested the effects of OLE on the expression of sugar transporters, GLUT2 and SGLT1, in intestinal Caco-2 cells, at sub-toxic concentrations achievable in the intestinal tract with supplementation. Caco-2 cells have a small intestinal phenotype and express high levels of both transporters. We demonstrated a dose-dependent decrease in the level of GLUT2 and SGLT1 mRNA in Caco-2 cells following a 24 hrs exposure to OLE or its phenolics (oleuropein and hydroxytyrosol). Polyphenol rich berry extracts induce a similar down regulation in GLUT2 and SGLT1 expression [127]. We then assessed the effects of OLE on radiolabelled glucose uptake in the Caco-2 cells under two distinct conditions: firstly, in the presence of sodium, thereby favouring glucose uptake via SGLT1, and secondly in the absence of sodium, favouring glucose uptake via GLUT2. In parallel to the decrease in glucose transporter expression, we observed a decrease in uptake of the glucose under both conditions with cells pre-incubated with OLE or its polyphenols. Green tea, strawberry and apple extracts are also shown to inhibit both SGLT1 and GLUT2 mediated uptake of

radiolabelled glucose into Caco-2 cells [128, 129]. It has been suggested that polyphenol-glucosides act primarily on sodium-dependent transport, while the corresponding aglycones regulate facilitated transport [130], however, we found that in this case the glycosides, OL and HT inhibited the expression of both transporters in a dose dependent manner (GLUT2 and SGLT1).

The strengths of this work are that we have explored a variety of potential mechanisms to directly explain the findings of an intervention study using a comparable dose as a treatment. Our data have demonstrated a number of mechanisms which might contribute to the in vivo anti-hyper-glycaemic activity, and these data are reinforced by similar findings for other polyphenol rich foods elsewhere in the literature. The weaknesses of this study are that we used *in vitro* models and that we did not account for the effects of digestive processes on the bioactivity of our compounds. However, oleuropein has previously been shown to be resistant to simulated gastric digestion by Ahmad-Qasim et al. although other olive phenolics were significantly degraded in that model [131].

Delaying the release of nutrients into the blood has implications for blood sugar, plasma lipids and appetite control, and may therefore attenuate cardiovascular and metabolic disease risk. Our data offer mechanistic support to intervention study demonstrating that OLE and its phenolics may confer protection from metabolic disease when consumed as part of a healthy diet and lifestyle.

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Chapter 4 Polyphenol Rich, Olive Leaf Extract Demonstrates Potential Hypotensive Activities *In Vitro*

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Abbreviations: ACE, Angiotensin-converting enzyme; **HUVECs**, Human umbilical vein endothelial cells; **IC_{50}**, concentration estimated to give 50% inhibition; **NO**, Nitric oxide; **OLE**, Olive leaf extract; **RAS**, Renin-angiotensin system

Keywords: ACE; HUVECs; Nitric oxide production; Olive leaf extract; Renin

Abstract

Previous studies have demonstrated an anti-hypertensive effect for polyphenol rich OLE in both human volunteers and in experimental animals. The mechanisms for this effect remain undefined. We hypothesised that olive phenolics may enhance NO production and/or inhibit angiotensin enzymes resulting in improved blood pressure control. To test these hypotheses, NO production was measured in HUVEC cells using an indirect method (Griess reagent method). Further, the inhibitory effects of OLE and its principal polyphenols, hydroxytyrosol and oleuropein, delivered at doses achievable in the bloodstream, were assessed against renin and against the angiotensin converting enzyme (ACE). OLE, and separately, the individual olive polyphenols, at 100 µM concentrations in cell culture carrier media, induced increases in NO production compared to untreated controls. In addition, OLE inhibited renin activity with an IC_{50} value of 63.08 ± 2.90 µM, oleuropein and to a lesser extent hydroxytrosol both also inhibited Renin activity. OLE extract also inhibited ACE activity, IC_{50} 60.86 ± 5.68 µM; oleuropein had no significant effect on ACE activity, but hydroxytyrosol induced a comparable level of ACE inhibition to OLE. These results demonstrate plausible mechanisms which might explain the observed hypotensive effects of consuming OLE. The benefits of OLE may be attributable to the synergistic presence of oleuropein and hydroxytyrosol acting on different components of the blood pressure regulation systems. The complete food supplement may be more useful for the prevention of hypertension related disease than the supplementation of either pure hydroxytyrosol or oleuropein.

4.1 Introduction

Hypertension is a major age-related risk factor for the development of cardiovascular diseases including stroke, kidney disease, heart failure and myocardial infarction [1]. The prevention and treatment of hypertension is a long standing public health priority. Anti-hypertensive drugs, include ACE inhibitors, angiotensin II receptor blockers, and calcium-channel blockers. The use of these drugs may be associated with undesirable side effects, including persistent cough, renal failure, postural hypotension, and angioedema [2]. Therefore, including anti-hypertensive foods and/or supplements in the diet might be both more cost effective and have fewer potential side effects. Polyphenol rich foods such as cocoa, tea, wine and olive oil are associated with lower blood pressure in epidemiological studies and with improved endothelial function in intervention study [3-6]. It is hypothesised that polyphenols may attenuate vasodilator activity by influencing the generation of NO in the vascular endothelium [7]. Red wine, and green tea (-) epicatechin for example have both been shown to induce vasorelaxation via a mechanism related to nitric oxide (NO) production [8].

The Mediterranean diet is associated with a reduced risk of CVD. A key feature of the diet is a moderate to high consumption of olives and of olive oil. Olive leaf is a by-product of the olive industry it is rich in polyphenols which can be extracted for use in supplements. In human intervention and animal model studies these extracts are shown to exhibit anti-hypertensive effects [9-12]. Understanding the mechanisms of action of bioactive food components is essential to proving efficacy and ensuring the public are not misled. The present study therefore aims to investigate the in vitro production of NO in cultured human umbilical vein endothelial cells (HUVECs) in response to OLE, and in response to the olive polyphenols contained within OLE, and further, to explore the effects of these compounds on the activity of RAS enzymes.

4.2 Materials and Methods

4.2.1 Olive leaf extract

Olive leaf extract was provided as an elixir by Comvita (Te Puke, New Zealand). This elixir contains Olea europaea (Olive) leaf extract (water, olive leaf solids) each 5 mL is equivalent to 5g of fresh olive leaf, standardized to 22 mg of oleuropein, and with a vegetable based glycerine stabiliser.

4.2.2 Chemical materials

OL, HT, captopril, aliskinrine, angiotensin converting enzyme (A6778 Sigma), sodium chloride, tris (hydroxymethyl) methylamine, hydrochloric acid, FAPGG – N-[3-(2-furyl) acryloyl]-Phe-Gly-Gly, glycerol solution and Nitrite/Nitrate Assay Kit, including NaNO2 Standard Solution (100 mM), NaNO3 Standard Solution (100 mM), Buffer solution (20 mM, pH 7.6), Nitrate reductase (lyophilized), Enzyme Co-factor (lyophilized), Griess Reagent A and Griess Reagent B were all purchased from Sigma-Aldrich (Poole, UK). The human recombinant renin inhibitor screening assay kit, including human recombinant renin, substrate (Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg) and Tris-HCl buffer (50 mM, pH 8.0, containing 100 mM NaCl) was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.). Human Umbilical Vein Endothelial Cells (HUVEC), Trypsin Neutralizer Solution, Low Serum Growth Supplement (LSGS), Trypsin/EDTA Solution (TE) and Medium 200 (Gibco™) were purchased from Fisher Scientific UK Ltd (Loughborough, UK). All other chemicals were obtained from the Department of Food and Nutritional science, University of Reading.

4.2.3 Cell cultures

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Cells were prepared in three 75-cm2 flasks in biologically independent manner (n=3) with a seeding density of 2.5 x 10^3 cells/cm^2 and they were grown in Medium 200 supplemented with Low Serum Growth Supplement (LSGS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture media was changed every other day thereafter, until approximately 80% confluent. Routine passage was carried out with phosphate-buffered saline solution (1x) containing 0.025% trypsin and 0.01% EDTA and neutralizing with phosphate buffered saline solution (1x) containing 0.5% new born bovine serum. HUVEC cells from passage 3 after purchase were used for experiments.

HUVEC cells were cultured in 12 well plates at a density of $1x10^5$ cells/well and treated with OLE, OL and HT (1-100 μM) for 24hrs. Medium was removed and stored at -80 °C until NO measurement.

4.2.4 Determination of Endothelial Nitric Oxide Production

Production of NO by HUVEC was measured as its stable oxidation product; nitrite, using colorimetric nitrite/nitrate assay kit (Sigma). Briefly, cell culture media was centrifuged at 1000 x g for 15 min to remove insoluble material. In a 96 well plate, 50 μL of the culture medium supernatant was diluted with 30 μL assay buffer and mixed with 10 μL of the nitrate reductase solution and 10 μL of the enzyme co-factors solution. The plate was incubated at 25°C for 2 hrs to convert nitrate to nitrite. Then 50 μL Griess reagent A (sulphanilamide) was added to all wells. After 5 minutes at 25°C of incubation with Griess reagent A, 50 μL Griess reagent B (naphthalene-ethylenediamine dihydrochloride) was added to all wells following 10 min incubation at 25°C. Total nitrite was measured at 540 nm absorbance using a Tecan micro plate reader.

4.2.5 Renin inhibition assay

Renin inhibitory activity was determined using a human recombinant renin inhibitor screening assay kit (Cayman, Michigan, USA). Prior to the assay, 50 μL of human recombinant renin enzyme solution was diluted with assay buffer consisting of 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl (assay buffer). A 95µM solution of renin substrate (Arg-Glu(EDANS)-Ile-His-Pro-Phe-Leu-Val-Ile-His-Thr-Lys (Dabcyl)-Arg) in dimethylsulfoxide (DMSO) (substrate solution) was ready to use as supplied. The positive control wells contained 150 µL of assay buffer and 10 µL of DMSO. Aliskiren (positive control), OLE and its phenolic compounds were dissolved in DMSO at a range of concentrations (1-100 µM). 150 µL of assay buffer and 10 µL of the test compound solution were prepared in a 96 well dark polypropylene plate. Assay blanks were prepared in wells containing 150 µL of assay buffer and 10 µL of DMSO. The reaction was initiated by adding 10 µL of renin enzyme solution to the control and test compound wells. The microplate was covered and shaken for 10 secs. The reaction plate was incubated at 37 °C for 15 min. The fluorescence intensity (FI) was determined on a fluorescence plate reader (Tecan micro plate reader), with an excitation of 340 nm and an

emission wavelength of 465 nm. The analyses were performed in triplicate. The renin inhibitory activity was calculated as follows:

Renin inhibitory activity (Inhibition%) = (FI(control)- FI(sample))/(FI(control)) × 100

The IC_{50} value was defined as the concentration of the inhibitor required to inhibit 50% of the renin activity, and calculated using the linear function of percentage renin inhibition versus the logarithm of the inhibitor concentration.

4.2.6 ACE inhibition assay

The method was adapted from a previous study with slight modifications [33]. Briefly, a substrate solution (0.88 mM FAPGG) was prepared by mixing 0.0176 g of N-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG), in 40 mL of 50 mM Tris-HCL buffer with 300 mM of NaCl, pH 7.5 (assay buffer). The enzyme solution (250 mU) was prepared by mixing ACE (1U) in 4 mL of 0.1 M Tris and 0.6 M NaCl in 86% glycerol solution. Captopril (positive control), OLE and its phenolic compounds were dissolved in assay buffer at the desired concentration (0.7-50 µM). In the first step, 150 µL of 0.088 mM FRAPGG and 10 µl of test compounds were mixed in each well. The mixture was incubated at 37 °C for 5 minutes. The reaction was catalysed by adding 10 µL ACE enzyme (250 mU). For the control, assay buffer was used instead of the test compounds. The product of the reaction was assessed at 340 nm after 30 min. The ACE inhibition was expressed as percentage of inhibition and calculated by the following equation:

ACEi%= = (1-(ρAinhibitor)/ρAcontrol) × 100

ρAinhibitor = slope with inhibitor

ρAcontrol = slope with control

The IC_{50} value was defined as the concentration of the inhibitor required to inhibit 50% of the ACE activity, and calculated using the linear function of percentage ACE inhibition versus the logarithm of the inhibitor concentration.

4.2.7 Statistical analysis

All data are the means of triplicate analyses and expressed as means ± standard deviation. Data were analysed using IBM SPSS Statistics version 21.0. A one-way analysis

of variance was applied to compare treatment effects. A p value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Determination of Endothelial Nitric Oxide Production

OLE and its associated polyphenols were tested for their ability to enhance NO production. There was a significant increase ($p < 0.001$) in the level of NO produced by HUVEC cells treated with OLE at a concentration equivalent to 100 µM of OL, as compared to the carrier control. At the highest concentrations tested (100 µM), both oleuropein and hydroxytyrosol induced significant increases in the level of NO produced compared to control ($p < 0.001$). Neither OL or HT performed significantly differently to the OLE (**Figure 32**). There appears to be a dose response but at the lower concentrations, the OLE and the polyphenols did not raise NO significantly above carrier control.

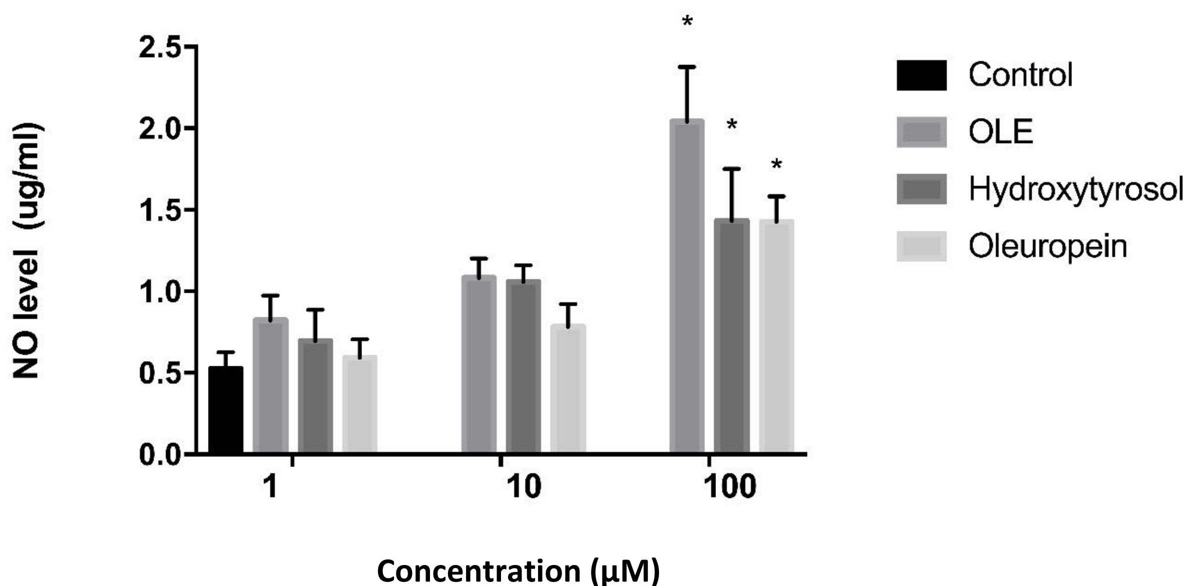
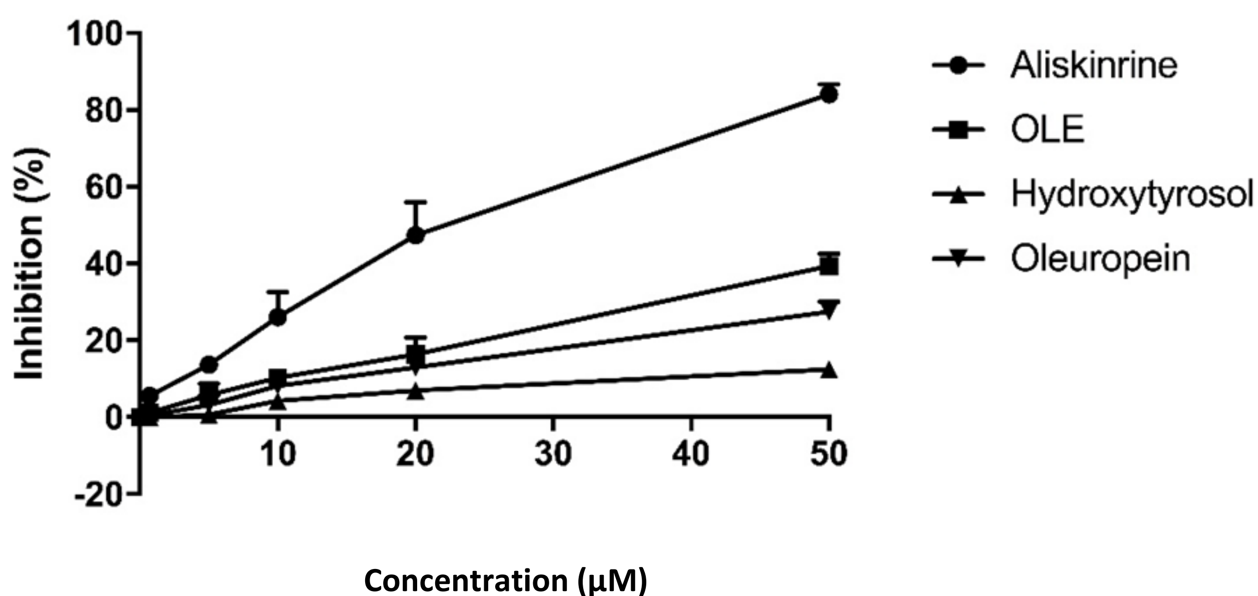


Figure 32 The effect of olive leaf extract and its phenolic compounds; OL and HT on NO production. HUVECs treated with 1-100 µM OLE, OL and HT for 24 hrs. Bar chart showing NO level in control, OLE, OL, and HT. Values are expressed as means ± SD of n = 4. *$p < 0.05$ versus control.

4.3.2 Renin inhibition assay

Aliskerin, the positive control, inhibited renin with high efficacy at low concentrations (IC_{50} value 24.50 ± 0.02 μM). OLE suppressed renin activity with less-efficacy than aliskerin, but in a dose-dependent manner (1-50 μM oleuropein equivalents). The percentage inhibition of OLE varied from 1.12-39.96% from the lowest concentration to the highest concentration tested (**Figure 33**) with an IC_{50} value of 59.63 ± 0.06 μM (expressed as equivalent oleuropein content) (**Table 9**); OL also independently inhibited renin in a dose dependent manner (IC_{50} 94.49 ± 4.71 μM) and may account for the bulk of the inhibition observed with the elixir. Hydroxytyrosol exerted some enzyme inhibition again in a dose dependent manner but much more weakly then OLE and OL (IC_{50} 193.6 ± 12.07 μM).



Concentration (μM)

Figure 33 Percent inhibition of renin by OLE and individual olive phenolics, oleuropein, and hydroxytyrosol. Aliskinrine was used as a reference renin inhibitor; the results are expressed as means ± SD (n=3).

Compounds	IC_{50} (µM) renin
Aliskinrine	26.01 ± 0.82
OLE	63.08 ± 2.90
Oleuropein	94.49 ± 4.71[a]
Hydroxytyrosol	193.6 ± 12.07 [a,b]

Table 9 IC_{50} of renin by OLE and individual olive phenolics, oleuropein, and hydroxytyrosol. Aliskinrine was used as a reference renin inhibitor; the results are expressed as means ± SD, (n=3). a: $p < 0.01$ as compared with aliskinrine. b: $p < 0.01$ as compared with OLE.

4.3.3 ACE inhibition assay

The percentage inhibition of ACE exhibited by OLE, and its derivatives are shown in **Figure 34**. Captopril was used as a positive control and was shown to inhibit ACE activity with high efficacy (IC_{50} 19.16 ± 0.89 µM) (**Table 10**). OLE induced a dose dependent inhibition of ACE activity with less efficacy than captopril (IC_{50} 60.86 ± 5.68 µM (expressed as equivalent oleuropein content)). Hydroxytyrosol inhibited ACE activity with similar efficacy to OLE, (IC_{50} value of 70.87 ± 6.49 µM) and thus may explain the bulk of ACE inhibitory activity of the OLE. Oleuropein relatively weakly inhibited ACE (IC_{50} 135.21 ± 14.52 µM) albeit in a dose dependent manner.

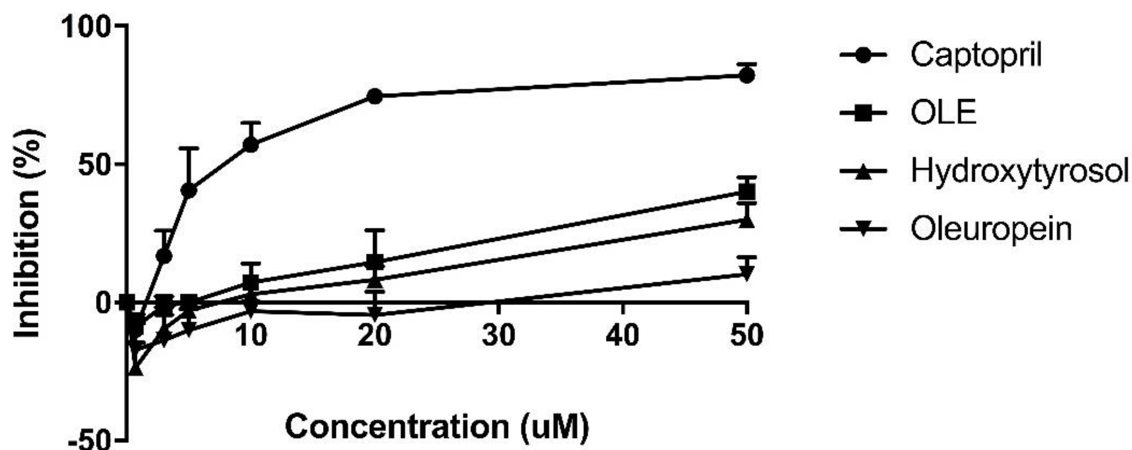


Figure 34 Percent inhibition of ACE by OLE and individual olive phenolics, oleuropein, hydroxytyrosol. Captopril was used as a reference alpha amylase inhibitor; the results are expressed as means ± SEM, (n=3).

Compounds	IC_{50} (µM) ACE
Captopril	19.16 ± 0.89 [b]
OLE	60.86 ± 5.68[a]
Oleuropein	135.21 ± 14.52 [a,c]
Hydroxytyrosol	70.87± 6.49 [a]

Table 10 IC_{50} of ACE by OLE and individual olive phenolics, oleuropein, and hydroxytyrosol. Captopril was used as a reference ACE inhibitor; the results are expressed as means ± SD, (n=3). a: $p < 0.05$ as compared with captopril. b: P<0.05 as compared with OLE. c: $p < 0.01$ as compared with OLE.

4.4 Discussion

A dietary intervention study with daily OLE containing 136 mg of OL has previously been shown to reduce blood pressure in pre-hypertensive male volunteers [10]. The purpose of this study was to understand the mechanism of action underpinning that observation. We

hypothesised that polyphenols present in OLE might stimulate NO production in endothelial cells and/or inhibit the enzymes involved in the renin-angiotensin system. In our *in vitro* studies, we have used concentrations of OLE extract which might be detectable in human plasma through supplementation. De Bock et al. demonstrated that following ingestion of OLE, containing 51.1-76.6 mg OL, plasma metabolites of OL and HT are detectable at 0.3-1 μM concentrations [13]. The HUVEC cell line is widely used as an NO producing model of vascular function [14]. We show that treating HUVEC cells with OLE and with the olive polyphenols, OL and HT, resulted in significantly increased NO production. This is consistent with a previous observation from Romero et al. who report endothelium-dependent, NO mediated vasorelaxation in rat aorta treated with OLE [12]. *In vitro* elucidating the active fraction of OLE responsible for inducing NO release in the HUVEC cells, and we therefore tested the effects of the two principle phenolic compounds present in OLE, oleuropein and hydroxytyrosol; both induced NO production. OL, HT and the minor olive phenolics, verbascoside, rutin and luteolin, have previously been shown to induce dose dependent increases in NO production [14-19]. These effects are seemingly not unique to olive polyphenols but are a characteristic response to an array of plant derived-phenolics; caffeic acid, resveratrol, quercetin, epigallocatechin gallate, and rutin have all been shown to significantly increase NO levels, inducing eNOS mRNA expression, protein synthesis, and eNOS activity [8, 14]. Storniolo et al, suggest that olive oil polyphenols modulate NO production through the control of Ser1177 eNOS phosphorylation and eliciting changes in [Ca^{2+}] in smooth muscle [20]. Intervention with a dose of OLE containing 136 mg OL is unlikely to achieve circulating OL or HT approaching the 100 μM concentration, and the biological activity of the individual metabolites present in blood has not been quantified. We therefore conclude that the NO stimulatory activity of the individual phenolics play relatively minor roles in the total effect of OLE consumption on blood pressure.

The renin-angiotensin system affects control over blood pressure via activation of angiotensin II, which regulates blood volume as well as vasodilation, independently of NO [21, 22]. Renin cleaves the N-terminus of angiotensinogen to yield angiotensin I, which may be further hydrolysed by ACE to release a potent vasoconstrictor (angiotensin II) [23]. Like NO synthase, the components of the renin-angiotensin system are frequently targeted for the therapeutic control of hypertension [22]. Here we examined the effects

of OLE and its phenolic compounds on the renin-angiotensin system as an alternative, or perhaps complimentary, mechanism of anti-hypertensive action to the effects on NO synthesis. OLE inhibited renin with an IC_{50} value of 63.08 ± 2.90 (µM oleuropein equivalent) versus 26.01 ± 0.82 µM for the pharmacological agent Aliskiren which was used as a positive control. It also suppressed ACE activity in a dose dependent manner (IC_{50} OLE; 60.86 ± 5.68 µM) although less efficiently than the positive control, and pharmacological agent captopril (IC_{50} captopril; 19.16 ± 0.89 µM).

Other polyphenol-rich foods have previously shown to influence the RAS system, and phenolic compounds including (-)-epigallocatechin gallate (IC_{50} = 44.53 µM), (-)-epicatechin gallate (IC_{50} = 619 µM) and (-)-epigallocatechin (IC_{50} = 2175 µM) are individually also effective against renin in vitro [24, 25]. Similarly, extracts of blueberry (IC_{50} = 46 µg/mL), Hibiscus sabdariffa (IC_{50} = 91 µg/mL), green tea (IC_{50} = 125 µg/mL), and Senecio inaequidens (IC_{50} = 192 µg/mL) have been shown to inhibit ACE activity. And both quercetin (IC_{50} = 0.42 mM), and tannic acid (IC_{50} = 0.23 mM) have been identified as ACE inhibitors in vitro [26-30]. We observed that OLE inhibits ACE in a dose-dependent manner; and further, that hydroxytyrosol (IC_{50}= 70.87± 6.49 µM) is more inhibitory than oleuropein (IC_{50}= 135.21 ± 14.52 µM). Hansen et al. also reported higher ACE inhibitory activity for hydroxytyrosol relative to oleuropein (95%; at 0.33 mg/mL versus 8% of ACE enzyme at the same dose [31]. The mechanism of ACE inhibition is uncertain but presence of the B-ring and the hydroxyl groups may be of importance [26]. Substitution of the hydroxyl groups for methoxyl groups decreased the ACE activity of rutin, luteolin and quercetin and kaempferol [32]. This may explain the differences in the functionality of hydroxytyrosol versus oleuropein in ACE inhibition.

The data we have presented suggests diverse and perhaps synergistic mechanisms which might explain the observed functional reduction in blood pressure. OLE extract may work directly on both NO release and on the renin-angiotensin system.

The weaknesses of this study are that it is entirely in vitro in nature and that we only report bioactivity data on the two principle olive leave phenolics; other small molecules present in the olive leaf extract may also have bioactivities, further we have not sought to determine the biological effects of the individual circulating metabolites of oleuropein and hydroxytyrosol.

The strengths of the study are that we are replicating the observations from a human dietary intervention study in established models of blood pressure control. It is important to provide mechanistic support explain to the observed anti-hypertensive effects of OLE. This mechanistic understanding adds strength to any guidance that may be given around the use of olive leaf extract in the management of hypertension. The use of a supplemental extract recovered from food production waste is attractive commercially and may be preferable to pharmacological intervention in the prevention of hypertension.

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Chapter 5 The synergistic anti-hypertensive effects of consuming olive leaf extract alongside increasing physical activity

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Abbreviations: 24-h **ABP**, 24-hour Ambulatory blood pressure; **BMI**, body mass index; **SBP**, Systolic blood pressure; **DBP**, Diastolic blood pressure; **OLE**, Olive leaf extract; **PA,** Physical activity

Keywords: 24-h ambulatory blood pressure/ Hypertension/ Olive leaf extract / Oleuropein/ Polyphenols/ Physical activity

Abstract

The olive leaf is rich in polyphenols that can be extracted (Olive Leaf Extract; OLE) for use as a food supplement or nutraceutical. Recent randomised control trials demonstrate hypotensive effects for OLE when consumed by pre-hypertensive volunteers. Advocating the consumption of a supplement for the prevention of hypertension is a sub-optimal public health approach as supplement usage can be used to justify an otherwise unhealthy lifestyle. Accordingly, evidencing the synergistic benefits of including a supplement as part of a healthy lifestyle is desirable. To evidence the synergistic benefits of consuming plant (and specifically olive) phenolics alongside being more physically active in individuals with elevated blood pressure. In this randomized, double blinded, four-arm parallel study, 63 pre-hypertensive overweight adults aged 25-70y were assigned to one of four treatment arms for 12 weeks: i) a placebo control group- with a daily capsule ii) an increasing physical activity and a placebo capsule group iii) an olive leaf extract arm (equivalent to 132 mg of oleuropein per day (OLE); iv) a physical activity and olive leaf extract arm. Individuals on the physical activity arms were equipped with pedometers and received weekly motivational guidance on gradually increasing physical activity over 12 weeks. The primary outcome measure for the study was the change in 24-h ambulatory blood pressure. Secondary outcome measures were arterial stiffness index, plasma HbA1C levels, fasted glucose and insulin, serum lipid profiles and body composition. 24-h systolic blood pressure (SBP) (-5.80 mmHg ± 7.63, p = 0.011) and day time SBP (-5.07 mmHg ± 7.16, p = 0.025) were significantly lower following OLE intake. The PA intervention alone resulted in lower 24-h SBP (-3.69 mmHg ± 9.39), p = 0.031) and daytime SBP (-4.19 mmHg ± 10.27), p = 0.041). Consuming the OLE alongside being increasing physical active reduced 24-h SBP (-3.88 mmHg ± 6.65), p = 0.027) from baseline. There were however, no effects on stiffness index, body composition, lipids, HbA1c, glucose and insulin in all groups after 12 weeks. Our data support previous research, showing that both OLE, and increasing PA, independently reduce systolic blood pressure in pre-hypertensive volunteers, there is no additive effect of combining PA with OLE.

5.1 Introduction

Hypertension affects 30% of the UK population and is causally implicated in the aetiology of renal disease, cardiovascular disease and stroke. Ageing, obesity, a poor diet and low levels of physical activity are all risk factors [1]. A meta-analysis of studies in the treatment of systolic hypertension demonstrated that for every 20 mmHg reduction in systolic BP there is a 40-45% reduction in CVD risk [2]. Adherence to a Mediterranean diet is protective against hypertension and its associated morbidities and olive oil is believed to be a key beneficially bioactive component of that diet [3]. As a source of lipids, olive oil is an unremarkable blend of monounsaturated, polyunsaturated and saturated fatty acids. It is however also rich in phenolic compounds, principally OL and HT, which may be of benefit to health. A recent randomised intervention trial, in predominantly hypertensive volunteers, showed that adherence to a Mediterranean diet supplemented with extra virgin olive oil reduced blood pressure and other measures of cardiovascular disease risk [4]. Olive phenolics can be extracted cheaply from the waste products of olive oil manufacture, such as the plant leaf for use as dietary supplements. In intervention studies in hypertensive or borderline hypertensive patients, olive leaf extract consumption has been shown to reduce blood pressure and improve vascular function [5-9].

Another intervention with established efficacy for improving blood pressure is to increase physical activity [10]. Engaging in regular PA is effective at preventing cardiovascular disease[11], type 2 diabetes [12], cancer [13], obesity [14], and mental health problems [15]. The 'Start Active, Stay Active', Chief Medical Officers report on physical activity recommends that adults achieve 150 minutes of moderate intensity physical activity per week, while data in that report suggest that fewer than 40% of adult men and 30% of adult women achieve these targets. Adherence to the physical activity guidelines may in fact be much worse in sub-sections of the population at higher risk of hypertension. Research evidencing best practice for population specific interventions is therefore needed [16, 17]. Home based interventions of moderate intensity, such as increased walking activity may be the most effective approach to increasing activity levels over the long-term [18]. One promising strategy for motivating PA is the use of pedometers [19]. Pedometers are relatively cheap, non-invasive, easy to use and can provide immediate personalised feedback [20]. Feedback from pedometer step counts has been shown to

prompt behaviour change as it raises awareness of current walking behaviours[21]. However, pedometers may be more effective as a motivational tool when pedometer feedback is used in combination with individualised goal-setting and self-monitoring methods (recording/PA per day)[22].

From a public health perspective, holistic guidelines for the prevention of hypertension, or its early diagnosis and management, based around a healthy diet and lifestyle are preferable to pharmaceutical intervention. Lifestyle interventions are economically favourable and they come with fewer side effects and perhaps wider health benefits than antihypertensive drugs. However, few intervention studies have considered the synergistic benefits of diet and exercise [23, 24]. This study therefore aims to evaluate the synergistic benefits of consuming olive phenolics alongside increasing physical activity in individuals with elevated blood pressure. Our hypothesis was that OLE and physical activity would reduce blood pressure and that a combination of the two would be more effective than either treatment alone.

5.1.1 Subjects

Volunteers residing in Reading, UK, were informed about the study via flyers, posters in community buildings, local shops and on the Reading University campus. Invitation letters, emails or phone calls were sent to Reading University students staff and volunteers from the Hugh Sinclair Unit of Human Nutrition volunteer database. All volunteers provided written informed consent prior to commencing the trial. Based on previous studies demonstrate hypotensive activity [6, 8, 25]for OLE in human volunteers, all focused on defined cohorts, but men and woman of all ages suffer hypertension, we are demonstrating the consistency of the effect across the life-course regardless of gender. Inclusion criteria were male or female, age 25–70 years, body mass index (BMI) ≥ 25 kg/m^2 and BP recorded at screening session between 120–160 mmHg systolic and/or 80–99 mmHg diastolic. Exclusion criteria were as follows: (1) smokers; (2) taking antihypertensive or lipid-lowering medications; (3) serious disease such as diabetes (4) pregnant or currently breastfeeding; (5) unwilling to wear the ambulatory blood pressure (ABP) monitor or to undergo BP monitoring for 24 hrs; (6) unwilling to maintain habitual diet; (7) unwilling to increase physical activity level and (8) inability to swallow capsules. During screening, a fasting blood sample was taken and BMI, waist circumference and BP were measured. Suitable individuals were invited to participate in the study.

5.1.2 Study design

This study was structured as a four-way parallel study design in pre-hypertensive volunteers, as shown in **Figure 35**. The study arms were: i) Placebo control, on which volunteers consumed a placebo capsule daily over 12 weeks. ii) Increased physical activity (PA): volunteers consumed a placebo control supplement and received guidance on achieving the PA guidelines. They were asked to wear an accelerometer daily and were asked to gradually increase their physical activity levels, working towards a target of achieving thirty minutes of moderate exercise on five days of the week over the 12-week period. iii) OLE supplement: volunteers consumed capsules of OLE over a 12-week period with no guidance on PA. iv) PA+ OLE supplement. Primary outcome for the study was change in 24-h ABP. Secondary outcome measures for the study were digital pulse wave velocity (as a measure of vascular function), plasma lipids, glucose insulin and body composition.

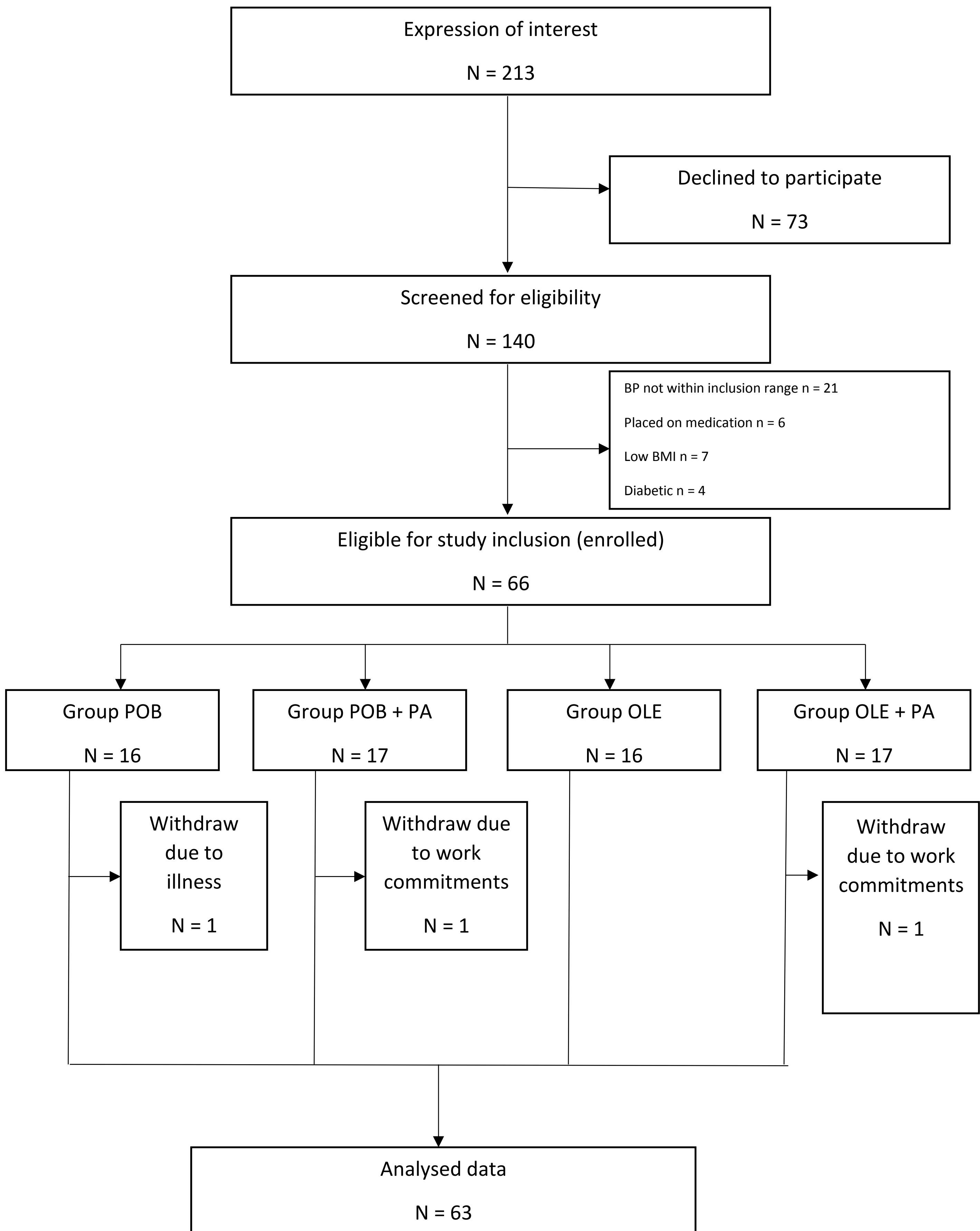

Figure 35 Consolidated Standards of Reporting Trials (CONSORT) diagram.

5.1.3 Supplement and Treatment Regime

The study product OLE and placebo capsules were provided by Comvita (Te Puke, New Zealand). The capsules were composed of an opaque chocolate brown soft gelatine. Each OLE capsule contained a total of 350 mg Olea europea leaf extract dry concentrate 10:1 (equivalent to 3.5 g of fresh olive leaf 3.5 g), delivering 66-79.2 mg oleuropein. Compositionally, OLE contained oleuropein, oleoside, hydroxytyrosol, luteolin-7-*O*-glucoside, tyrosol, verbascoside, apigenin-7-*O* glucoside, rutin, vanilic acid, vanilin and luteolin (Comvita, Limited). The placebo capsule contained 350 mg of maltodextrin per capsule and were identical in appearance to OLE capsules. Subjects were instructed to take 2 tablets daily, preferably after breakfast or the first meal of the day, and to record each intake in the assigned supplement diary. If a dose was missed, participants were requested to indicate the reason for missing and not to carry over the dose to the next day. Any changes in medication, dietary supplement intake, general well-being, bowel movements, and/or any adverse effects during the intervention were to be self-reported in the supplement diary.

5.1.4 Physical activity intervention

The physical activity intervention was designed to encourage a progressive increase in physical activity over 12 weeks. Volunteers were asked to wear an Omron hip mounted pedometer (Omron Walking Pro.2). The data from this instrument can be uploaded onto an online database and the subjects were encouraged to record the data with the ultimate goal of achieving 10000 steps per day. They received weekly motivational hint sheets by mail that also encouraged continued engagement. These sheets were structured such that activity was increased progressively over the duration rather than setting potentially unachievable and therefore demotivating targets.

5.1.5 Randomization and Masking

Randomized allocation was done using the Covariate Adaptive Randomization programme. At screening, individuals were categorised by gender, age group and BP range to ensure well-balanced groups in the intervention phase. Both researchers and subjects were 'blinded' to the contents of capsules being taken. The products were labelled using A and B and provided in identical bottles made from opaque plastic. The investigational product was safely stored in a locked and limited access area. The code was kept by an independent third party, and was not released until after statistical analysis.

5.1.6 Assessment Protocol

Enrolled participants were required to visit the Hugh Sinclair Unit of Human Nutrition on 5 occasions (screening visit, familiarisation visit, baseline visit, 6-week visit and 12-week visit) during the study period, as shown in **Figure 36**. On the measurement days, subjects attended having fasted for at least 12 hrs before the visit. A 'familiarisation visit', was conducted to minimise the impact of stress on the subsequent (baseline) assessments as well as to equip/instruct the subjects with 24-h ABP monitors and the accelerometers. At baseline 24-hour ambulatory blood pressure was assessed using the A&D Medical Ambulatory Blood Pressure Monitor (Model TM-2430, Scan Med, A&D Medical, UK). Blood samples were taken and anthropometric measures were made. Subjects were given the relevant capsules and instructed to maintain their habitual diet and physical activity throughout the intervention, unless otherwise directed. The measurements of 24-h ABP, physical activity, clinical BP, arterial stiffness, anthropometrics and the collection of blood samples were conducted at baseline, and at week 6 and week 12.

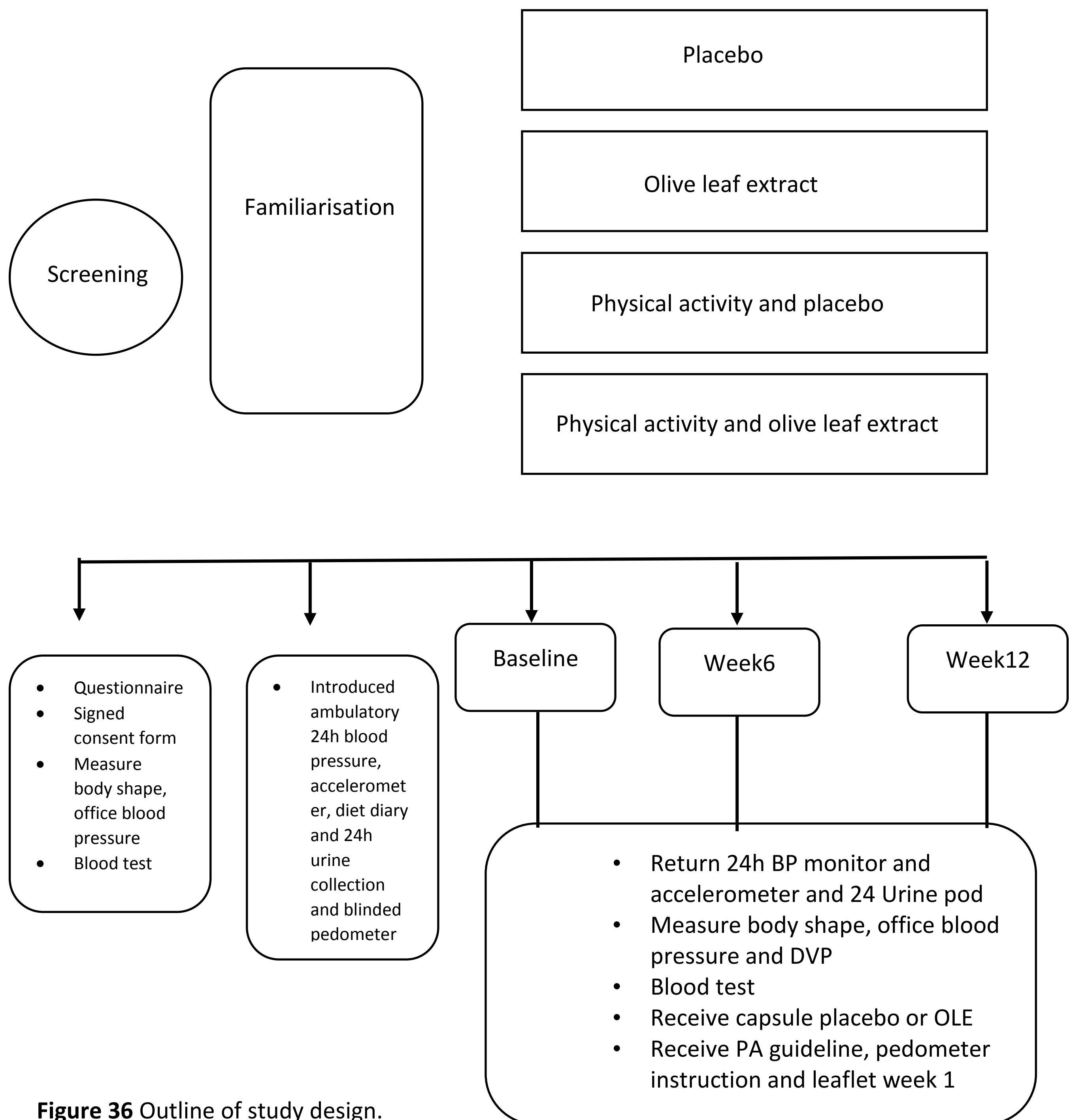

Figure 36 Outline of study design.

5.1.7 Outcome Assessments

Compliance measures

At the end of 12 weeks, the treatment bottle containing any unused tablets was returned with a participant diary (self-report), noting adverse effects, and/or reasons for missed doses. At the end of the trial, all tablets were manually counted and tallied with the corresponding diary records to monitor compliance. 24-h urine and blood samples were collected from volunteers at each visit (baseline, 6 week and at 12 week), and they will be

analysed for phenolic compounds deriving from the OLE via LC-MS to assess subject compliance Adherence to the PA arm was assessed through the assessment of pedometer and accelerometer data.

24-h ambulatory blood pressure

The description of methods is presented in chapter two (section 2.5.1). 24h ambulatory BP monitoring was carried out prior to each clinical assessment (baseline, week 6, week 12).

Arterial stiffness

The description of methods is presented in chapter two (section 2.5.2). Arterial stiffness was carried out at baseline, week 6, week 12.

Anthropometric measurement

Standing height and body weight were measured before vascular assessments commenced. Height was measured using a wall-mounted Stadiometer (Medical scales and measuring system, Seca Lt., UK). A Tanita BC-418MA Body Composition Analyser (TANITA, UK, Ltd.) was used to measure weight and body composition following the instructions of the manufacturers. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Waist circumference was measured using a flexible non-stretchable tape.

Blood collection

Fasting blood samples were collected at baseline 6 weeks and after 12 weeks. Venous blood samples were collected in serum lithium heparin, EDTA and serum separator vacutainers (SST; Greiner Bio-one, USA) with no additives. Blood collected in SST was allowed to coagulate at room temperature for 30 min before further centrifugation. Then all samples were centrifuged at 1800 g for 15 min at 4°C to separate plasma. Plasma was stored in 2 ml screw cap microtube (Sarstedet Ltd, Leicester, UK) at - 80°C until analysis.

Biochemical measures

HbA1C was measured by HPLC at Royal Berkshire Hospital, Reading. Total cholesterol (TC), HDL cholesterol (HDL-C), triglycerides (TAG), non-essential fatty acid (NEFA) and fasting glucose were enzymatically measured using an automated ILAB 600 clinical chemistry analyser (Instrumentation Laboratory, Warrington, UK). Low-density

lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula: ([LDL-C] = [TC] – [HDL-C] – ([TAG]/2.2). Plasma insulin was measured using an ELISA assay kit (Dako Ltd, Denmark) following standard instructions provided by the manufacturer. Insulin resistance (HOMA-IR) was calculated using the formula: (glucose x insulin)/22.5 and insulin sensitivity (QUICKI) was evaluated by using standard equations: 1/ (log insulin + log glucose).

5.1.8 Sample size and Statistical analysis

Sample size was calculated by using G * Power 3 program [26]. A power calculation was based upon data from a crossover study investigating the effect of the same OLE on blood pressure in 45 pre-hypertensive volunteers. In that study, the mean difference in 24-hour systolic blood pressure between treatment (dose OLE equivalent to 136.2 mg oleuropein per day) and placebo was -3.33 mm Hg with an sd of 7.9, thus giving an effect size (Cohen's d) of 0.422 with the cut off for α at 0.05 and with 85 % power, predicted total sample size is 112 volunteers; 28 per group, or 30 per group allowing for drop outs.

Due to time and resource the study was cut short with 63 volunteers per group. A post hoc power analysis given the mean and the between-groups comparison effect size observed in the present study (Cohen's d= 1.03), where n= 63 gave a statistical power greater than 99% to detect a mean difference in 24-h SBP of -9.23 (±17.76) mmHg between treatment and placebo.

All statistical analyses were performed using SPSS statistic software version 24 for Windows (IBM Corporation, USA). Descriptive statistics are presented as means and standard deviations. Data were initially checked for normality using the Kolmogorov-Smirnov and Shapiro-Wilk test. One-way ANOVA was used to determine difference in treatment for the groups. The least significant difference was used as Tukey's HSD *post hoc* test. There were sufficient numbers in each treatment arm to additionally perform a parallel comparison; independent t-tests were used to compare pre-post treatment differences in four arms during the first intervention phase. P-values below 0.05 were deemed to be statistically significant. In addition, we determined whether there were any treatment differences between sexes, as there was a good gender split.

5.1.9 Results

Compliance

Sixty-six eligible subjects were randomised in to the four arms of the trial (Figure 1). Three subjects dropped out of the study prior to the assessment point in week 6. One subject fell ill (abdominal pain) and two subjects withdrew due to work commitments. Consequently, 63 subjects completed all assessment time points and were included in the primary and secondary outcomes analyses. Compliance with consuming the study capsules was greater than 90 % as measured by counting capsules in returned containers for all groups (compliance rates were 96.67 % (±4.23) of placebo group, 95.98 % (±5.16) for the placebo + PA group, 96.43 % (±3.25) for the OLE group and 91.76 % (±6.02) for OLE + PA group. Subsequently all 63 datasets were included in the analysis. Total steps taken/day was significantly increased during the intervention period by an average of 64.41% to 9,499 (±587) for Placebo+PA group ($P < 0.001$). Subjects in OLE+PA group significantly increased their total steps/day by an average of 40.21% to 8,043 (±723) throughout the study ($p < 0.001$). There was no significant difference in total steps count/day between Placebo+PA and OLE+PA. The step count in the non-PA groups remained unchanged during the 12-week intervention, as measured by accelerometer data.

Baseline characteristics of the subjects

Baseline characteristics of the subjects can be found in **Table 11-12**. All subjects were overweight (range 27.1-30.1 kg/m^2), with a mean age of 45.7 years (range 42.18-49.28). Mean daytime BP at baseline was 133/80 mmHg, and all were deemed pre-hypertensive [27] Subjects mean triglyceride and HDL-C levels were within the ideal range set by NHS choices guideline UK (1.3 and 1.4 mmol/L vs. guideline values of <1.7 and ≥1 mmol/L, respectively). Mean total cholesterol and LDL-C levels were slightly above the ideal lipid values (5.5 mmol/L and 3.5 mmol/L vs. guideline values of <4 mmol/L and <2 mmol/L, respectively). There were no differences in baseline characteristics between those randomised initially to the four treatments ($p > 0.05$) (**Table 12**).

Table 11 Baseline characteristics of subjects (Total)

Variable	Mean (SD)
Gender (male/female)	29/34
Age (years)	45.7 (±14.1)
Weight (Kg)	81.1 (±12.9)
BMI (kg/m^2)	28.6 (±6.0)
% Body Fat	32.2 (±10.4)
Total cholesterol (mmol/L)	5.5 (±1.1)
LDL cholesterol (mmol/L)	3.5 (±0.9)
HDL cholesterol (mmol/L)	1.4 (±0.3)
Triglycerides (mmol/L)	1.3 (±0.6)
TC/HDL cholesterol ratio	1.0 (±0.5)
HbA1C (mmol/mol)	35.4 (±3.9)
Fasting Glucose (mmol/L)	5.4 (±0.5)
Fasting Insulin (mU/L)	6.9 (±6.4)
QUICKI	0.7 (±0.1)
HOMA-IR	1.5 (±1.4)
24-h SBP/DBP (mmHg)	129 (±12)/78 (±9)
Daytime SBP/DBP (mmHg)	133 (±13)/80 (±9)
Night time SBP/DBP (mmHg)	114 (±13)/67 (±8)

BMI body mass index, *TC* Total cholesterol, *LDL* Low-density lipoprotein cholesterol, *HDL* High-density lipoprotein cholesterol, *TG* Triglycerides, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *HOMA-IR* homoeostasis model assessment-estimated insulin resistance, *QUICKI* quantitative insulin sensitivity check index

Table 12 Baseline characteristics of the subjects (each group)

Variable	Placebo (n=15)	Placebo+PA (n=16)	OLE(n=16)	OLE+PA (n=16)	$p^{ǂ}$
Gender (male/female)	6/9	8/8	8/8	7/9	0.934
Age (years)	49.6 ± 13.4	42.1 ±14.4	46.4 ± 14.6	45.0 ±14.1	0.530
Weight (Kg)	78.6 ± 12.4	85.6 ± 9.6	77.3 ± 12.1	82.4 ± 16.3	0.275
BMI (kg/m2)	28.8 ± 5.2	30.3 ± 5.4	26.1 ± 8.2	29.1 ± 3.9	0.243
TC (mmol/L)	5.7 ± 0.5	5.4 ± 0.5	5.3 ± 0.4	5.5 ± 0.4	0.176
LDL cholesterol (mmol/L)	3.6 ± 1.11	3.1 ± 1.0	3.3 ± 0.9	3.8 ± 1.0	0.178
HDL cholesterol (mmol/L)	1.5 ± 0.2	1.4 ± 0.3	1.3 ± 0.3	1.4 ± 0.4	0.358
Triglycerides (mmol/L)	1.2 ± 0.6	1.2 ± 0.5	1.4 ± 0.6	1.3 ± 0.6	0.650
TC/HDL cholesterol ratio	0.8 ± 0.5	0.9 ± 0.5	1.1 ± 0.5	1.0 ± 0.6	0.453
HbA1C (mmol/mol)	35.2 ± 3.5	35.9 ± 4.6	34.4 ± 4.4	35.9 ± 3.2	0.691
Glucose (mmol/L)	5.4 ± 0.5	5.5 ± 0.5	5.3 ± 0.4	5.5 ± 0.4	0.686
Insulin (mU/L)	6.6 ± 3.8	4.7 ± 0.7	8.0 ± 10.5	8.0 ± 1.5	0.507
QUICKI	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.417
HOMA-IR	1.7 ± 1.0	1.9 ± 2.4	1.1 ±0.5	1.9 ± 1.2	0.487
24-h SBP/DBP (mmHg)	125 ±11/76±10	131 ±11 /76 ±7	132 ± 11 /76 ±7	128 ± 16 /78 ± 11	0.361/0.421
Daytime SBP/DBP (mmHg)	128 ±11/78±10	135 ± 12 /83 ±8	135 ±11 /79 ± 8	132 ± 17 /81 ± 12	0.391/0.478
Night time SBP/DBP (mmHg)	108 ±7 /63 ± 7	116 ± 14 /68 ± 8	117 ±14 /67 ± 7	115 ± 14 / 68 ± 9	0.245/0.250

BMI body mass index, *TC* Total cholesterol, *LDL* Low-density lipoprotein cholesterol, *HDL* High-density lipoprotein cholesterol, *TG* Triglycerides, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *PA* physical activity,*OLE* olive leaf extract, *HOMA-IR* homoeostasis model assessment-estimated insulin resistance, *QUICKI* quantitative insulin sensitivity check index

Data points are mean ± SD

ǂ Mean value was significantly different between all treatment ($p < 0.05$)

Blood Pressure and vascular function

24 hour blood pressure

Mean 24-h, daytime and night-time BP at baseline, 6-weeks and at the end of the 12-week intervention are summarised for each group in **Figure 37-38**. When compared with placebo, mean 24-h SBP was significantly reduced in the increasing physical activity (Placebo+PA) group at the 6 week time-point (-7.07 mmHg ± 5.26; $p = 0.048$) and at the 12 week point (-9.05 mmHg ± 6.39; $p = 0.001$). Those consuming the OLE with no physical activity guidance (OLE group) also showed a significant reduction in 24-h SBP of 10.69 mmHg ± 7.56 and 11.16 mmHg ± 7.88 at 6 and at 12 weeks ($p = 0.031$ and $p = 0.006$). Amongst those consuming the OLE with physical activity instruction (OLE+PA group) the mean 24-h SBP was reduced by 9.23 mmHg ± 6.53 at 12 weeks ($p = 0.027$) relative to control, although the reduction at 6 weeks (6.31 mmHg ± 4.45 did not reach significance ($p = 0.118$). In the OLE group the 24-h SBP fell relative to baseline at 12 weeks by -5.8 mmHg ± 7.6 ($p = 0.011$) and in the OLE+PA the reduction from baseline at 12 weeks was −3.9 mmHg ± 6.7 ($p = 0.034$).

Daytime blood pressure

Daytime SBP was lower at 12 weeks compared to control following both the physical activity guidance intervention by -4.19 mmHg ± 10.27, $p = 0.041$ and the consumption of OLE intervention by -5.07 mmHg ± 7.16, $p = 0.025$; however, the mean daytime SBP did not significantly decrease from baseline to 12 weeks in either group **(Figure 37-38)**. There was a near-significant decrease in the mean rate of daytime SBP ($p = 0.087$), night time SBP ($p = 0.094$), and DBP ($p = 0.076$), following OLE+PA compared with the control. There were no significant differences between the effects of the four treatments on the mean change in 24-h DBP, or daytime DBP. However, a within group analysis revealed a significant decrease from baseline to 12 weeks in 24-h mean DBP −3.6 mmHg ± 6.3, $p = 0.011$ and −1.1 mmHg ± 5.3, $p = 0.034$) over 12 weeks subsequent to placebo+PA and OLE+PA treatment, respectively. No significant effects on arterial stiffness were found for any group ($p > 0.05$) (**Figure 39**).

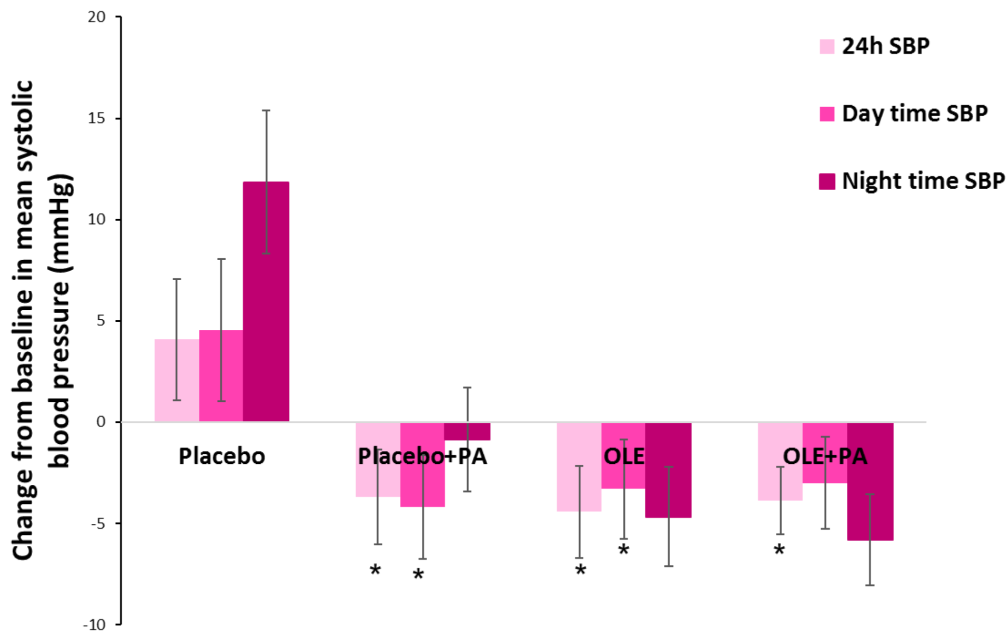

Figure 37 Changes in 24-h, Day-time and night-time systolic ambulatory blood pressure at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment ($p > 0.05$).

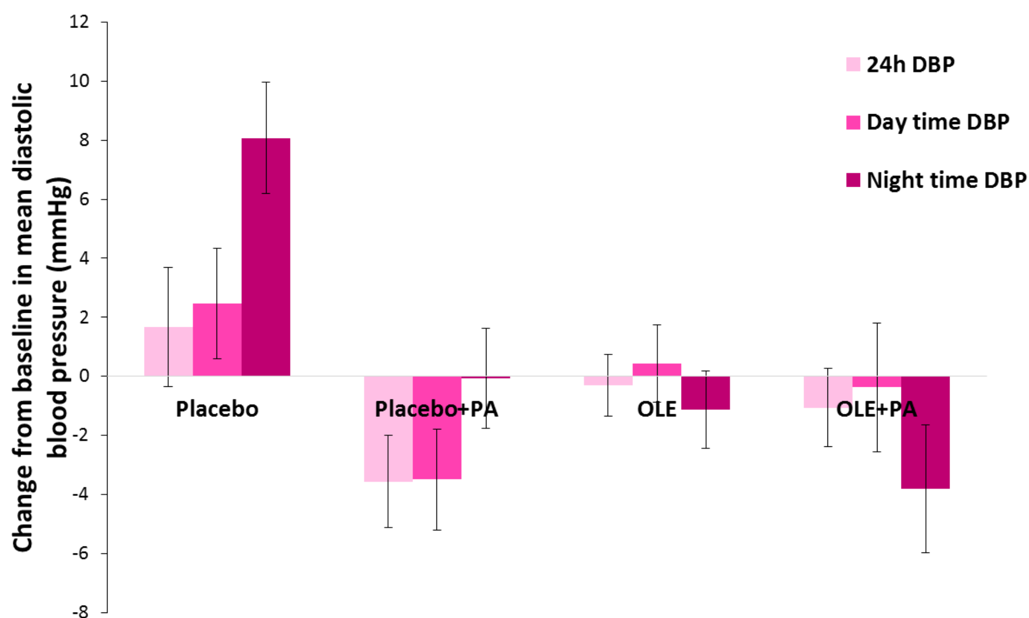

Figure 38 Changes in 24-h, Day-time and night-time diastolic ambulatory blood pressure at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment ($p > 0.05$).

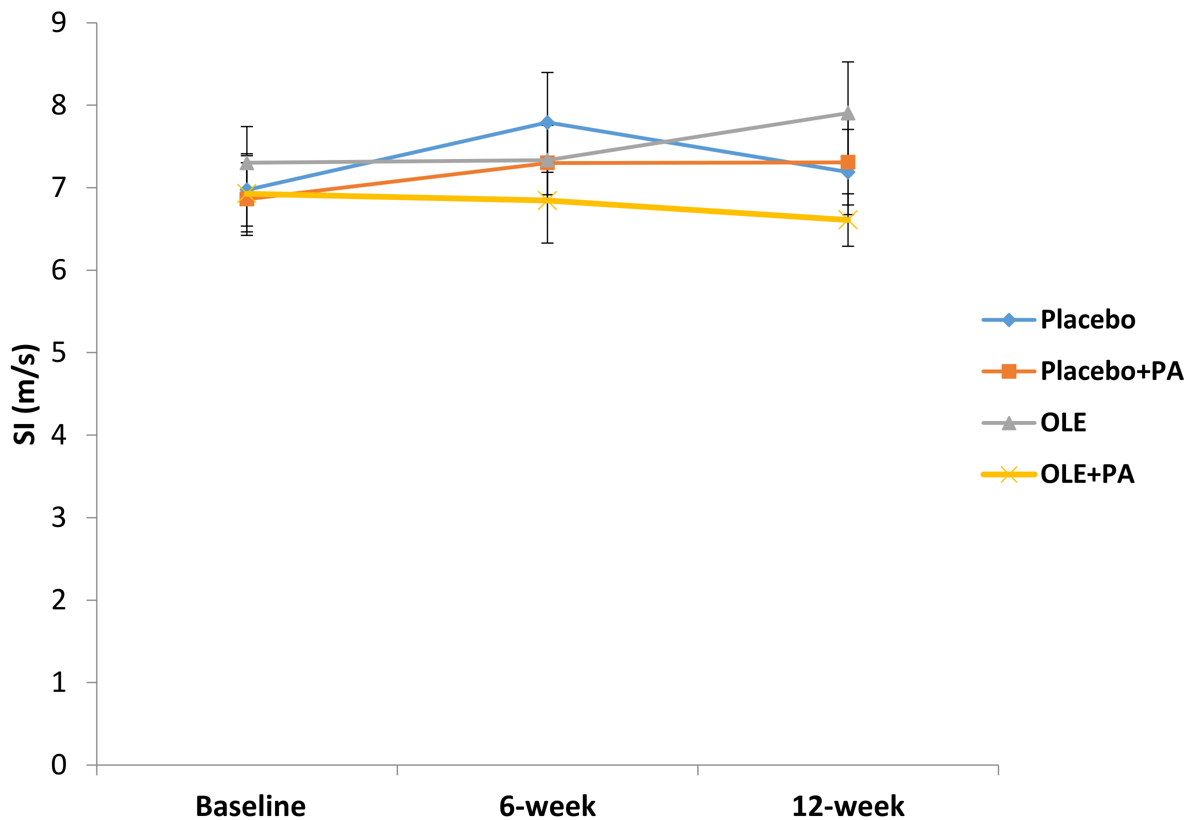

Figure 39 Arterial stiffness at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

Body composition and biochemical analysis

There was no impact of any treatments on body weight, waist circumference, relative fat mass or fat-free mass ($p > 0.05$) (**Figure 40-43).** Compared with placebo, treatment with increasing PA alone, or OLE alone, or with OLE +PA did not significantly alter fasting serum total cholesterol and LDL-C level ($p > 0.05$. HDL-C was significantly increased from baseline to 12 weeks following consumption of OLE alone ($p = 0.024$); however, there were no significant differences in HDL-C at 12 weeks compared to the control. Conversely, OLE intake alone significantly reduced TAG ($p = 0.012$) and TC/HDL-C ratio (*p*

= 0.005) from baseline to 6 weeks, but there were no significant differences in TAG (p = 0.366) and TC/HDL-C ratio (p = 0.350) relative to control) **(Figure 44-49)**. The OLE+PA group had a significantly reduced HbA1C (p = 0.045) from baseline to 6 weeks; however, there were no significant effects on HbA1C compared to the control (p = 0.939). There were no effects of OLE intake and/or PA on fasting glucose, insulin or calculated HOMA-IR or QUICKI indices compared with placebo ($p > 0.05$) (**Figure 50-54**).

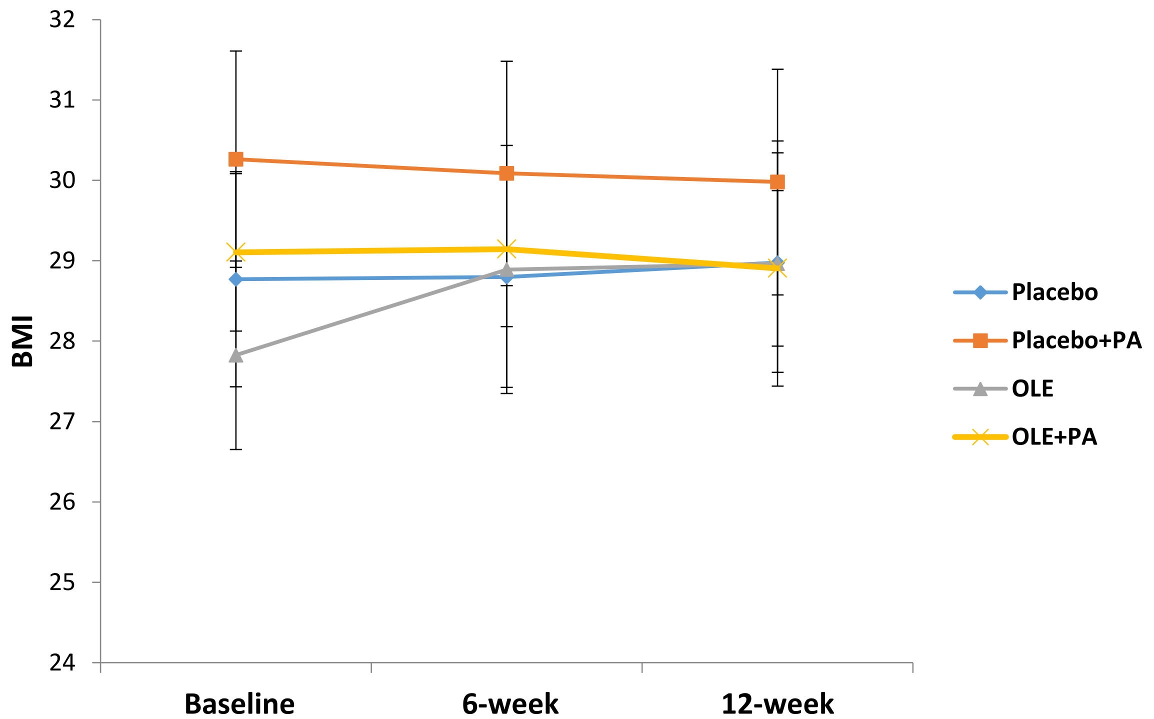

Figure 40 BMI at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

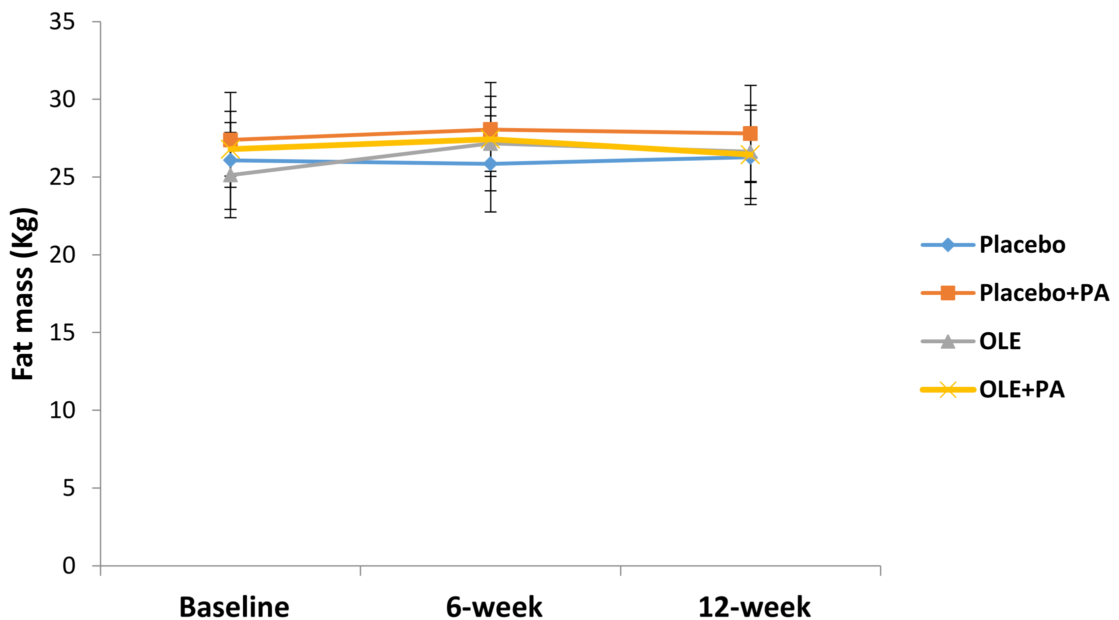

Figure 41 Fat mass at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

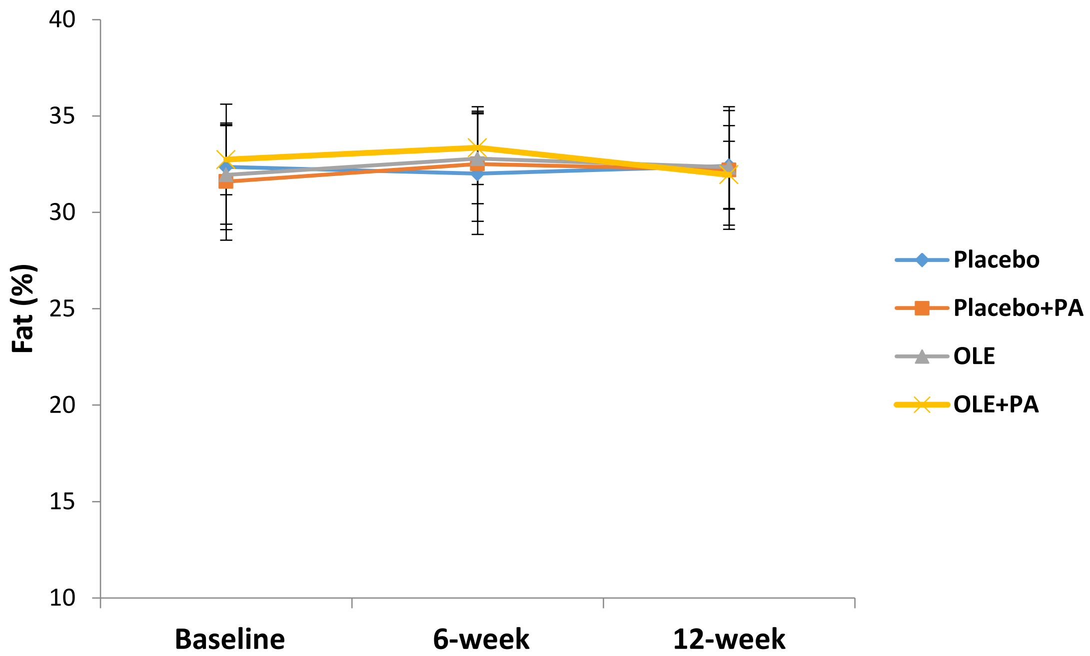

Figure 42 Percentage fat at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

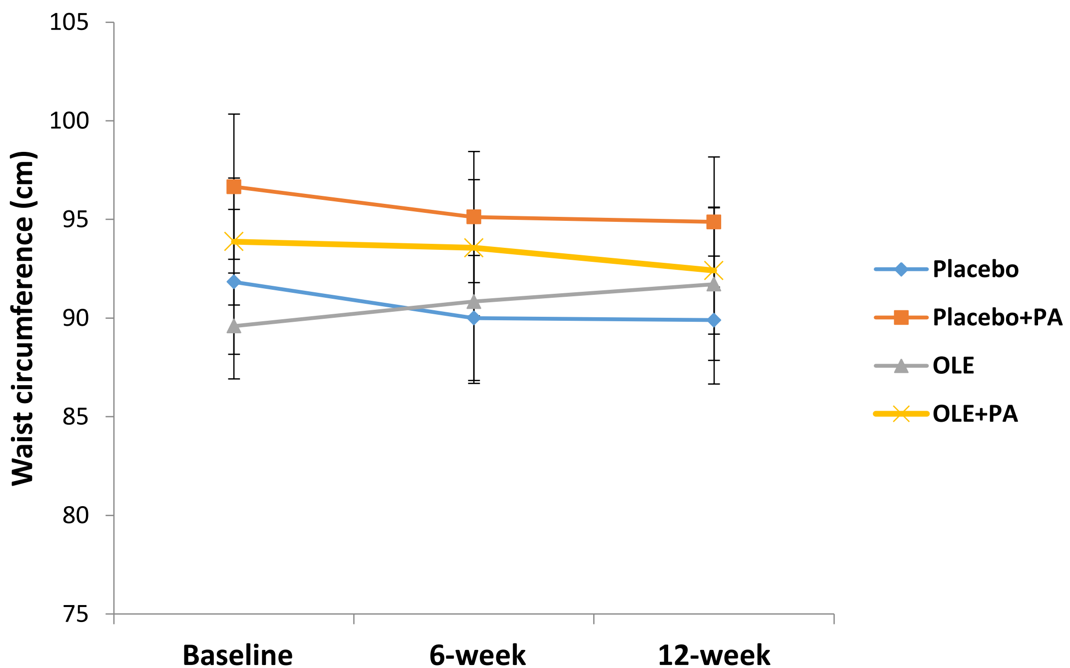

Figure 43 Waist circumference at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

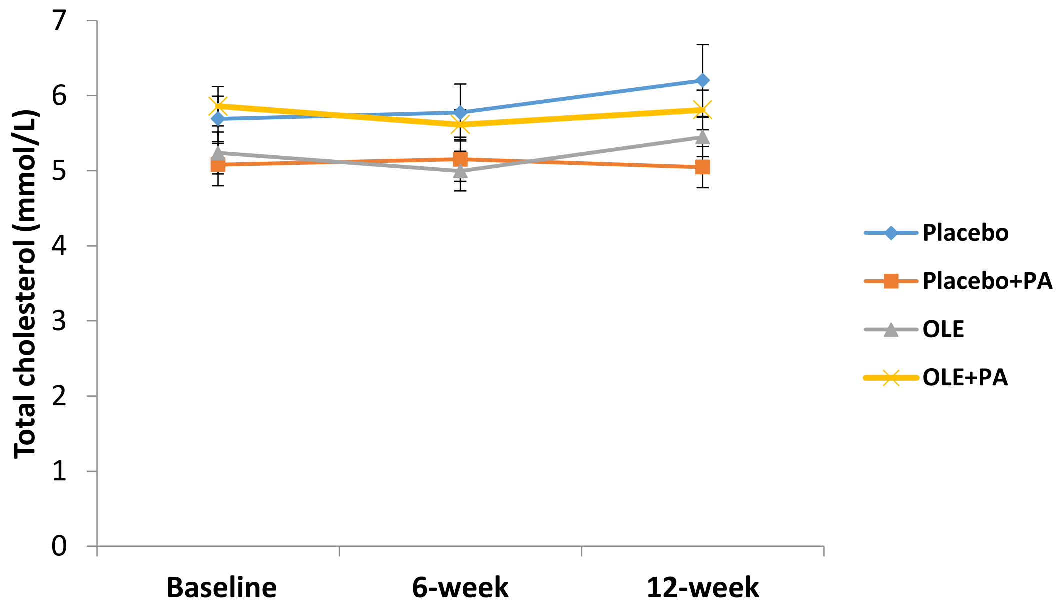

Figure 44 Total cholesterol level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

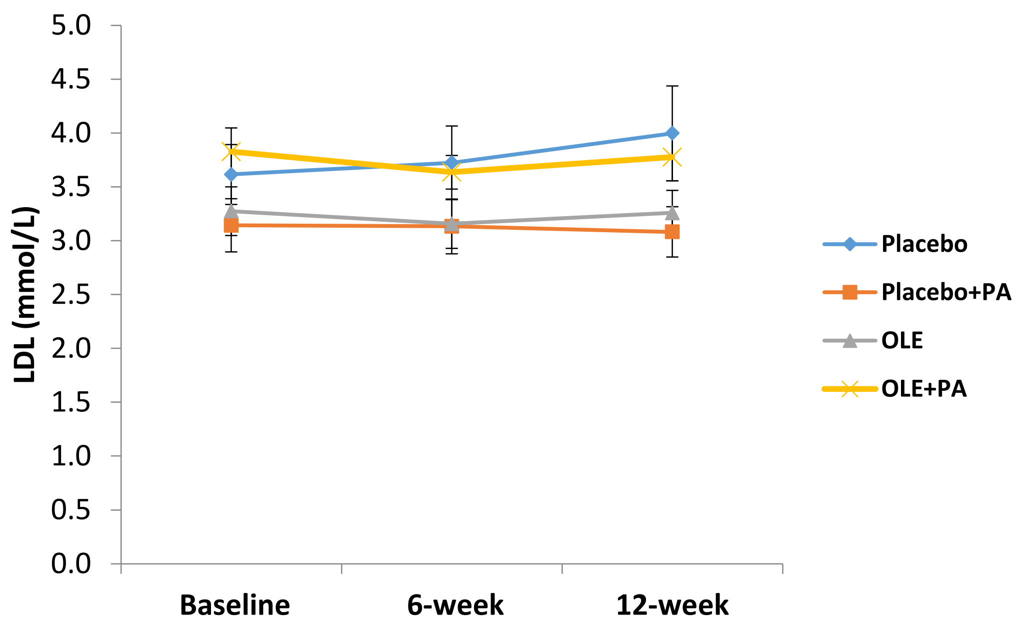

Figure 45 Low-density lipoprotein cholesterol level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

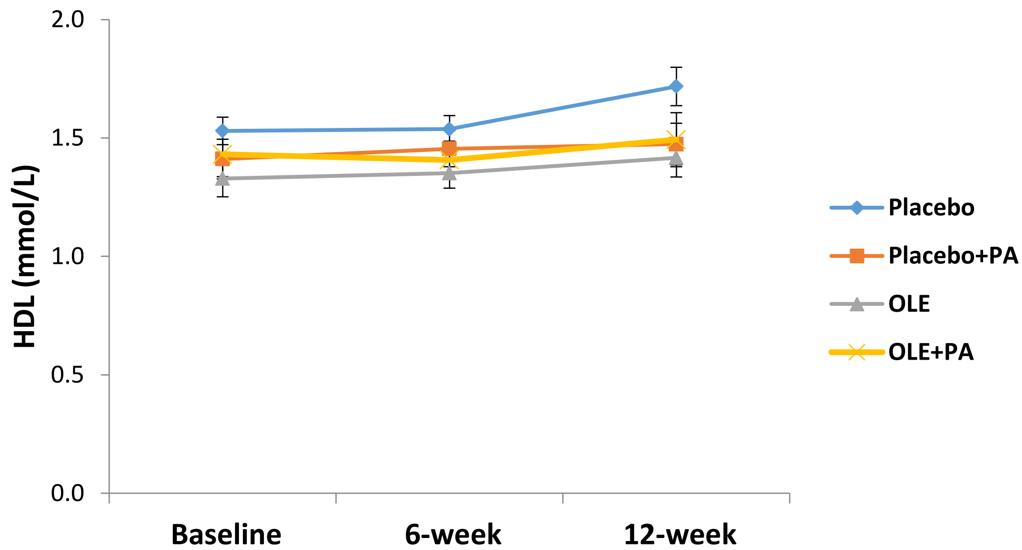

Figure 46 High-density lipoprotein cholesterol level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment ($p > 0.05$).

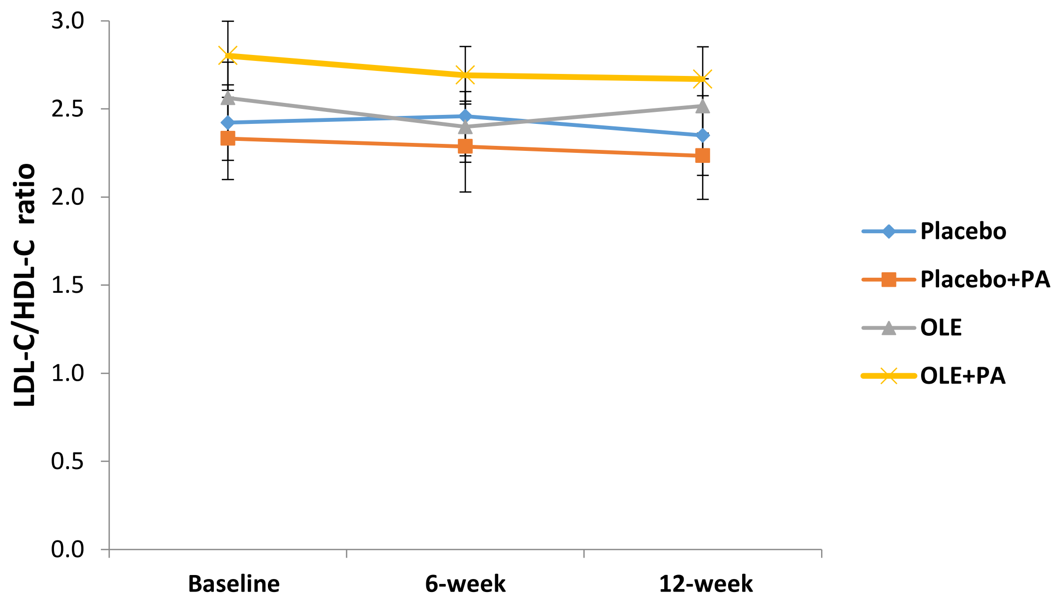

Figure 47 LDL-C/HDL-C ratio at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment ($p > 0.05$).

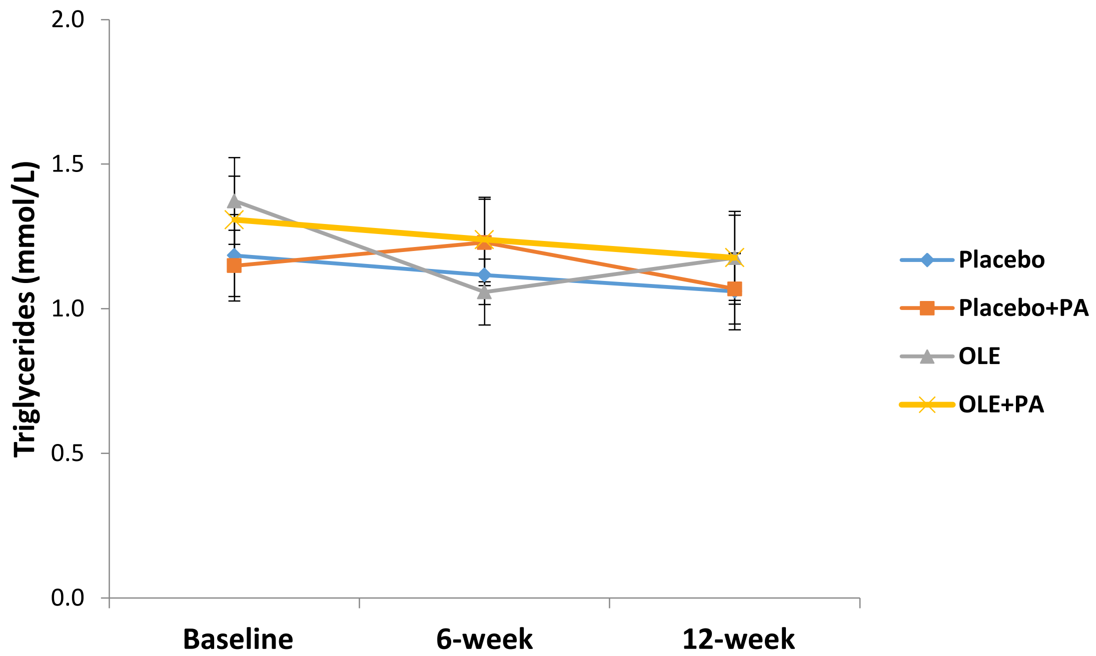

Figure 48 Triglycerides level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment ($p > 0.05$).

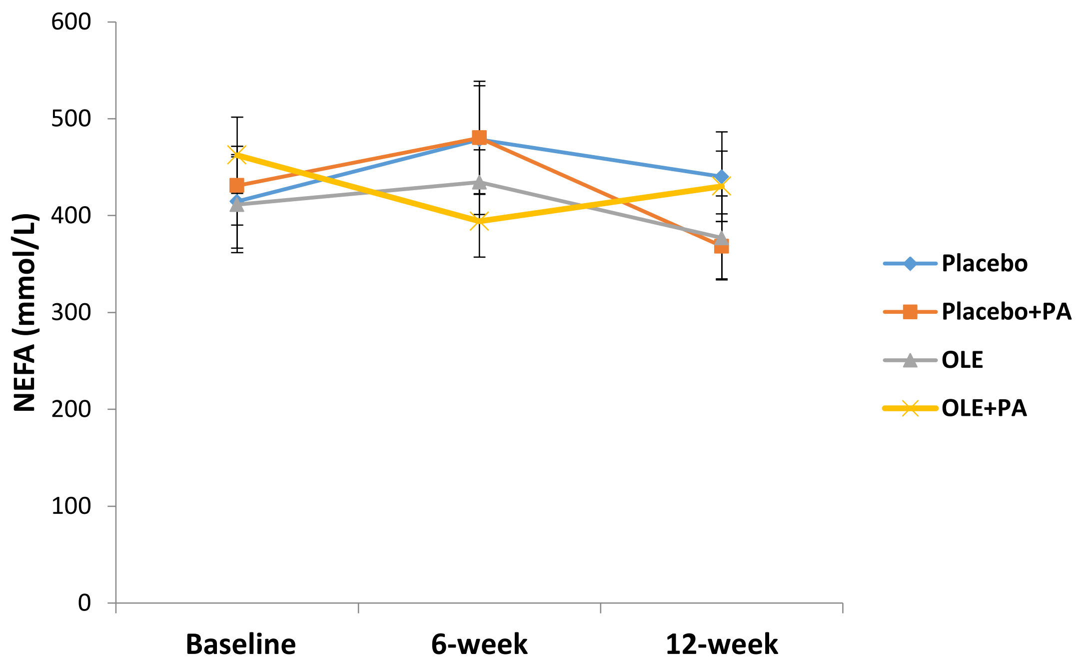

Figure 49 Non-esterified fatty acid level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment ($p > 0.05$).

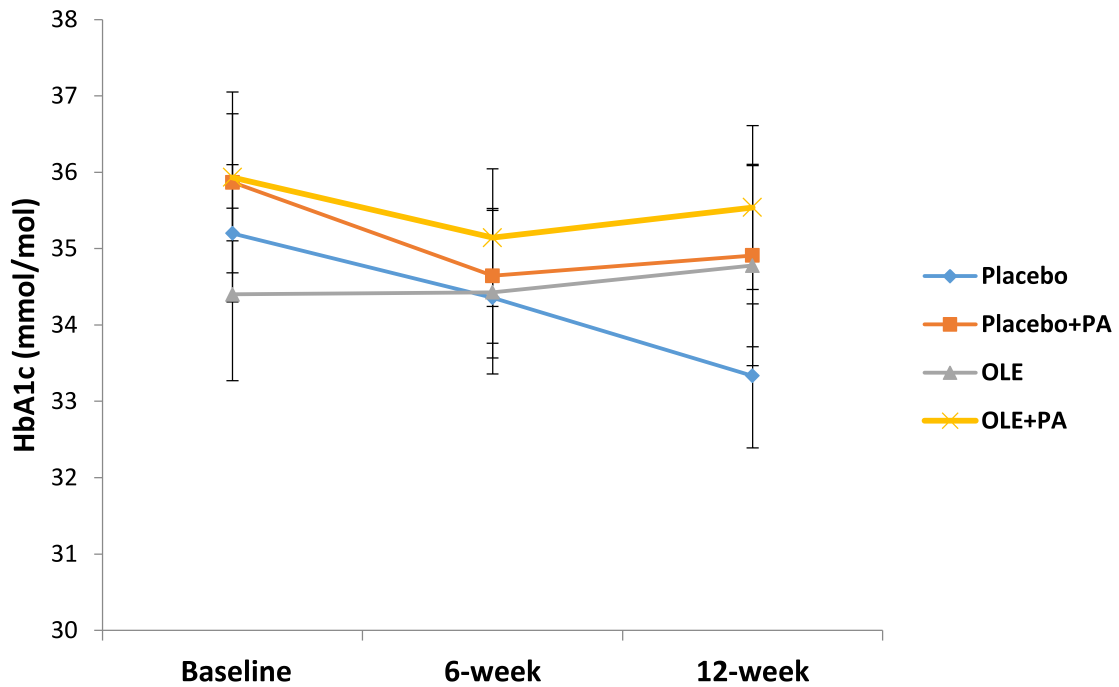

Figure 50 HbA1c level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

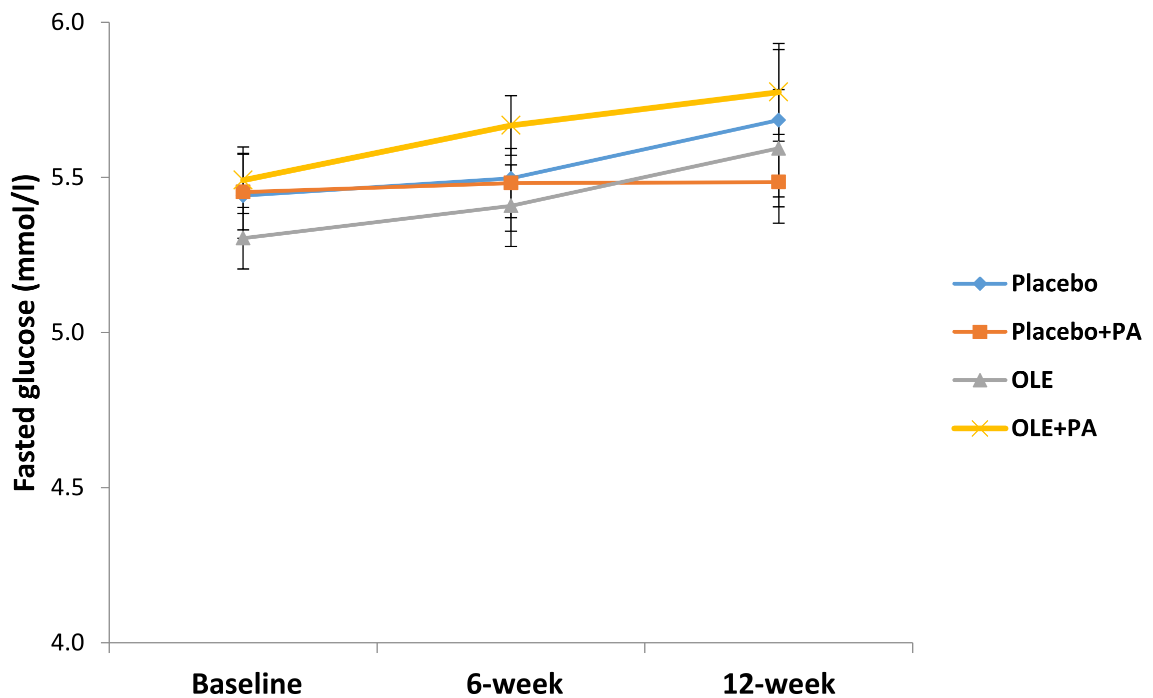

Figure 51 Fasted glucose level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

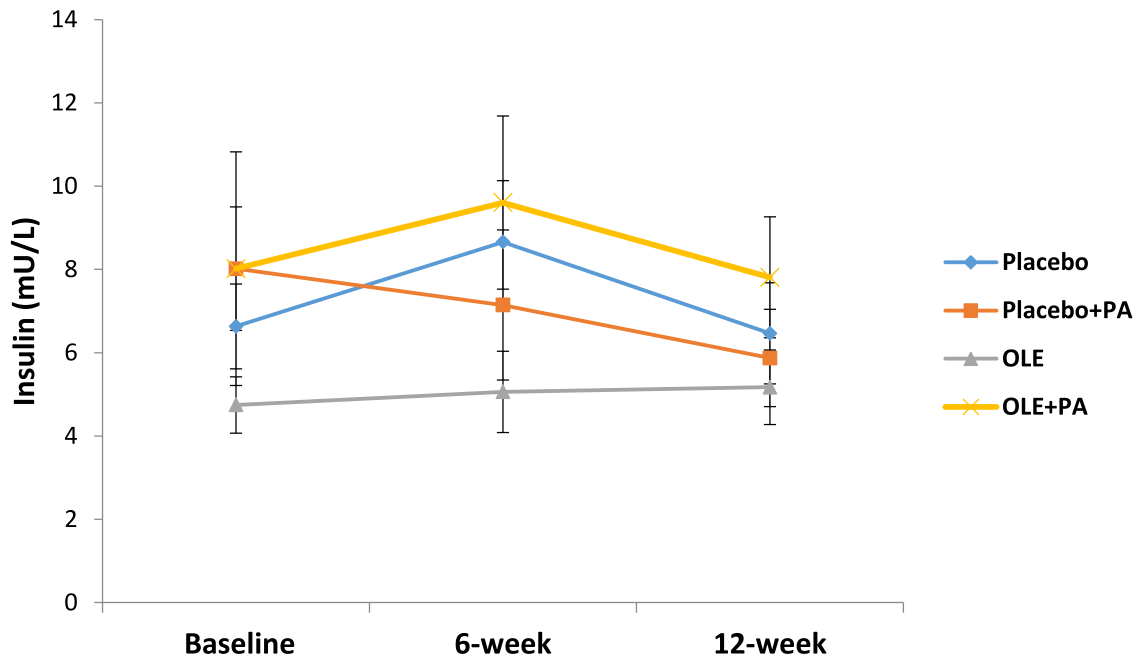

Figure 52 Insulin level at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

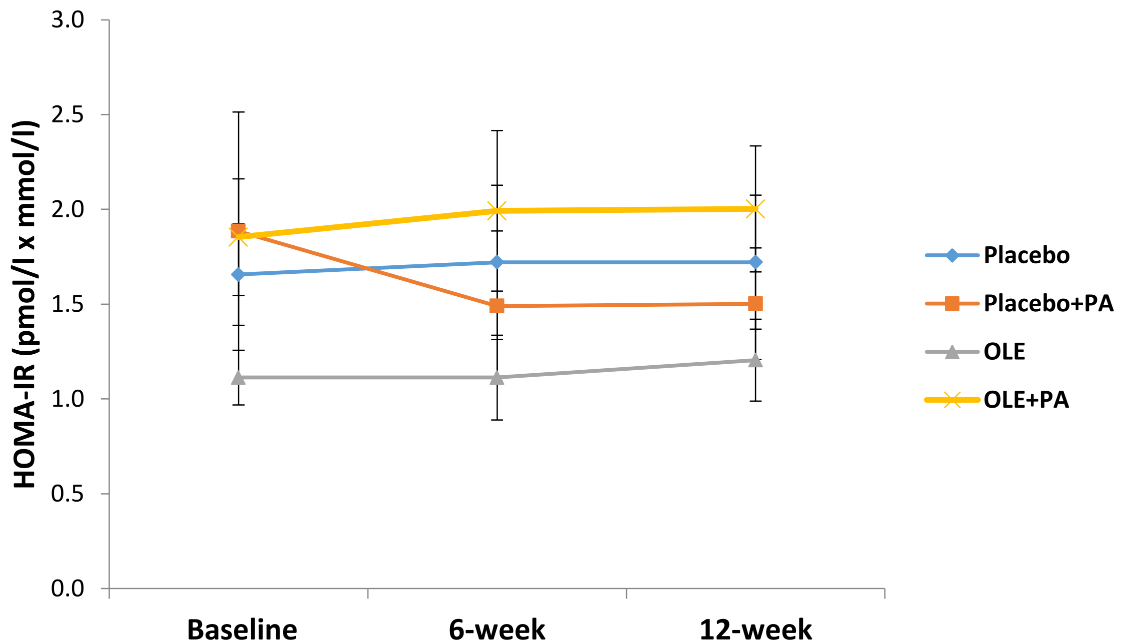

Figure 53 Homoeostasis model assessment-estimated insulin resistance at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

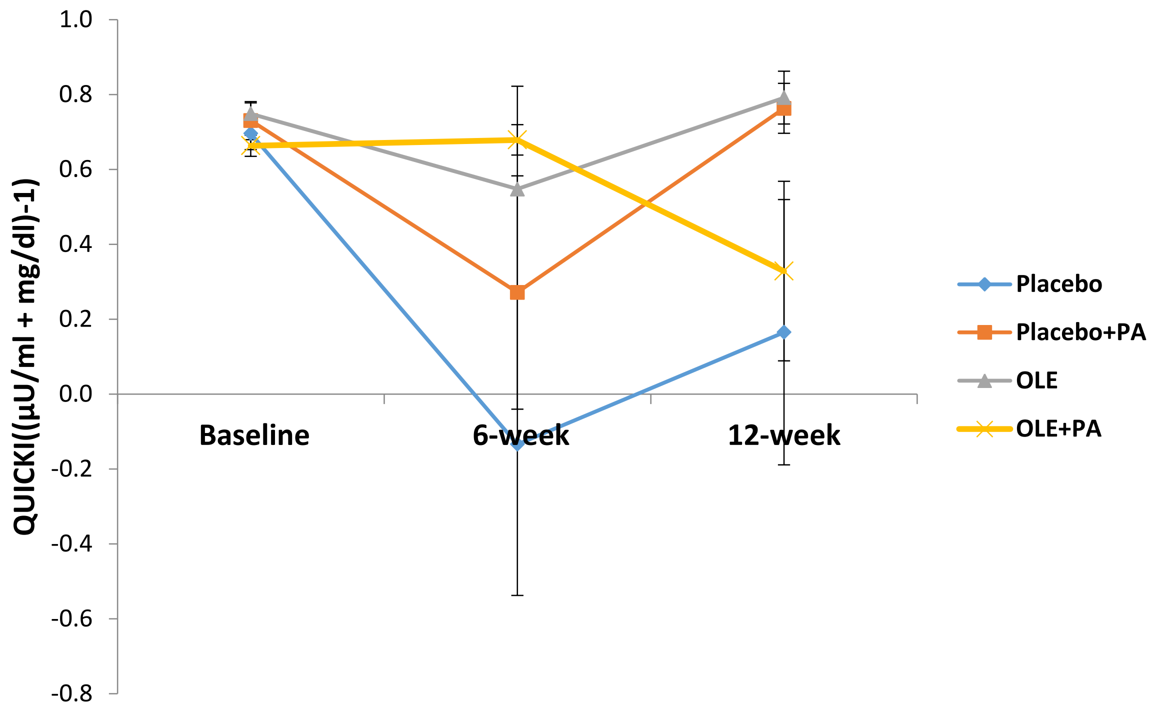

Figure 54 Quantitative insulin sensitivity check index at baseline, 6-week and at the end of the 12-week intervention in OLE, OLE+PA, placebo+PA and placebo groups. Data points are mean ± SD (n = 63). *Mean value was significantly different between all treatment (p > 0.05).

5.2 Discussion and Summary

We hypothesised that consuming OLE, in combination with increasing physical activity, would lower blood pressure to a greater extent than either intervention alone in pre-hypertensive British adults. As expected, we observed a reduction in blood pressure amongst those consuming the OLE alone, this has now been shown consistently in chronic intervention studies with OLE in pre-hypertensive volunteers. Lockyer et al. demonstrated that OLE at dose of 136.2 mg oleuropein/day has the potential to significantly reduce 24-hour SBP (3.33 mmHg) and daytime SBP (3.95 mmHg) and 24-hour DBP (2.42 mmHg) and daytime DBP (3.00 mmHg) relative to control [8]. In addition, OLE at dose of oleuropein, 200 mg/day resulted in mean reductions in SBP and DBP of 12 and 5 mmHg, respectively, in hypertensive patients [25] and 13 and 5 mmHg, respectively, in pre-hypertensive MZ twins [6]. These reductions in BP have not been reported in intervention studies with normotensive subjects [28, 29]. In the present study, the OLE intervention led to a reduction in 24-hour systolic blood pressure, from baseline, of 6 mm Hg at 6 weeks, and of 5.8 mm Hg at 12 weeks. In contrast, the non-physical activity placebo group, showed an actual increase in 24-hour systolic blood pressure from baseline at both time-points. The magnitude of the reduction in SBP with treatment is clinically meaningful; each 2 mm Hg reduction in SBP is associated with 7% reduction in coronary artery disease and a 10% reduction in the risk of stroke [30]. Curiously however we observed no reduction in DBP, although this was an average not elevated at baseline, it is thought that SBP may be a more valuable measurement in evaluating health outcomes related to hypertension [31].

The intervention with the physical activity guidance plus the placebo capsule also led to a reduction in 24-hour systolic blood pressure, amounting to -2.8 and -3.69 mmHg at weeks 6 and 12 respectively. This clinically meaningful reduction in blood pressure was precipitated by an average increase in total steps taken per day of 65% (3722 steps) above that observed at baseline. A recent meta-analysis of 9 studies suggested achievable reductions in SBP amongst hypertensive patients, guided towards increasing PA over a sustained period, of between 4-10 mmHg, and with more modest reductions of between 1 and 6 mmHg for diastolic pressure [32]. These and our own data support current physical activity guidelines for hypertension [33, 34]. The underlying mechanisms responsible for the exercise-induced reduction in blood pressure may include decreased

sympathetic nervous system activation [35], decreased renin activity and increased endothelial nitric oxide synthase activity [36], improved endothelium mediated vasodilatation and systemic arterial compliance in large vessels [37], and reduced peripheral vascular resistance [38].

Those volunteers who consumed both the OLE and who were given the PA guidance, showed a reduction in 24-hour systolic blood pressure from baseline in 24-h SBP, which was only observed at week 12. Further this reduction at -3.9 ± 6.7 mmHg was significantly less pronounced than that observed for the OLE group, and was of a comparable magnitude to that observed for the PA and placebo capsule group. Thus, our original hypothesis, that combining physical activity with the consumption of olive polyphenols would lead to greater reductions in blood pressure than either individual intervention alone, is not supported. It is however worth noting that the OLE+PA group were slightly less adherent to the physical activity intervention, they achieved an increase from baseline in their total step count of 40 % (2307 steps) versus 65% (3722 steps PA+placebo). In addition to moving less than the PA + placebo group, these volunteers adhered less well to the capsule consumption, with a compliance rate of (91.76%) versus their sedentary peers (96.43%) Further, the mean baseline 24-hour systolic blood pressure was slightly lower in the OLE+PA cohort (128 mmHg) than both the OLE group and placebo+PA group (131 mmHg and 132 mmHg, respectively). There is some argument that interventions with both physical activity and diet are not more effective for BP control than diet alone [38, 39], however the merits of adding physical activity to a polyphenol rich diet need further investigation.

The BP-lowering benefits of consuming plant polyphenols are hard to discern in studies with whole foods, where the effects can be masked by the hypotensive activity of the fibre or monounsaturated fatty acid - rich foods that they are found in [40-43]. In the present study, we observed that supplementation with an OLE, with a high dose of phenolics (equivalent to 132 mg of oleuropein), in the absence of fibre and lipids, significantly reduced 24-h and daytime SBP relative to control. The possible mechanisms of hypotensive activity for these phenolics include inhibition ACE enzymes [44], inhibition of calcium channel blockers [45] and the induction of NO synthesis [46]. Proof of these principles in in vivo systems is still needed.

In our analysis of secondary endpoints, we observed a reduction in the mean TC/HDL-C ratio (-0.290, p = 0.005) and TAG (-0.33 mmol/L, p = 0012) from baseline at 6 weeks for volunteers consuming the OLE, and an increase in the HDL- C (0.13 mmol/L, p = 0.024) from baseline at 12 weeks. HbA1C was reduced by OLE+PA combination after 6 weeks compared to baseline (−1.15 mmol/mol, p = 0.045). Other endpoints measured namely vascular stiffness index, body composition, lipids, glucose or insulin were not significantly affected by any of the treatments over 12 weeks These findings mirror previous work in hypertensive patients prescribed either OLE or an increased physical activity intervention. In these studies improvements in blood pressure were observed without changes in the vascular stiffness index [47, 48], BMI [8, 39, 49] or in the metabolism of glucose [6], or lipids [49].

In contrast to Wainstein et al., Lockyer et al., Salisu et al., and Ammar et al., we did not observe any significant changes in total cholesterol, LDL-C and HDL relative to control [6, 8, 25, 50]. The mean baseline cholesterol was in the high normal category for all study groups. Our physical intervention was not of a high enough intensity to expect significant changes in body composition over the study timeframe and our participants had good initial blood sugar control.

Study strengths and limitations. This study has several strengths. We used a randomised double-blind (to the OLE intervention), four-arm, parallel-group controlled trial design, and we 24-h ambulatory blood pressure as a primary endpoint. This is a well validated measure causally implicated in disease risk [51]. The compliance with respect to OLE consumption was excellent. We conducted a familiarisation visit for subjects in order to minimise the impact of stress on the subsequent (baseline) assessments. All subjects were free of antihypertensive drugs, as potential confounding factors. However, this study has several limitations. The starting 24-h DBP levels of the subjects were not elevated. We did not implicitly control the amount and intensity of the physical activity prescription, and none of the participants achieved the national physical activity guidelines. The volunteers adherence to the physical activity intervention also decreased over time. We did not measure the effects of the intervention arms on habitual diet. The subject of the study included both men and women; variance in the female cycle and oral contraceptives in pre-menopausal women might be a possible confounding factor in our study. And finally, we were unable to recruit our original target of 112 study subjects.

To conclude, OLE consumption, increasing physical activity and the combination of these interventions significantly improved 24-h SBP. Both interventions may be considered as beneficial in the control of blood pressure, consumption of supplemental olive leaf phenolics may be easier for a free living otherwise sedentary pre-hypertensive population. Further work is needed to establish the benefits which might be achieved with a higher dose of physical activity.

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Chapter 6 General discussion

6.1 Summary and discussion

Diets rich in plant foods are strongly associated with reduced risk of chronic diseases [1]. This effect may be mediated through a combination of mechanisms; plant foods are high in fibre which of itself is protective, they are nutrient dense and act as low energy displacers of other foods associated with weight gain and adverse health outcomes. They are also a rich source of biologically active phytochemicals including the polyphenols.

A suggested intake of polyphenols as contributors to health and lifespan might be based on the amount of polyphenols in '5-a-day'[2]. A significant percentage of the global population consume low quantities of dietary polyphenols as a result of inadequate fruit and vegetable intake, despite public health messages encouraging fruit and vegetable consumption [3]. Given the persistent public health failures in achieving the recommendations on fruit and vegetables, the development of dietary supplements which contain high doses of beneficial polyphenols is desirable.

Polyphenol supplements are increasingly popularity with the consumer, as they are presumed to be safe, and are freely available without prescription to all. The UK market for dietary supplements is worth more than £670 million, with consumers believing that they can provide a useful vehicle for increased consumption of dietary phytochemicals which may be otherwise under-consumed in the western diet [4]. However, effective supplements have the greatest potential to improve health and/or help prevent certain diseases when taken as part of a balanced diet and a healthy lifestyle. Consumers must realize that functional foods and supplements are not a "magic bullet" or a panacea for poor health habits. There are not good and bad "foods," only good and bad dietary patterns [5].

Currently, olive oil polyphenols are the only polyphenols to have achieved a positive opinion for a health claim in Europe. There has been a 1.7-fold increase in the global consumption of olive oil between 1991 and 2015 due to its associations with health [6]. Nevertheless, the consumption of phenolic-rich, lipid-free valorised olive leaf products may be a preferential approach to ingesting these beneficial compounds. Supplemental OLE may be a sensible source of olive polyphenols negating the need to consume large amounts of energy dense olive oil [7]. Intervention studies with EVOO consumed in the Mediterranean diet at quantities of up to 50g per day deliver a 5-20x lower dose of the

phenolics than can be delivered in an olive leaf extract supplement [8]. Directly replacing traditional spreads with EVOO in the UK diet (10g per day) would beneficially increase MUFA consumption, but would not substantially change total polyphenol intake. Although, there is a growing body of research demonstrating health benefits from olive polyphenols, there are currently no approved claims in regard to OLE. Publications arising from this thesis may therefore provide further evidence for the assessment of health claims by olive producers in the European market.

Recent randomised control trials demonstrate hypotensive, hypolipidemic [9-11] and anti-glycaemic activity [12, 13] for OLE in human volunteers. The mechanisms for these effects are still uncertain. In this thesis, we provide mechanistic support for the previous intervention studies, an understanding of the biological modes of action is a key component of the Bradford-Hill criteria when it comes to proving efficacy. In chapter 3, we showed that OLE and its phenolics may exert anti–glycaemic effects via the inhibition of porcine pancreatic α-amylase and lipase activity and the suppression of cellular glucose transporters hence impeding glucose uptake. Anti-diabetic and anti-obesity drugs like acarbose and orlistat are widely used, but may induce side effects, including abdominal distension, bloating, and flatulence, which are linked to excessive inhibition of the digestive enzymes [14]. OLE showed lower, but potentially clinically meaningful inhibition of digestive enzymes, thus it may be a safer, cheaper and more acceptable intervention [15]. In chapter 4, we explored mechanisms which might explain the antihypertensive activities of OLE and its polyphenols. We showed that polyphenols present in OLE might act through stimulating NO production in endothelial cells; and we also showed that they inhibited the enzymes involved in the renin-angiotensin system. Again, the OLE had lower efficacy than the standard antihypertensive drugs, but these weaker effects in a pre-hypertensive population might also be clinically meaningful.

A potential public health draw back which may be associated with the consumption of supplements is the demotivating influence on the individual towards pursuing other healthy lifestyle choices such as physical activity. However, research investigating the synergistic benefits of including a specific dietary supplement as part of a healthy lifestyle is limited. Physical inactivity is an established major global health risk, and few adults in the UK meet current Physical Activity Guidelines [16]. Stronger scientific evidence might better steer current public health guidelines and help raise awareness, particularly

amongst people at high risk of chronic disease. In our intervention study, we therefore aimed to combine the consumption of phenolics with increasing physical activity with a view to maximising the benefit to the pre-hypertensive participants. Getting free-living people, especially those at high risk of disease to change their physical activity behaviours was a particular challenge. We used a combination of goal setting and the availability of immediate feedback via a pedometer to motivate this intervention. Analysis of the physical activity data showed that our PA intervention successfully induced behaviour change in this UK cohort. However, the PA was not universally adhered to by the participants, and the degree of behaviour change was not quite as strong as we had hoped for. Moreover, our study also confirmed that higher physical activity is associated with a reduction in blood pressure. This result has been observed before but adds to the body of evidence, and helps raise awareness of the importance of physical activity. In addition, we assessed the synergistic benefits of consuming OLE alongside being more physically active in individuals with elevated blood pressure for 12 weeks. We observed that OLE consumption, increasing physical activity and the combination of these interventions significantly improved 24-h SBP. We observed a significant decrease in 24-h SBP after consumption of OLE alongside being increasing physical activity (OLE+PA). The magnitude of SBP change observed in this thesis would suggest that regular OLE intake as part of healthy lifestyle may be associated with a 9-14 % reduction in CHD risk and a 20-22 % reduction in risk of stroke. According to the results presented in chapter 5, our original hypothesis, that combining physical activity with the consumption of olive polyphenols would lead to greater reductions in blood pressure than either individual intervention alone, is not supported. There is some argument that interventions with both physical activity and diet are not more effective for BP control than diet alone [17, 18], however the merits of adding physical activity to a polyphenol rich diet need further investigation. A higher dose of physical activity than we employed may exert a different interaction with the hypotensive activity of the OLE. In the present study, OLE consumption, in the absence of increasing physical activity, led to a more optimal blood pressure profile, however the benefits of increasing physical activity are wide and we would continue to recommend it to OLE consumers.

6.2 Future perspectives

The interaction between habitual diet and the consumption of olive polyphenols has not been well studied; it is probable that supplementation has the greatest effects in low polyphenol consumers. In our intervention study we collected data on habitual dietary intakes using a food diary which have not yet been analysed. An immediate priority for us is to leverage these data and to consider differences in response to the intervention according to habitual intake.

Our in vitro studies (Chapter 3-4) showed that OLE inhibits key enzymes involved in macronutrient digestion (α-amylase and lipase), and also enzymes involved in hypertension (renin and ACE). In the intermediate term we intend to expand on this work through in silico molecular modelling, we will study the interactions of the olive phenolics and their in vivo metabolites with the amino acids in the active sites of these enzymes [19]. This will allow us to better understand the human observational study data.

The biological properties of dietary polyphenols may depend on their absorption in the gut and their bioavailability [20]. A priority for future work is to investigate the effects of digestive processes on the bioactivity and bioavailability of individual OLE phenolics. We have developed in vitro digestion and colonic fermentation models which might be leveraged to help us understand the concentrations of these molecules likely to directly interact with the vascular endothelium [21]. Further research is also needed to elucidate which specific OLE polyphenols exert the most potent effects, here we only considered the major phenolics (OL and HT) other related molecules present in OLE might have a considerable physiological influence. There remains a lack of knowledge about the biological effects of the individual circulating metabolites of OLE. Thus, future intervention studies might seek to quantify these in appropriate samples [22].

The work presented in this thesis adds to growing evidence demonstrating the benefits of OLE for cardiovascular health; from the human intervention trials we can now be fairly confident of the anti-hypertensive effects, anti-glyceamic effects and anti-oxidant effects of OLE, although mechanisms of action still need to be fully elucidated. Therefore, in the long term the strategy for olive polyphenol research may be to focus on wider benefits, improved cardiovascular function may have links to cognition which could be explored. Extra virgin olive oil consumption has been shown to improve cognitive function [23] and enhances learning and memory [24].

The influences of OL polyphenols on macronutrient absorption and glycaemia might have consequences for appetite regulation, again this is an exciting and timely area for potential research. Polyphenol rich grape extract consumed with a high starch-based meal, induced post-meal CHO malabsorption, enhancing satiety and reducing food intake at a later meal [25].

6.3 Concluding remarks

Taken together, our in vitro studies suggest potential mechanisms involved in the anti-hyperglycaemic activities and anti-hypertensive activities of OLE polyphenols. OL phenolics may influence glucose metabolism by inhibiting enzymes involved in glucose and fat digestion in the small intestine, and by inhibiting glucose uptake by the gut epithelia. In vitro, OLE phenolics enhanced NO production and inhibit angiotensin enzymes albeit at supra-physiological doses, this mechanism may help explain the observed improvements in blood pressure, but further studies are needed. It is possible that in vivo observations are due to the synergistic effect between the various polyphenols in OLE mixture; and this mixture is poorly understood.

Our data add weight to growing evidence that olive phenolics may be useful as supplements for the prevention of chronic diseases. Moreover, the current research achieved its objectives of providing evidence that OLE consumption, increasing physical activity and the combination of these interventions significantly improved 24-h SBP. Both interventions may be considered as beneficial in the control of blood pressure, consumption of supplemental olive leaf phenolics may be easier for a free living otherwise sedentary pre-hypertensive population. It is hoped that publications arising from this thesis may provide further evidence for the assessment of health claims made by olive producers for the European market, thus this thesis will have economic and social impact.

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Appendix: Human studies Ethics Application

SECTION 1: APPLICATION DETAILS

1.1

Project Title: Olive leaf extract as part of a healthy lifestyle in the reduction of blood pressure

Date of Submission: December 2014 Proposed start date: March 2015 Proposed End Date: End of September 2017

1.2

Principal Investigator: Dr Daniel Commane
Office room number: 3.43 Internal telephone: 0118 378 7108

Email address d.m.commane@reading.ac.uk
Alternative contact telephone: mobile phone: 07834423112
(Please note that an undergraduate or postgraduate student cannot be a named principal investigator for research ethics purposes. The supervisor must be declared as Principal Investigator)
Other applicants

Name: Miss Bandhita Saibandith (PhD Student) Department of Food and Nutritional Sciences
Email: B.Saibandith@pgr.reading.ac.uk; mobile phone specific for study: 07475285282
Name: Prof Jeremy Spencer. Department of Food and Nutritional Sciences
Email: J.P.E.Spencer@reading.ac.uk

1.3 **Project Submission Declaration**

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed…………………………… (Principal Investigator) Date:…………

…………………………… (Student) Date:…………

…………………………… (Other named investigators) Date:…………

…………………………… (Other named investigators) Date:…………

…………………………… (Other named investigators) Date:…………

1.4

University Research Ethics Committee Applications

Projects expected to require review by the University Research Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission.

Signed…………………………… (Chair of School Committee) Date:……………………

Signed…………………………… (Head of School) Date:……………………

SECTION 2: PROJECT DETAILS

2.1

Lay summary

Please provide a summary of the project in non-specialist terms, which includes a description of the scientific background to the study (existing knowledge), the scientific questions the project will address and a justification of these. Please note that the description must be sufficient for the committee to take a reasonable view on the likely scientific rigour and value of the project.

Hypertension affects about 30% of the UK population and is causally implicated in the aetiology of renal disease, cardiovascular disease and stroke. Ageing, obesity, a poor diet and low levels of physical activity are all risk factors [1].
Studies have shown that adherence to a Mediterranean diet is protective against hypertension and its associated morbidities; olive oil is believed to be a key beneficially bioactive component of that diet [2]. As a source of lipids olive oil is an unremarkable blend of monounsaturated, polyunsaturated and saturated fatty acids; it is however rich in phenolic compounds, principally oleuropein and hydroxytyrosol, which may be of benefit to health. A recent randomised intervention trial in predominantly hypertensive volunteers showed that adherence to a Mediterranean diet supplemented with extra virgin olive oil, reduced blood pressure and other measures of cardiovascular disease risk [3]. Olive phenolics can be extracted cheaply from the waste products of olive oil manufacture, such as the plant leaf and these are used as dietary supplements. In intervention studies in hypertensive or borderline hypertensive patients, olive leaf extract consumption has been shown to reduce blood pressure [4-6].
Another intervention with established efficacy for improving blood pressure is to increase physical activity (7). The 'Start Active, Stay Active', Chief Medical Officers report on physical activity recommends that adults achieve 150 minutes of moderate intensity physical activity per week, while data in that report suggest that fewer than 40% of adult men and 30% of adult women achieve these targets. Adherence to the physical activity guidelines may in fact be much worse in sub-sections of the population at higher risk of hypertension.

From a public health perspective, holistic guidelines for the prevention of hypertension, or its early diagnosis and management, based around a healthy diet and lifestyle are preferable to pharmaceutical intervention. Lifestyle interventions are economically favourable and they come with fewer side effects and perhaps wider health benefits than antihypertensive drugs. This study therefore aims to evidence the synergistic benefits of consuming plant (and specifically olive) phenolics alongside achieving the recommended guidelines for physical activity in individuals with elevated blood pressure.

The proposed study is funded by Comvita Ltd. It will be structured as a four way parallel study design in pre-hypertensive volunteers. The study arms are: i) A placebo control arm, on which volunteers consume a placebo capsule (Maltodextrin) daily over 12 weeks. ii) An increased physical activity arm, volunteers consume a placebo

control supplement and receive guidance on achieving the physical activity guidelines, they will be asked to wear an accelerometer daily and be asked to gradually increase their physical activity levels working towards a target of achieving thirty minutes of moderate exercise on five days of the week over the 12 week period, the accelerometer will be used both to monitor adherence but also as a motivational tool with the data up-loadable on to a community space, and a discussion/online support network created to establish a motivational competition for participants. iii) An olive leaf extract arm; volunteers will consume capsules of olive leaf extract (132 mg of oleuropein per day suspended in olive leaf extract) for consumption over the 12 week period with no guidance on physical activity. iv) And finally a physical activity and olive leaf extract arm, volunteers will receive the same intervention as the physical activity arm plus the OLE supplement over the 12 week period.

The intervention will last for 12 weeks and adherence to the treatment arms will be assessed via the analysis of plasma for metabolites of olive extract and through the assessment of accelerometer data.

Primary outcomes for the study will be changes in 24 hour ambulatory blood pressure and digital pulse wave velocity (as a measure of vascular function). Secondary outcome measures for the study will be the plasma HB1AC levels, (a measure of chronic blood sugar control). Volunteers will need to attend the NRG clinical unit on five occasions to facilitate screening and the collection of samples.

No adverse effects are anticipated with any of the treatments.

Public health guidelines generally advocate a healthy diet and increasing physical activity for the prevention of disease, few intervention studies have considered the synergistic effects of both. If successful this study may therefore be used to support that broader public health message.

(This box may be expanded as required)

2.2

Procedure

Please briefly describe what the study will involve for your participants and the procedures and methodology to be undertaken (you may expand this box as required).

This study is designed to test the hypothesis that olive phenolics and physical activity will induce independent and synergistic improvements to blood pressure in non-medicated pre-hypertensive volunteers in a chronic intervention.

The study will aim to recruit volunteers with above average blood pressure, but without clinically managed stage 2 hypertension (systolic 120 -160 and diastolic 80-99)

Suitable participants will be further defined as

- Men and women
- 25−70y
- BMI >25kg/m^2

•not having suffered a myocardial infarction/stroke in the past 12 months
•not diabetic (diagnosed or fasting glucose > 7 mmol/l) or suffer from other endocrine disorders
•not suffering from renal or bowel disease or have a history of choleostatic liver or pancreatitis
•not on drug treatment for hyperlipidaemia, hypertension, inflammation or hypercoagulation
•no history of alcohol misuse
•not planning or on a weight reducing regime
•not taking any fish oil, fatty acid or vitamin and mineral supplements
•non smokers

Females who are breast-feeding, who may be pregnant, or if of child-bearing potential and are not using effective contraceptive precautions will be excluded.

Interested volunteers will be provided with outline information about the study (Appendix D) and asked to complete a medical and lifestyle questionnaire (Appendix E). Volunteers who meet the inclusion criteria will be asked to attend a screening session during which a fasting blood sample will be taken and their BMI, waist circumference and blood pressure measured. The screening blood sample will be analysed for total cholesterol (TC), high density lipoprotein (HDL) cholesterol, triacylglycerol and glucose; and will also be used to determine blood count and kidney and liver function. By screening a blood sample, the general state of health of the volunteer may be ascertained by identifying if certain conditions are present, such as anaemia, hyperlipidaemia, diabetes and cirrhosis of the liver. The total pre-screening volume of blood collected will be 15mL (approximately 1 tablespoon). Individuals who are anaemic (Hb <14g/dl male, 11.5g/dl female) or who have 'abnormal' blood biochemistry based on standard clinical cut-offs, will be automatically excluded. Suitable individuals (please see page 4) will be invited to participate in the study. With the participants' permission, a letter informing the General Practitioners of each volunteer's study participation will be sent Appendix F).

At all stages of the study each participant will be identified by a unique code number.

During an initial two−week run−in period no intervention will be provided to any of the volunteers. However, during this period subjects will attend the Hugh Sinclair Unit of Human Nutrition for a 'familiarisation visit' in order to have their blood pressure (BP) measured and vessels elasticity determined via the Digital Volume Pulse (DVP) as a surrogate marker of blood flow. Stress is a major determinant of BP and vascular function; anxiety caused by unfamiliarity with the procedures may cause atypical values on the first visit (white coat effect). Our proposal to include measurement of these parameters in the run–in (as a dummy measure) aims to familiarise the participants with the techniques in order to minimise the impact of stress on the subsequent (baseline) assessments.

Volunteers will then be randomised to one of the four study arms.

i) A placebo control arm. These volunteers will be asked to consume a placebo capsule (maltodextrin) daily over 12 weeks.
ii) An increasing physical activity arm. Volunteers will be given a placebo control supplement and receive guidance on the physical activity guidelines, they will

be asked to wear an accelerometer daily and given the target of achieving thirty minutes moderate exercise on five days of the week over the 12 week period, the accelerometer will be used to monitor adherence but also as a motivational tool with the data up-loadable on to a community space, and a discussion/online support network created to establish a motivational competition for participants.

iii) An olive leaf extract supplement arm: volunteers will be given capsules of the olive leaf extract (containing 132 mg of oleuropein in olive leaf extract) for consumption once daily over the 12 week period with no guidance on physical activity.

iv) A physical activity and olive leaf extract combined arm: Volunteers will receive the same instruction and support tools regarding exercise as the physical activity arm plus they will be asked to consume the OLE supplement capsule daily over the 12 week period

Each volunteer will be required to record information daily about their general well-being, bowel movements, any medication they take during the trial and any adverse effects, in diaries distributed during visits to the unit (Appendix H).

Motivating physical activity:

Volunteers on the physical activity arm of the study will be asked to wear an Omron hip mounted pedometer. The data from this instrument can be uploaded onto an online database and we will provide the individuals with a diary in which they can track their daily step count. Volunteers will receive weekly motivational hint sheets (Appendix K) through the post to encourage continued engagement. These sheets are structured such that activity is increased progressively over the duration rather than setting potentially unachievable and therefore demotivating targets.

Volunteers will be required to attend the clinical unit for a total of five clinical visits, screening, familiarisation, baseline, 6 weeks and at 12 weeks, volunteers will visit the clinical nutrition unit having fasted overnight, in order to provide samples and have measurements taken (visits 1-5, Figure1).

The primary endpoint is 24 hour ambulatory blood pressure measured using the ScanMed Oscillometric ABP device (www.scanmed.co.uk). Volunteers will be asked to wear the device which will be programmed to record BP measurements every 30 minutes during the day (7am-10pm) and every hour by night (10pm-7am). Measurements will be made in accordance with British Hypertension Society guidelines. Subjects will be asked to complete a diary card of activities during the 24 hour measurement period.

Vascular reactivity will also be assessed using a technique called Digital Volume Pulse (DVP) during the clinical visit. The DVP is recorded by placing an infra–red light–transmitting unit on the participants’ finger and measuring the changes in the volume of blood in the fleshy part of the finger caused by participants’ normal pulse. The measurements are painless, and will take 10 seconds. Three measurements will be carried out at 5 minute intervals.

Blood samples will be collected from the cannula and used for assessing:

- Average plasma glucose concentration (a measure of chronic blood sugar control) by measuring of glycated haemoglobin (HbA1c)

- Plasma biomarkers of endothelial function including nitric oxide, vascular cell adhesion molecule (VCAM), Inter-Cellular Adhesion Molecule (ICAM), E-selectin, von Willebrand factor

- Fasting lipid profile including measures of total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol, triglycerides and non-esterified fatty acids

- Indices of insulin resistance derived from fasted measures of glucose, insulin and non-esterified fatty acids (revised QUICKI statistical analysis) (Brady *et al*., 2004)

- Haemostatic factors including Plasminogen activator inhibitor-1 (PAI-1)

- Inflammatory biomarkers including the acute phase proteins, C-reactive protein (CRP), tumour necrosis factor alpha (TNFα), Interleukin-6 (IL6)

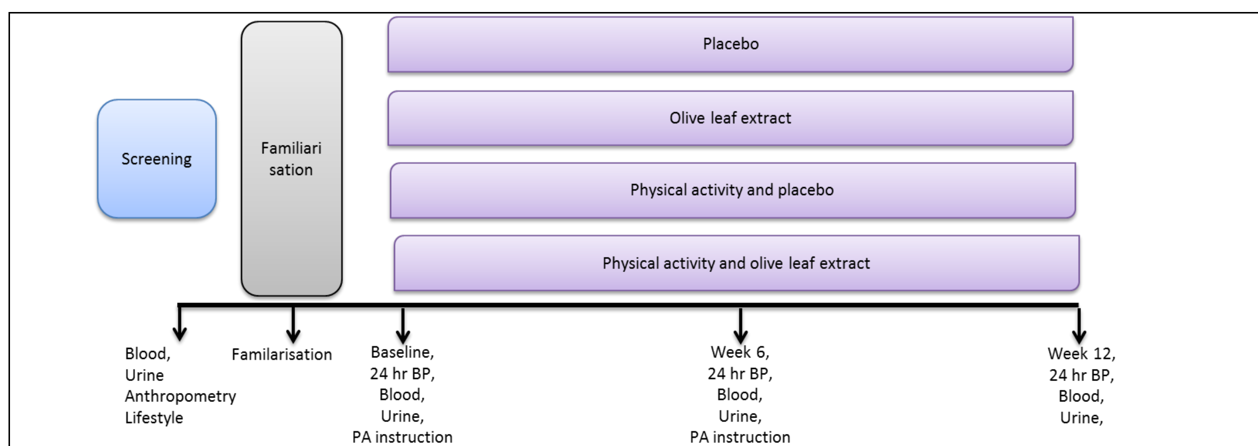
- Serum will be extracted by centrifugation of fasting, venous blood collected into lithium heparin tubes immediately after collection at each time point and stored at -80°C before being analysed by 1H-NMR-based metabolomics for serum low molecular weight metabolite profiles, and used for assessing adherence to the supplement intervention.

A total of 65 mL of blood shall be collected at each visit.

Urine samples will be analysed for phenolic compounds deriving from the OLE via LC-MS to assess subject compliance

During each visit anthropometric measurements will be taken and recorded

- Supine blood pressure
- Weight
- Waist circumference
- Body fat composition (using electrical bioimpedance)



Setting: study visits will take place at the Hugh Sinclair Human Nutrition Unit which has significant experience of performing dietary intervention studies. The Department of Food and Nutritional Sciences is fully licensed under the Human Tissue Act 2004 and as such will adhere to the guidelines necessary for the storage of faeces and blood. A detailed log will be kept to record when the samples were taken, the place of storage, when analysis was conducted on the samples and how and when the samples were disposed of.

Assessment of physical activity

The new lifestyles NL1000 is a hip worn pedometer that can be worn daily for the duration of the study it reports steps taken directly to the wearer and can therefore be used to motivate participants as well as to capture physical activity data. Individuals on the physical activity arms of the study will be asked to wear these for the duration. These measures will be validated against data collected using the wGT3X actigraph http://www.actigraphcorp.com/product-category/activity-monitors/ Volunteers will be asked to wear the actigraph, periodically 4 x 3 days over the duration of the study.

Details of the ambulatory blood pressure measurement

Ambulatory blood pressure will be measured using the ScanMed Oscillometric ABP device (www.scanmed.co.uk). This device has been validated independently according to the British Hypertension Society (BHS, www.bhsoc.org) Protocol (A/A grade). The software of the device issued to the volunteers will be programmed to record BP measurements every 30 minutes during the day (7am-10pm) and every hour by night (10pm-7am). Measurements will be made in accordance with British Hypertension Society guidelines. Subjects will be asked to complete a diary card of activities during the 24 hour measurement period.

- Details of the procedure of Digital Volume Pulse (DVP)

The DVP is recorded by placing an infra-red light-transmitting unit on the participants' finger and measuring the changes in the volume of blood in the fleshy part of the finger caused by participants' normal pulse. The digital volume pulse (DVP) is recorded by measuring the

transmission of infra-red light absorbed through the finger. The amount of light is directly proportional to the volume of blood in the finger pulp. It has been shown that the stiffness index (SIDVP) that can be derived from the DVP waveforms is highly correlated to arterial stiffness. For measurement of the SIDVP, a photoplethysmograph (Micro Medical, Gillingham, Kent, UK) is placed on the index finger of the right hand to obtain the DVP. Blood pressure and DVP waveforms will be recorded 3 times over 10 second periods with 5 minute intervals between measurements. The DVP waveform has two components. The first part of the waveform (systolic component) is formed as a result of pressure transmission along a direct path from the aortic root to the finger. The second part (diastolic component) is formed by pressure transmitted from the ventricle along the aorta to the lower body where it is reflected back along the aorta to the finger. The average peak-to-peak time (PPT) for each measurement will be calculated using the PulseTrace Softwave (version 1.0, Micro Medical) and SIDVP index will be calculated by dividing the PPT value into height in m and multiplying the result by 1000 to convert the result into m/sec. The reflection index (RI) is calculated as the height of the diastolic peak of the DVP relative to that of the systolic peak.

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(Note: All questionnaires or interviews should be appended to this application)

2.3 Where will the project take place?

The project will take place at the Food Biosciences building (Department of Food and Nutritional Sciences), within the Hugh Sinclair Unit of Human Nutrition.

If the project is to take place in Hugh Sinclair Unit of Human Nutrition, please confirm that you have informed Mrs Sarah Hargreaves(s.e.penn@reading.ac.uk)

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? Yes

If Yes, please give details:
Comvita Ltd are funding this study

Please note that *all* projects, (except those considered as low risk, questionnaire-based studies which require Head of School approval) require approval from the University Research Ethics Committee.

2.5

Ethical Issues

Could this research lead to any risk of harm or distress to the participants? Please explain why this is necessary and how any risk will be managed.

There are no reported side effects of the study treatment, the supplement is a commercially available extract of olive leaf and all procedures (venepuncture, Digital Volume Pulse) will be performed by trained researchers. However, in case of an emergency (e.g. fainting, blood spillage) researchers will take immediate action according to Hugh Sinclair Unit of Human Nutrition relevant SOP that describes in detail the actions that need to be taken by. Also, a Departmental First Aider will be available in the building when blood is being taken. In case of emergency, the research scientists will call the emergency services (999).

(*this box may be expanded as required*)

2.7

Payment

Will you be paying your participants for their involvement in the study? Yes
If yes, please specify and justify the amount paid

Based on standard remuneration to volunteers on other similar studies a total of £170 will be paid, to cover time and travel expenses incurred (5 visit days). There is no payment for the initial screening visit.

Early dropouts will have payment pro-rated according to time spent. Reserve volunteers will be paid £20 if they are not required to participate in the treatment phases of the trial.

Note: excessive payment may be considered coercive and therefore unethical. Travel expenses need not to be declared.

2.8

Data protection and confidentiality

What steps will be taken to ensure participant confidentiality? How will the data be stored?

Confidentiality will be maintained by allocating volunteers an identification code, which will be used to identify all samples and data obtained. Volunteer's names will not be used in any reports or publications. All data from the study will be held securely throughout the duration of the project, however, a record of the names of the volunteers will not be held on the same file. Information matching volunteer's names with identification code will be kept by a departmental secretary and the investigators will only use identification codes. The only time data will be matched with volunteer's names is for those volunteers that request to have their personal results discussed with them. A request for individual results to be discussed will include a review of all sample results for the individual volunteer.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

Interested volunteers will be provided with outline information on the study and will be asked to complete a medical and lifestyle questionnaire. Volunteers who meet the inclusion criteria will be asked to attend a screening session during which consent will be asked for (Appendix C). Following agreement, a fasting blood sample will be taken and BMI, waist circumference and blood pressure measured. The screening blood sample will be analysed for total cholesterol (TC), high density lipoprotein (HDL) cholesterol, triacylglycerol; and glucose and will also be used to determine blood count and kidney and liver function. Individuals who are anaemic (Hb <14g/dl male, 11.5g/dl female) or who have 'abnormal' blood biochemistry based on standard clinical cut-offs, will be automatically excluded. Suitable individuals will be invited to participate in the study.

Please note that a copy of consent forms and information letters for all participants must

be appended to this application.

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined?
No

Please note that a copy of all information sheets on the implications of determining the specific genotype(s) to be undertaken must be appended to this application.

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

Data from an investigation of the effect of the same extract on blood pressure in 45 pre-hypertensive volunteers with a crossover study design was used to predict effect size. In that study the mean difference in systolic blood pressure between treatment and placebo was -3.33 mm Hg with an sd of 7.9, thus giving an effect size (cohens d) of 0.422
With the cut off for α at 0.05 and with 85 % power, predicted total sample size is 112 volunteers. 28 per group, or 30 per group allowing for drop outs.

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)? No

If yes, how will you ensure these participants fully understand the study and the nature of their involvement in it and freely consent to participate?

(Please append letters and, if relevant, consent forms, for parents, guardians or carers). Please note: information letters must be supplied for all participants wherever possible, including children. Written consent should be obtained from

children wherever possible in addition to that required from parents.

3.3

Will your research involve children under the age of 18 years? No
Will your research involve children under the age of 5 years? No

3.4

Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? No

Please note that if your research involves NHS patients or Clients of Social Services your application will have to be reviewed by the University Research Ethics Committee and by an NHS research ethics committee.

3.5

Recruitment
Please describe the recruitment process and append all advertising and letters of recruitment.

The Hugh Sinclair Unit in the Department of Food and Nutritional Sciences has extensive experience, and uses a variety of approaches, in the recruitment of subjects for dietary intervention studies. The approaches to be used here will include:

- posters, gumtree and newspaper advertising, articles and radio interviews
- advertising the study by e-mailing all staff members in large local organisations and companies i.e. Reading Borough Council, The Prudential, Oracle and ING Direct, the University of Reading
- Invitation letters to be sent to potential volunteers from the sensory dimensions volunteer database
- Invitation letters, emails or phone calls to be sent/conducted to volunteers from The Hugh Sinclair Unit of Human Nutrition volunteer database containing approximately 1000 names
- Email to be sent to organisations managers to be displayed within the premises of large local organisations, companies and clubs.
- Advertisement around the university, notices on staff and student webpages

These approaches have proved very successful in the past as a means of recruiting specific population groups for challenging dietary interventions.

(see appendix I Invitation letter and Invitation by email Appendix J and Volunteer recruitment poster Appendix K)

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and have been taken and all the appropriate documentation has been appended.

2. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the Form in Appendix B.

Appendix A: Application checklist

This must be completed by an academic staff member (e.g. supervisor)

- Please tick to confirm that the following information has been included and is correct.
- Indicate (N/A) if not applicable:

- **Information Sheet**

Is on headed notepaper

Includes Investigator's name and email / telephone number

Includes Supervisor's name and email / telephone number

Statement that participation is voluntary

Statement that participants are free to withdraw their co-operation

Reference to the ethical process

Reference to Disclosure

Reference to confidentiality, storage and disposal of
personal information collected

Consent form(s)

- **Other relevant material**

Questionnaires

Advertisement/leaflets

Letters

Other (please specify)

☑

Expected duration of the project
(months)

Name (print) ……………………………………… Signature ………………………………

Appendix B: Project submission form

Title of Project: **Olive leaf extract as part of a healthy lifestyle in the reduction of blood pressure**

Proposed start date: End of March 2015
Brief description of Project: A 12 week diet and lifestyle intervention study investigating the effects of consuming olive leaf extract, in conjunction with achieving the guideline daily physical activity levels, on blood pressure.

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.
I confirm that if this project is an interventional study, a list of names and contact details of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.
Signed:
…………………………………………………….. Date: …………………………………
(Investigator)
…………………………………………………….. Date: …………………………………

(Head of School or

Authorised Head of Department)

…………………………………………………….. Date: …………………………………

(Student -where applicable)

Version – September 2010

Checklist

1. This form is signed by my Head of School (or authorised Head of Department) ☑
2. The Consent form includes a statement to the effect that the application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct ☑
3. I have made, and explained within this application, arrangements for any confidential material generated by the research to be stored securely within the

University and, where appropriate, subsequently disposed of securely.

☑

4. I have made arrangements for expenses to be paid to participants in the research, if any, OR, if not, I have explained why not.

☑

5. EITHER

(a) The proposed research does not involve the taking of blood samples; ☐

OR

(b) For anyone whose proximity to the blood samples brings a risk of Hepatitis B, documentary evidence of protection prior to the risk of exposure will be retained by the Head of School or authorized Head of Department.

☑

Signed:

………………………………………….. Date……………………

(Head of School or

Authorised Head of Department)

6. EITHER

(a) The proposed research does not involve the storage of human tissue, as defined by the Human Tissue Act 2004; ☐

OR

(b) I have explained within the application how the requirements of the Human Tissue Act 2004 will be met.

☑

7. EITHER

(a) The proposed research will not generate any information about the health of participants; ☐

OR

(b) In the circumstance that any test reveals an abnormal result, I will inform the participant and, with the participant's consent, also inform their GP, providing a copy of those results to each and identifying by name and date of birth;

☑

OR

(c) I have explained within the application why (b) above is not ☐

appropriate.

8. EITHER

(a) the proposed research does not involve children under the age of 5;

☑

OR

(b) My Head of School (or authorised Head of Department) has given details of the proposed research to the University's insurance officer, and the research will not proceed until I have confirmation that insurance cover is in place. ☐

Signed:

…………………………………………. Date…………………..…

(Head of School or

authorised Head of Department)

This form and further relevant information (see Sections 5 (b)-(e) of the Notes for Guidance) should be returned to:

Mr Nathan Helsby

Planning Support Office

Room 321

Whiteknights House

Tel: 0118 378 6972

Email: n.e.helsby@reading.ac.uk

- **both electronically and in hard copy**

You will be notified of the Committee's decision as quickly as possible, and you should not proceed with the project until then.

Appendix C: Consent Form

Volunteers /name

General Practitioner/ name

1st line of Address...
....
Town...
County..
Postcode...

1st line of Address...
....
Town...
County..
Postcode...

(Tick boxes if in agreement)

1. I have read and had explained to me by ……………………………………………..
the accompanying Information Sheet relating to the project on **A study of diet and lifestyle behaviours in the control of blood pressure**

2. I have had explained to me the purposes of the project and what will be required of me, and any questions I have had have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation.

3. I understand that participation is entirely voluntary and that I have the right to withdraw from the study at anytime, without giving reason and that this will be without detriment

4. I consent to an initial blood sample being taken for screening purposes, followed by a series of blood samples throughout the study at times indicated on the Participant Information Sheet.

5. I authorise the investigator to inform my General Practitioner concerning my participation in this study

6. I authorise the investigator to notify myself and my General Practitioner in the event of any abnormal results and to provide a copy of the results to both.

7. This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

8. I have received a copy of this Consent Form and of the accompanying Information Sheet.

9. I give consent for a copy of this form to be kept on a database for a minimum of 5 years (a copy of which will be made available to the volunteer)

10. I wish to receive a summary of the results once the study is complete and analysed statistically.

Yes ☐ ☐

Print name..Date of birth...

Signed...
Date...

Witnessed by

Name..

Date ....

(This form is to be retained by the Head of School or authorised Head of Department. A copy should be made available to the person providing the sample(s) if he or she wishes)

Appendix D: Volunteer Information Sheet

Olive leaf extract as part of a healthy lifestyle in the reduction of blood pressure

You are invited to take part in a volunteer research trial at the Department of Food and Nutritional Sciences.

Why is this study being carried out?
High blood pressure is a leading cause of kidney disease, cardiovascular disease and stroke. It may be precipitated by dietary and lifestyle choices. In this study we will consider the potential for extracts from the olive plant to reduce blood pressure when consumed as part of a healthy lifestyle.

Before you decide whether to take part in the study, please read the following information carefully. If you want to know anything more about the study, or want to discuss further, please contact an investigator

What will I be asked to do?
We will first invite you for a 30 minute screening visit to determine your eligibility to take part in this study. During this visit we will ask you questions about your health we will measure your body shape including your height, weight, waist circumference and blood pressure. We will take a fasted blood sample (15 mL ~ 1 tablespoon in volume; which our trained staff can collect on site) so that the fat and cholesterol levels in your blood can be measured. The levels of insulin and blood glucose will also be assessed - along with measures of kidney and liver function. We will also ask you to complete a lifestyle questionnaire. From this information we can assess your suitability for the study against the inclusion and exclusion criteria detailed below.

Individuals who meet these **Exclusion Criteria** will be unsuitable for this study:

- Use of antibiotics within the previous 6 months
- History of alcohol or drug abuse
- Intake of any experimental drug within 4 weeks of the start of the study
- Excessive alcohol consumption (more than 21 units/wk male, 15 units/wk female)
- Females who are breast-feeding, may be pregnant, or of child-bearing potential and not using effective contraceptive precautions
- Have had recently (in the last 5 years) major surgery, which might limit participation in, or completion of, the study.
- On drug treatment for high blood fats, high blood pressure and blood clotting.
- Physical or mental diseases that are likely to limit participation or completion of the study
- Diabetic (diagnosed or fasting glucose > 7mmol/l) or suffer from endocrine disorders
- Suffer from renal or bowel disease or have a history of choloeostatic liver or pancreatitis

- Have suffered from a myocardial infarction/stroke in the past 12 months
- Anaemic (haemoglobin men >14 g/dl; women >11.5 g/dl)
- Taking any dietary supplements including dietary antioxidants, other phytochemicals, fatty acid supplements including fish oil
- Aged below 20 or over 70 years
- Have a BMI of less than 25 kg/m^2
- Lactose allergy or intolerance
- Calorie restriction or other special diet (e.g. Atkins diet, Montignac diet) 6 weeks prior to the start of the study
- Smokers

If none of the above apply then to participate you also need to satisfy these **inclusion criteria**

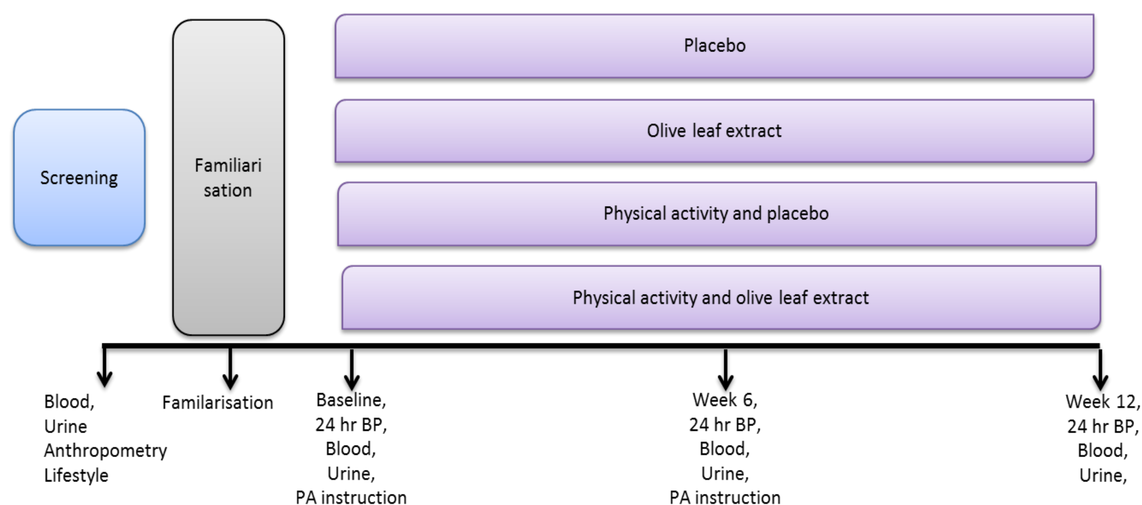
- Signed consent form
- The study will aim to recruit volunteers with above average blood pressure but without clinically managed stage 2 hypertension (systolic 120 -160 and diastolic 80-99)
- Body mass index – 25 kg/m^2 inclusive
- Age 20– 70 years
- Men and women

If deemed suitable, and if you consent to participate in the study, we will invite you to attend the clinic once more for a familiarisation visit; during this visit we will introduce to the processes by which we measure physical activity, blood pressure and digital pulse wave velocity, we will also ask you to take a 3 day food diary away with you for completion prior to your next visit and we may equip you with an accelerometer to measure how much you move. The familiarisation visit will last no more than 1.5 hours. You will then be asked to return to the clinic in a fasted state between 8 and 10 am for the baseline visit, we will ask you to bring with you a 24 hour urine sample on this day and during this visit we will take measures of blood pressure, pulse wave velocity, and physical activity in addition to collecting blood samples. We will then assign you to a study treatment arm. The treatment will involve a daily dose of four capsules containing either 51.1 mg of the active test compound (oleuropein) in safflower oil or four capsules of a safflower oil control. These should be consumed as a single dose once daily with a glass of water.

A second part of the study is to assess the effects of increasing physical activity in conjunction with the supplement and so you may, or may not, also be asked to increase your physical activity levels in line with targets laid out in the Physical Activity for Health report. The recommendations are equivalent to achieving at least 30 minutes of moderate-intensity physical activity on five days per week. If you are on a physical activity arm of the intervention we will encourage you to progressively increase your physical activity levels over the 12 weeks towards these targets. We will ask you to wear a hip mounted physical activity monitor and to record your step count each day. Where appropriate we will give you guidance on strategies to achieve desirable levels of activity based around increasing walking behaviour and decreasing sedentary leisure time

behaviour. If you are not on the physical activity arm of the study we would encourage you to continue with your normal activity levels.

Your participation in the intervention will last for 12 weeks and will involve a total of 5 site visits, including the screening visit, familiarisation, baseline, 6 weeks and 12 weeks. The screening visit will last approximately 30 minutes, and the other 4 visits approximately 1.5 hours.



Study visit schedule

The final two study visits to the Department of Food and Nutritional Sciences will be scheduled between 8 and 10 am in the morning before breakfast. You will be asked to attend in a fasted state (i.e. no food or drink apart from water for the 12 hours prior to your visit). You will need to provide:

- fasted (12 hour) venous blood (40 mL, which is the equivalent to ~3 tablespoons in volume) (collected by our trained staff)
- a 24 hour urine sample (collected over the 24 hours prior to the visit using the water-tight vessel provided for you)
- If you are equipped with an accelerometer, we will use the study visits to log that data.

During each of the study visits we will equip you with a blood pressure monitor which will need to be worn for a 24 hour period, and for which we will then arrange for you to return by post.

Diet, lifestyle and medication during study

Throughout the study you will be asked to keep a Volunteer Diary of your general well-being

- Please record any period of sickness or feelings of unwell during the study

- It is important that you adhere to your usual diet and lifestyle unless otherwise advised through the duration of the study.

General

•This study has been subject to ethical review by the University of Reading Research and Ethics Committee and has been given a favourable ethical opinion for conduct.

•Your participation in this study is purely voluntary. You may leave the study at any time without giving a reason and without detriment.

•If you would like to receive further information or book an appointment to complete the pre-study medical questionnaire then please contact me using the details at the end of this information sheet.

Throughout the trial you will be asked to provide a total of 6 urine samples, and a total volume of 340 mL of blood which is about 23 tablespoons in volume (see attached timetable). During study visits you will be issued questionnaires that you will be asked to complete containing some basic information that will help us evaluate the treatment products.

Are there any risks?
It is not envisaged that the volunteers will experience any adverse effects, the olive leaf extract is commercially available and previous trials have shown it to be safe.

Confidentiality
Your records will be kept strictly confidential. Your study results will be recorded in a case record form for the study investigator but your name will not appear in this or on any report/publication of the results. The investigator or government authorities may need to look at your records to check if the information has been correctly collected, but at no stage will you be identified by name. All records will be kept for 5 years before complete destruction.

Other information
You will receive £170 for completing the trial if deemed eligible after screening. Volunteers that drop out will have their payment pro-rated to cover the part of the study completed.

You are free to ask the investigator for more information about this study before you give your consent to take part.
The general results from the study and those relating to an individual may be of interest to volunteers. Each volunteer may only request their own individual results and not those of any other volunteer on the study. Only at the time of result dissemination will data be matched with volunteer's names. Volunteers that request information on the trial will be sent information on the results of their participation, including their blood parameters and urine data from the sampling periods although this will not be until after the full completion of the study.

If you have any queries, please do not hesitate to contact the study team

Investigators
Miss Bandhita Saibandith, B.saibandith@pgr.reading.ac.uk
Dr Daniel Commane, d.m.commane@reading.ac.uk
Prof Jeremy Spencer, j.p.e.spencer@reading.ac

Study Timetable

Trial Day	***Stage of Study***	*Treatment*
-28	Screening Visit 1	• *Briefing about the study* • Screening blood samples Weight, height, waist circumference and blood pressure measurements will be taken • Habitual diet recorded
-14 to -7	Familiarisation Visit 2	• Digital Volume Pulse • Blood and urine sample collection • 24 hour ambulatory blood pressure fitting and instruction
1	• Base-line samples Visit 3	• Digital Volume Pulse • Blood and urine sample collection • 24 hour ambulatory blood pressure fitting and instruction Allocation to study arm, and instruction for study
39-49	6 weeks Visit 4	• Digital Volume Pulse • Blood and urine sample collection • 24 hour ambulatory blood pressure fitting and instruction
80-89	12 weeks Visit 5	• Digital Volume Pulse • Blood and urine sample collection • 24 hour ambulatory blood pressure fitting and instruction

***Please note breads can be collected on a weekly basis for ease of storage – please arrange with investigator**
Note: Volunteers will take all treatments during the course of the study but not at the same time.

Appendix E: Questions to be asked during recruitment

Olive leaf extract as part of a healthy lifestyle in the reduction of blood pressure

Medical and Lifestyle Questionnaire

<table>
<tr><td colspan="2">Name:</td><td>Title:</td></tr>
<tr><td colspan="3">Address:</td></tr>
<tr><td>Daytime Tel:

Mobile no:

Best time to phone:</td><td colspan="2">DOB:

Include if born after 1981

Exclude if born before 1941</td></tr>
<tr><td colspan="3">E-mail:</td></tr>
</table>

How did you hear about the study?

....

Height....................................... (cm)

Weight....................................... (kg)

Waist circumference................... (cm)

Medical questions

- *Please delete as appropriate.*

1. Do you have Coeliac disease or Gluten Allergy YES/NO

Exclude if yes

2. Have you been diagnosed as having any of the following illness? Please give details.

a) High blood cholesterol YES/NO

medication_...
....

....
....

b) High blood pressure YES/NO

medication...
....
....
....

c) Hyper coagulation (excessive blood clotting) YES/NO

medication..
....
....
....

Exclude *if they are on blood pressure, hyperlipidaemia, or hyper coagulation medication*

d) Thyroid disorder or any other endocrine disorders? YES/NO

If 'YES', please give details on how long since it was first diagnosed?

....
....
....
....

e) Heart problems, stroke or any vascular disease in the past 12 months YES/NO

If 'YES', please give details

....
....
....
....

f) Diabetes or other hormone disorders YES/NO

g) Inflammatory diseases such as rheumatoid arthritis, psoriasis YES/NO

If 'YES', please give details, including if you take any anti-inflammatory drugs or any other medication?

....
....
....

h) Renal or bowel disease or have a history of liver disease or pancreatitis YES/NO

If 'YES', please give details

………
………
……

***Exclude** if suffering from conditions **d**) to **h**).*

3. Do you suffer from any conditions involving dermatological/connective tissue? YES/NO
(if yes please provide details)
………
………
……………

4. Do you suffer form any conditions involving head, ears, nose and throat? YES/NO
(if yes please provide details)
………
………
…………………

5. Do you suffer from any respiratory conditions? YES/NO
(if yes please provide details)
………
………
…………………

6. Do you suffer from any abdominal conditions? YES/NO
(if yes please provide details)
………
………
…………………

7. Do you suffer from any urogenital/rectal conditions YES/NO
(if yes please provide details)
………
………
…………………

8. Do you suffer from any lymphatic conditions? YES/NO
(if yes please provide details)
………
………
…………………

9. Do you suffer from any neurological conditions? YES/NO
(if yes please provide details)

………
………
………………………

10. Have you been diagnosed as suffering from any other illness which causes you to take any long term medication? YES/NO

If 'YES', please give details

Illness and medication

………
………
…………………

11. Are you due to undergo major surgery which might limit participation in, or completion of the study YES/NO

………
………
…………………

12. Have you used antibiotics within the previous 6 months YES/NO

13. Do you have electronically sensitive support systems e.g. pace-maker? YES/NO

***Exclude** if YES to questions 11-14*

14. Do you smoke? YES/NO

If 'YES', please give details. (e.g. how many do you smoke on average a day?)

………
………
……………………………

15. Have any of your close relatives (e.g. mum, dad, sister, brother, son, daughter) suffered from cardiovascular disease (myocardial infarction) before the age of 55 years (for males) or before the age of 65 (for females), or been diagnosed with type 2 diabetes? YES/NO

16. (a) Do you take any pain killing medication (for example, aspirin, paracetemol, Nurofen, Ibuprofen) daily or more than 4 times per week? YES/NO

b) If you take aspirin is it on prescription? YES/NO

***Exclude** aspirin users if use is prescribed.*

If taking non-steroid anti-inflammatory drugs (NSAID), ask question c below

c) If you were to participate in our study, would you be willing to use paracetemol as an alternative during the course of the study? YES/NO

Exclude *if not willing.*

17. Do you suffer from any type of allergies including food and pollen or any intolerance? YES/NO

If 'YES', please give details of condition and medication use).

……
……
……
………………………

Exclude *if severe asthma and on regular anti-inflammatory medication or use of asthmatic inhalers more than twice per month. In case of lactose or glucose- intolerance or allergy exclude*

Females please answer/complete question 19; males go to question 20

18. a) Are you premenopausal, perimenopausal or postmenopausal?

If 'postmenopausal':

i) Do you remember the approximate time of your last menstruation period?

Less than a year ☐
1-2 years ☐
2-5 years ☐
More than 5 years ☐
Don't know (HRT) ☐
Can't remember ☐

ii) Are you on hormone replacement therapy (HRT)?
YES/NO

If 'YES', how long have you been on HRT?

………
………………………………………………………

b) I hope you don't mind the following questions but I need to ask these since hormones may affect your lipids and other measurements.

Are you on contraception? YES/NO

If 'YES' please give details including, if relevant, the name of the contraceptive pill)

……………………………………………………………………………………………….

If you are breast-feeding, may be pregnant, or of child-bearing potential and not using effective contraceptive precautions you will be excluded from the study

Dietary questions

19. Are you a vegan or vegetarian? YES/NO

20. (a) Do you take any form of prebiotics or probiotics? (i.e. fructooligosaccharides or live yoghurts) YES/NO

(b) To participate in the study volunteers should not have consumed these products within four weeks of the start date, and also during the study, would this be an issue for you?

YES/NO

If 'YES' for b exclude from the study

21. Do you drink alcohol?

YES/NO

If '*YES*' Roughly how much do you drink per week?

A unit of alcohol is half a pint of beer/lager, a single pub measure of spirits (e.g. gin/vodka or a small glass of wine (125 mL).

Exclude *those who appear to drink more than 15 units of alcohol (for women) or more than 21 units (for men) per week.*

Have you ever suffered from alcohol misuse?
YES/NO

Exclude *if 'YES'.*

22. Are you currently on or planning to start a weight reducing or other diet? YES/NO

If *'YES'*, please give details

……
……
………

a) If you were to participate in our study, would you be willing to postpone the weight reducing or other diet for after the end of the trial?

YES/NO

Exclude *if on or not willing to postpone the diet for after the end of the trial.*

Do you currently consume laxatives on a regular basis?

YES/NO

If *'YES'*, please give details

………
………………………………………………………

a) If you were to participate in our study, would you be willing to cease consumption of these products until the end of the trial?

YES/NO

Exclude *if not willing to cease laxative consumption*

23. Are you taking any dietary antioxidant, phytochemical, fatty acid or fish oil supplements?

YES/NO

Lifestyle questions

24. Have you taken part in any other nutrition study or clinical trial or been a blood donor in the last three months?

YES/NO

If yes, what kind of trial was that?

……
……………………………………………………..

If 'YES', when will this study be completed?

Exclude *if the study will be completed in more than 1 month*

25. Do you travel regularly for work? YES/NO

26. Do you plan to take a holiday in the next 6 months? YES/NO

List holiday period:

Researcher: ______________________

This is the end of the questionnaire. Thank you for your time. All information provided will remain confidential at all times.

NOTE: IF SUITABLE FOR THE STUDY THEN BOOK A DAY FOR SCREENING AND REMIND THEM NOT TO WEAR TIGHTS (females - for Tanita) AND VERY TIGHT LONG SLEEVES (for blood pressure) AND TO COME IN FASTED.

Appendix F: Letter inform GP

Dear *Name of GP*,

Name of Patient, DOB, has expressed an interest in participating in a dietary intervention trial at The University of Reading. The trial will aim to study the effects of consuming olive leaf extract in conjunction with achieving the recommended physical activity guidelines on the reduction of blood pressure**.**
This human study is funded by the Comvita Ltd and has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been given a favourable ethical opinion for conduct.

The trial will commence on (*Trial start date)*

We have enclosed a subject Information sheet for your information. If you have any questions about the trial please do not hesitate to contact me.

Yours sincerely,

Bandhita Saibandith

Appendix H: Volunteer diaries

Volunteer Diary

Page 1

Volunteer No. ________________________

Period No. ______________________ **Day No. to Day No.**

Please fill in the diary carefully and completely for each day. If you are unsure how to answer, please give the best information you can. Please return completed diary to Bandhita Saibandith on your next visit.

To be filled in by investigator only!

Date started at:

Next visit at:

Medication

Please enter the intake of any medication taken during the study with name, dosage per day, date started and date stopped.

Medication	Dosage	Date started - stopped
e.g. paracetamol	**500mg twice**	**15/1 – 15/01**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		

Volunteer No. ________________

Adverse Events

Please enter the occurrence of all adverse events with start and end date and time.

Adverse Event	Date – started	Date – stopped
e.g. headache	**15/01**	**15/01**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		

Volunteer No. _______________

Final Questions

Did you experience any of the following after taking the supplement?

	yes	no
Did you experience an after taste?		
Did you experience a feeling of fullness?		
Did you experience difficulty in taking the product?		
Did you NOT consume any of the products?		

If the answer to the last question above is "yes" then please state numbers of slices or quantity of orange juice not consumed and the date and the reason for non-consumption.

	Date missed	Reason for non-consumption

Appendix I: Letter

Dear.............................

I am writing to inform you about a new study that is taking place at the Department of Food and Nutritional Sciences at the University of Reading that maybe of interest to you. The aim of this study is to investigate the synergistic benefits of olive polyphenols and physical activity in the control of blood pressure.

If you would like to take part in this study we can determine your eligibility by asking you to give a brief medical history and lifestyle questionnaire and measurements for height, weight, waist circumference and blood pressure. We are looking for individuals who are at above average blood pressure but who are otherwise healthy. Therefore after giving consent, we will ask you to supply a fasted blood sample (not ingesting anything but water for 12 hours) so that the fat levels in your blood, blood count and kidney and liver function can be determined to assess your suitability for inclusion in this study

The study will begin in June 2015, your participation would be required over 12 weeks. During the study you will be asked to wear a physical activity monitor and to consume either a supplement capsule or a placebo daily for 12 week study, also you may or may not be encouraged to increase your physical activity levels to meet the national guidelines.

If you wish to find out more about this study please contact me on the details provided.

Yours sincerely

Miss Bandhita Saibandith, PhD student

The University of Reading
Department of Food and Nutritional Sciences
PO Box 226
Whiteknights, Reading
RG6 6AP
Tel:
E-mail: B.Saibandith@pgr.reading.ac.uk
Note: If using Satellite Navigation to find the University please enter RG6 6UR for the campus.

Appendix J: Email

Dear.............................

I am writing to inform you about a new study that is taking place at the Department of Food and Nutritional Sciences at the University of Reading that maybe of interest. The aim of this study is to investigate the synergistic benefits of olive polyphenols and a healthy lifestyle in the control of blood pressure.

If you would like to take part in this study we can determine your eligibility by asking you to give a brief medical history and lifestyle questionnaire and measurements for height, weight, waist circumference and blood pressure. We are looking for individuals who are at above average blood pressure but who are otherwise healthy. Therefore after giving consent, we will ask you to supply a fasted blood sample (not ingesting anything but water for 12 hours) so that the fat levels in your blood, blood count and kidney and liver function can be determined to assess your suitability for inclusion in this study

The study will begin in June 2015, your participation would be required over 12 weeks. over the course of the study you will be asked to consume a supplement or placebo pill daily for the 12 week study period and you may or may not be encouraged to increase your physical activity levels.
If you wish to find out more about this study please contact me on the details provided.

Yours sincerely

Miss Bandhita Saibandith, PhD student

The University of Reading
Department of Food and Nutritional Sciences
PO Box 226
Whiteknights, Reading
RG6 6AP
Tel:
E-mail: B.Saibandith@pgr.reading.ac.uk

Note: If using Satellite Navigation to find the University please enter RG6 6UR for the campus.

Appendix K: OLE-2 study poster

Volunteers needed

Olive leaf extract as part of a healthy lifestyle in the reduction of blood pressure

This study is looking to recruit:

- Male and female, age 25-70y
- BMI >25kg/m²
- Non smokers

(subject to inclusion and exclusion criteria)

Volunteers will be <u>reimbursed for their time and expenses</u>

For more information, please contact Bandhita Saibandith on (074)75285282 or email: qt004491@reading.ac.uk

OLE Study
(Bandhita Saibandith)
(074)75285282
qt004491@reading.ac.uk

OLE Study
(Bandhita Saibandith)
(074)75285282
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(074)75285282
qt004491@reading.ac.uk

OLE Study
(Bandhita Saibandith)
(074)75285282
qt004491@reading.ac.uk

Appendix K: Weekly Physical activity sheets

Get

Active

OLE STUDY

Volunteer name: Weekbeginning:

OLE STUDY

Get active with us

[Week 1]

Being physically active increases lifespan

British researchers in the mid twentieth century compared the risk of dying for bus conductors versus bus drivers, and for postmen and women to sorting office workers. In the US, similar studies were conducted comparing dock workers to desk based staff. These studies all strongly suggested that individuals with more physically active occupations had a lower risk of death.

Be more active

Many of us drive to work, we park close to our workplace and sit at our desks for much of the day. To increase your activity levels try walking to work, or park a five or a ten minute walk from the office. These extra steps all count towards improving your health and a short walk can be a great way to kick start your day.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
Monday		
Tuesday		
Wed		
Thursday		
Friday		
Saturday		
Sunday		

Volunteer name: Weekbeginning:

Get active with us

[Week 2]

Being physically active protects against diabetes

There are over 3 million diabetics in the UK, and this number is growing due to our unhealthy lifestyles. A study in the British Medical Journal showed engaging in regular moderate physical activity (such as walking) may reduce your risk of diabetes by 40%. Amongst those diagnosed with diabetes, walking a mile a day may reduce risk of death by 50%

Be more active

There are lots of opportunities to increase our activity levels. Consider a brisk walk at lunchtime. Talk to your colleagues face to face as opposed to over the internal phone, take the stairs instead of the lift.

Remember every little bit helps.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
Monday		
Tuesday		
Wed		
Thursday		
Friday		
Saturday		
Sunday		

Volunteer name: Weekbeginning:

Get active with us

[Week 3]

Being physically active can be a great opportunity to socialise

Whether its playing a friendly game of tennis, squash or bowls or simply meeting up for a walk, taking exercise can be a great opportunity to catch up with friends or to make new ones.

Be more active

Consider joining a walking club, a sports group ,or just taking an exercise class. These are all fantastic ways to make a physically active lifestyle also an enjoyable lifestyle.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer

	Pedometer step count	Use this box to record other activities.
Monday		
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Volunteer name: Weekbeginning:

Get active with us

[Week 4]

Being inactive for long periods of the day is bad for your health

A study carried out at the University of Sydney found that adults who were sedentary (sitting or lying down) for more than 10 hours a day, were three times more likely to die prematurely than the most active people.

Be more active

Fitting activity into a daily routine can be easy. Whether it is gardening, walking the dogs, or playing with the kids or grandkids in the park, physical activity should be something that you enjoy. Look for activities that make you feel good

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
Monday		
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Volunteer name: Weekbeginning:

Get active with us

[Week 5]

Being physically active can benefit the environment and that is good for everyone.

Our cars are a leading cause of greenhouse gas emissions, they also emit nasty chemicals which negatively everybody's health. Walking or cycling are much greener alternatives.

Be more active

Consider taking opportunities to leave the car at home on occasion, walking cycling or even catching the bus is great for the environment and it boosts your health.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer

	Pedometer step count	Use this box to record other activities.
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Volunteer name: Week beginning:

Get active with us

[Week 6]

You are half way through the OLE study, time to pat yourself on the back

Are you moving more than you were when you started this project? We hope that you are more active now and also that you feel better for it. What are the benefits of moving more to you?

Be more active

Much of our leisure time is spent watching television, or increasingly using computers and other electronic devices which promote sedentary behaviour. Consider rationing how much time you give to these activities.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
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Volunteer name: Weekbeginning:

Get active with us

[Week 7]

Being physically active makes us feel better, it may also improve brain performance

Daily exercise improves mood, relieves depression and is associated with better sleep patterns. It also improves performance on cognitive tests, with physically active volunteers scoring higher for measures of understanding and memory.

Be more active

Plan each day allowing for some time for exercise, walking, swimming, cycling or lifting weights, it all counts and the ideal balance would be a combination of aerobic and resistance exercise

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
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Volunteer name: Weekbeginning:

Get active with us

[Week 8]

Being physically active slows weight gain and burns calories

We are all prone to putting on a few pounds as we get older, studies show that those of us who are the most physically active are the least likely to gain weight.

Be more active

Exercise will change your body composition, the rate of these changes are dependent on the intensity and nature of the exercise that you do, but as they occur you may start to feel more confident in your appearance, this confidence translates into many areas of peoples lives.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

	Pedometer step count	Use this box to record other activities.
Monday		
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Volunteer name: Weekbeginning:

Get active with us

[Week 9]

Being physically active boosts your general energy levels

As you get fitter your heart, lungs and muscles become more efficient and effective. This makes every day activities like household chores, gardening and carrying shopping easier. And as these activities become less taxing, will have more energy for other things.

Be more active

The talk test is a good way of understanding the intensity of your physical activity. If you are doing moderate-intensity activity you can talk, but not sing. If you can sing whilst walking you probably aren't moving quick enough.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

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Volunteer name: Weekbeginning:

Get active with us

[Week 10]

Just 2 weeks to go, take another pat on the back

We hope you have been enjoying a greater level of activity over the course of the study. We hope that you feel better for it, the competition is heating up; lets set some goals to see if we can stretch you a little further over the remaining two weeks

Be more active

The guidelines for physical activity are that we aim for ten thousand steps per day, How many steps have you been averaging each week up to this point, can you get closer to the target levels over the next two weeks?

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer

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Volunteer name: Weekbeginning:

Get active with us

[Week 11]

Being physically active reduces ill health in the overweight

Carrying too much body fat is bad for our health, yet studies show that being physically fit whilst overweight may protect against many of the health problems associated with excess weight. In fact, the obese but fit have a lower risk of death than people who are very thin but unfit.

Be more active

Increasing physical activity levels, will boost your physical fitness. Consider setting yourself goals to test whether your fitness is improving. These can be small personal goals, like how out of breath you feel after climbing the stairs at work, or during a brisk walk or how quickly you complete the same walk over time.

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer

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Volunteer name: Weekbeginning:

Get active with us

[Week 12]

The final push

Regular physical activity protects against cardiovascular disease, obesity, diabetes, hypertension and cancer. It may also improve bone health and mechanical strength as we age.

Be more active

You have been doing really well over the last 12 weeks! We hope this isn't the final push and that you can maintain an increasingly active lifestyle beyond this week, but lets see how many steps we cab achieve in this last study week

How active are you? Use the step count diary below to track how many steps you complete in a day. You can collect this data from your pedometer.

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