Universidade de Aveiro Departamento de Química
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Virgínia Carvalho Lemos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, especialização em Métodos Biomoléculares, realizada sob a orientação científica da Professora Doutora Sílvia M. Rocha, Professora Auxiliar do Departamento de Química da Universidade de Aveiro e do Professor Doutor Zbigniew Krejpcio, Professor Associado do Departamento de Nutrição e Saúde Humana da Poznan University of Life Sciences, Polónia.

Dedico este trabalho aos meus pais por me terem proporcionado a oportunidade de estudar, que sempre me apoiaram e aconselharam.

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presidente

Professor Doutor Pedro Miguel Dimas Neves Domingues
Professor Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro

arguente

Professora Doutora Marcela Alves Segundo
Professor Auxiliar do Departamento de Ciências Químicas da Faculdade de Farmácia da Universidade do Porto

orientadora

Professora Doutora Sílvia Maria da Rocha Simões Carriço
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

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palavras-chave

Arbutus unedo L., Medronheiro, composição química, atividade biológica, compostos bioativos, compostos fenólicos, diabetes, STZ, high-fat, diabetes tipo 2, modelo animal para diabetes tipo 2

Resumo

O medronheiro (*Arbutus unedo* L.) é um arbusto endémico que cresce em toda a região Mediterrânica. Em Portugal, pode ser encontrado em todo o território continental, principalmente na zona sul do país. O fruto é utilizado na produção de compotas, marmeladas e aguardente, pelo que a composição química deste é relativamente bem conhecido quando comparado com as folhas. Actualmente, existem poucos estudos sobre a composição química das folhas, sendo que a maioria visa apenas o seu conteúdo em compostos fenólicos.

Esta planta tem sido utilizada na medicina popular para tratar diversas doenças tais como distúrbios gastrointestinais, gastrite, problemas dermatológico, urológicos, renais e cardiovasculares. No entanto, até ao momento existem poucos estudos *in vivo* que atestem estas propriedades medicinais, o que justifica a realização deste tipo de ensaios. No caso da diabetes, que afecta milhões de pessoas em todo o mundo, existem apenas dois estudos sobre as propriedades antidiabéticas do extrato aquoso das raízes de *A. unedo*.

O presente trabalho de mestrado avaliou o potencial antidiabético dos extractos aquosos das folhas e frutos do medronheiro. O estudo foi efectuado em ratos diabéticos (diabetes induzida por Estreptozotocina - STZ) alimentados com uma ração rica em gordura. Primeiramente foram optimizados os parâmetros de extracção para a obtenção do extrato aquosos do fruto. De seguida os extratos aquosos de folhas e frutos foram caracterizados em termos de teor em fenóis totais. O extrato aquoso das folhas apresentou uma maior concentração de fenóis totais do que o extrato aquoso do fruto.

Ratos Wistar do sexo masculino foram injectados com STZ após serem alimentados durante 2 semanas com uma dieta rica em gordura. Desta forma induziu-se diabetes tipo 2, não dependente de insulina. Após a confirmação da condição diabética dos ratos, a dieta rica em gordura continuou a ser administrada aos animais. No entanto, as dietas foram suplementadas com extrato aquoso ou do fruto ou das folhas de *Arbutus unedo* L., num tratamento com a duração de 4 semanas. Os extratos foram administrados nas seguintes doses: 1.25g /kg peso corporal/dia no caso do extrato da folhas 0.5g /kg peso corporal/dia no caso do extrato do fruto. Efectuaram-se análises a parâmetros do sangue, hematológicos, à homeostasia mineral dos tecidos e à expressão de determinados genes após os animais serem sacrificados.

O extrato do fruto apresentou uma tendência a regular os níveis de glucose no sangue. Outros efeitos benéficos foram atribuídos a este extrato, nomeadamente ao nível da regulação da expressão do gene de TNF-α a nível muscular. Este extrato também apresentou alguns efeitos ao nível da regulação da homeostase renal de zinco, embora não muito significativos. O extrato da folha provou ser mais eficiente a regular os níveis de insulina, a melhorar a resistência à insulina (HOMA-IR). Também apresentou uma tendência a regular os níveis de glucose no sangue. Ambos os extratos aumentaram de forma eficiente a sensibilidade à insulina (embora o extrato da folha o tenha feito de forma mais pronunciada). Obtiveram-se ainda bons resultados preliminares, uma vez que estes extratos demostraram que ocorreu uma tendência em termos de diminuição da concentração de creatinina e/ou fosfatase, de aumento do valor do parâmetro de Amplitude de Distribuição dos Eritrócitos medido como Desvio Padrão (RDW-SD), de aumento da concentração de linfócitos e diminuíram. Para ambos os extratos foram detectados efeitos antidiabéticos e anti-inflamatórios.

Os resultados obtidos confirmam que *A. unedo* apresenta alguns efeitos terapêuticos, embora ligeiros. Os extratos desta planta melhoraram significativamente a sensibilidade à insulina mas não conseguiram regular significativamente o metabolismo da glucose.

Este estudo foi o primeiro, num modelo animal de diabetes tipo 2 que avaliou o potencial antidiabético dos extratos aquosos dos frutos e folhas de *Arbutus unedo* L.

Keywords *Arbutus unedo* L., Strawberry tree, chemical composition, biological properties, folk medicine, bioactive compounds, phenolic compounds, diabetes, STZ, high-fat; type 2 diabetes, type 2 diabetes animal model

Abstract

The strawberry tree (*Arbutus unedo* L.) is an endemic shrub, which grows in the Mediterranean region. In Portugal, it can be found through all the country but mostly in the south. The fruit is used to produce hams and jellies and firewater, reason why the fruit's chemical composition is relatively well known, when compared to the leaves. Until now, there are only a few studies concerning the leaves' chemical composition and the existing mainly focus on their phenolic content.

This plant has long been used in folk medicine to treat a vast amount of illnesses like gastrointestinal disorders, gastritis, dermatologic and urological problems, kidney problems and cardiovascular diseases. However, very few studies *in vivo* have been performed about these medicinal properties until now. Diabetes is a pandemic disease that affects millions of people. There are only two studies about *A. unedo* root's aqueous extract antidiabetic properties.

This study evaluated the anti-diabetic effect of strawberry tree (*Arbutus unedo* L.) fruit and leaf aqueous extracts in streptozotocin (STZ) induced diabetic rats fed with a high-fat diet. Firstly the optimal parameters to obtain the fruit aqueous extract were established. After that, the leaves and fruits aqueous extracts were characterized in terms of total phenolic content (TPC). Leaf aqueous extracts presented a higher TPC concentration than the fruit aqueous extract.

Male Wistar rats were injected with STZ, after a 2 week feeding with high-fat diet in order to induce non-insulin dependent type 2 diabetes. After confirmation of the rats' diabetic state, the animals were fed with *Arbutus unedo* L. fruit aqueous extract or leaf aqueous extract added to the high-fat diet for 4 weeks. The extracts were given in the following doses: 1.25g /kg b.w./day for leaf extract and 0.5g /kg b.w./day for fruit extract. After the animals were sacrificed analyses were perform for blood indices, haematological indices, mineral tissular status and gene expression.

Arbutus unedo L. fruit extract presented a tendency to lower blood glucose levels. More beneficial effects were seen for this extract in terms of regulating TNF-α gene expression in muscular tissue. Some effects in zinc renal homeostasis were also shown, although they were not very significant. The leaf extract, appeared to be more efficient in regulating insulin levels and improving HOMA-IR index. It also presented a tendency towards lowering blood glucose levels. Both extracts proved to efficiently improve insulin sensitivity (leaf extract in a larger extent). In this study, there were also some good preliminary results, as the groups supplemented with this extracts showed a tendency in decreasing serum creatinine and/or phosphatase concentration, improving the Red Cell Distribution Width measured with standard deviation (RDW-SD) and lymphocytes concentration. Positive anti-diabetic and anti-inflammatory aspects were found for both aqueous extracts. The results obtained confirm the mid-therapeutically effects of *A. unedo* as the plant's material effectively improved insulin sensitivity (HOMA-IR index) although failed to significantly modulate glucose metabolism.

This study was the first in vivo trial on a type 2 diabetes animal model that evaluated the antidiabetic potential of *Arbutus unedo* L. fruits and leaves aqueous extracts.

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Abbreviations

A. unedo	*Arbutus unedo* L.
f.w.	Fresh weight
kcal	Kilocalorie
d.w.	Dry weight
(w/w)	(weight/weight)
Na	Sodium
K	Potassium
Mg	Magnesium
Ca	Calcium
Fe	Iron
Cu	Copper
Mn	Manganese
Zn	Zinc
TPC	Total Phenolic Content
GAE	Gallic Acid Equivalents
CoA	Co enzyme A
NADPH	Nicotinamide adenine dinucleotide phosphate
EC_{50}	Efficient concentration
DPPH	2,2-diphenyl-1-picryl-hydrazyl
IC_{50}	Inhibitory concentration
ESI	Electrospray ionization
MS	Mass spectrometry
LC–DAD	Liquid Chromatography with Diode Array Detection

STZ	Streptozotocin
ROS	Reactive oxygen species
NOS	Nitric oxide synthase
TNF-α	Tumour Necrosis Factor α
IRS2	Insulin receptor substrate 2
GLUT-2	Glucose transporter 2
GLUT-4	Glucose transporter 4
IL-6	Interleukin 6
b. w.	Body weight
C	Control diet
HF	High-fat
HF + FE	High-fat with fruit extract (1.25g/kg)
HF + LE	High-fat with leaf extract (0.5g/kg)
WBC	White Blood Cell Count
RBC	Red Blood Cell Count
HGB	Haemoglobin
HCT	Haematocrit
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
PLT	Platelets
RDW-SD	Red Cell Distribution Width Based on Standard Deviation
RDW-CV	Red Cell Distribution Width Based on Coefficient of Variation
PDW	Platelet Distribution Width

MPV	Mean Platelet Volume
P-LCR	Platelet Large-Cell Ratio
PCT	Procalcitonin
NEUT	Neutrophils
LYMPH	Lymphocytes
MONO	Monocytes
EO	Eosinophils
BASO	Basophils
LDL	Low density lipoprotein
HDL	High density lipoprotein
TAG	Triacylglycerols
ALT	Alanine Aminotransferase
ASP	Aspartate Aminotransferase
ALP	Alkaline phosphatase
CRP	C-reactive protein
HOMA-IR	Homeostasis model assessment of Insulin resistance
b. m.	Body mass
AU_L	High-fat diet with *Arbutus unedo* L. aqueous Leaf Extract (0.5 g/kg)/STZ injected
AU_F	High-fat diet with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected

CHAPTER I – Introduction

1. Strawberry tree - *Arbutus unedo* L.

1.1. Origin, characteristics, and botany

Arbutus unedo L., commonly known as the strawberry tree, is a genus that belongs to the *Vaccionioideae* subfamily and a member of the *Ericaceae* family [1, 2]. It is an evergreen shrub, native of the Mediterranean region, being found in western, central and southern Europe, north-eastern Africa, Canary Islands and Palestine [3, 4]. In the Mediterranean region there are only 4 species and 2 hybrids [3]. This tree can live up to 200 years [4].

The strawberry tree is characterized by being a busy shrub of 1.5m to 3m tall, so it is sometimes used as an ornamental plant [4, 5]. However, it can sometimes reach 9-12m in height as it has a spreading habit, but often this tree is much shorter, for example in the USA, it rarely exceeds 2.5-3.7m [2, 4, 5]. The plant is evergreen and broadleaved [2] and its geographical distribution can range from an altitude of 700-1000m above the sea [4, 5]. In northern Portugal, it usually ascends to 1200m above sea level [4, 6].

Strawberry tree prefers mild climates, where snow or hails do not occur very often, but it also needs a climate where the summer temperature and dryness is not very intense. Because of this, it is found mainly in coast and land areas where the climate is sub humid. Regarding the soil where it usually grows, this plant prefers siliceous or carbonated substrates [3]. Being considered the temperate area of Europe, the Mediterranean basin is the place that has all these characteristics and where this shrub better adapts. The Strawberry tree is thereby found in Portugal, Spain, France, Italy, Albania, Greece, Croatia, Bosnia, Serbia including the islands Balearic, Corsica, Sardinia, Sicily and Crete, spreading from the north of the Iberian Peninsula, going along the west coast of France until it reaches Ireland [3].

In terms of natural habitat, the tree prefers holm, cork and mixed forests, cliffs and canyons near rivers. It is also found in rocky soils although the shrub likes them fresh, deep and loose. It also tolerates industrial pollution and can prosper even if it is growing near the sea. The pH of the soil does not affect its growing but *Arbutus unedo* L. likes to be full exposed to light, needs some humidity and resists low temperatures (-15ºC) and hail [4].

This evergreen shrub has a short trunk with dense foliage. The bark is fissured (that reveals a red coloured young bark beneath) and usually peels off in small flakes,

which are mostly dull brown [4, 7]. The leaves (Figure 1a) which grow from the tree´s 5mm long hairy pinkish stalks are alternate and simple, usually 5 to 10cm long with a glossy and brilliant green colour (can sometimes be dark green) [2, 7, 8]. They can also have a margin that is more or less serrated, usually 2–3 times as long as wide, glabrous with a petiole of 10 mm or less [7].

Arbutus unedo L. usually starts flowering in Autumn (September-October) and continues until the end of the winter, normally in February [2, 4]. The flowers from this plant are small, not having more than 5cm long and 8mm in diameter; bell-shaped and hermaphrodite (Figure 1 b). They are a bit like the blueberry flowers, having a white, pinkish or greenish colour. They assemble drooping panicles and have a soft honey scent [2, 8].

s b) c) d)

Figure 1: *Arbutus unedo* L. a) leaves b) flowers with pink or white color c) fruit (mature) d) fruit and flower exist at the same time [2].

Strawberry tree mature fruits and flowers are existent at the same time, as the fruit takes 9-12 months to ripen. The coexistence of fruits and flowers gives the plant an immense beauty (Figure 1 d) [2, 4], reason why the strawberry tree can be used as an ornamental plant. The fruits look somewhat like strawberries as their colour ranges from yellow to scarlet and dark red. Their berries are spherical with about 2cm in diameter, covered in conical swellings (Figure 1 c). The fruit´s pulp is edible (although the taste is somewhat astringent) , soft and yellow and has seeds inside [4]. The resemblance between strawberries and this fruit only applies to looks, as the taste is very different [2, 9]. Even though these fruits are edible, their taste is not very appreciated by the consumer, so they are rarely eaten as fresh fruits [2]. The fruit´s own name may suggest that, because the

Latin word "*unedo*" means "I eat one (only)". So this can mean that the fruit is not very tasty or that it so delicious that each person only needs to eat one [2].

1.2. Production in Portugal

The shrub is implanted all over the Portuguese territory (Figure 2), with exception of the northern regions which are colder. It exists mainly in the south area of the country, but it does not grow in very dry areas of that region [4].

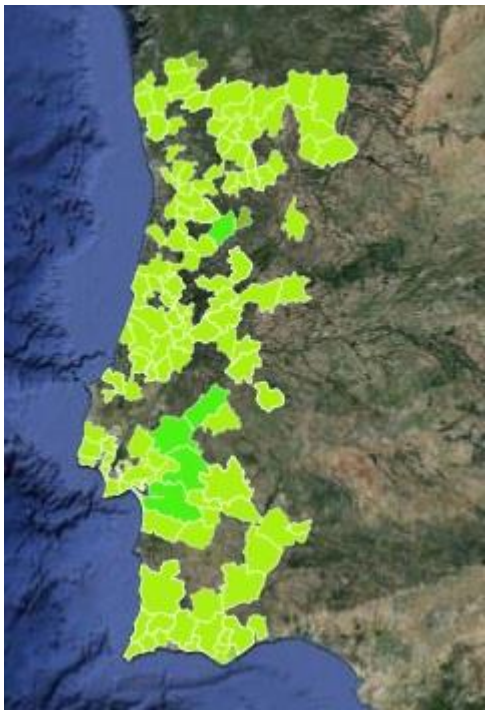

Figure 2: *Arbutus unedo* L. distribution in the Portuguese territory recorded until 30/08/2011 (adapted from [10]). Areas in green represent the areas where the species was observed in its natural state. Yellowish green – que findings were validated internally by a collaborator of the database by photography or non-specialized publication. Green- the findings were validated by an expert. Dark green – the findings were validated by scientific work(s).

Its presence is more relevant in some regions like the south of *Rio Tejo* namely in the region of *Serra do Caldeirão* and *Serra de Monchique* and the *Algarve* region [1, 9, 10]. However, since this is an endemic plant and it is spread all over the country, it can also be found in the North-eastern part of Portugal, namely *Trás-os-Montes* [1, 11]. There are also large plantations of the strawberry tree in *Perocabeço, Oleiros* and *Pampilhosa da Serra*, with an extension of 15 and 36 hectares respectively [1, 12]. In recent years, the

region of *Rio de Alva* in *Penacova* has also been known by its vast Strawberry tree plantation. This tree exists wildly across this region in woodlands near *Rio Alva* [13].

1.3. Strawberry tree related products

Even though the fruits are edible, they are not usually consumed or appreciated by people. The fruits are usually consumed as jams, marmalades, honey or distilled liquors. In rural areas, the fruit-based products are a source of complementary income. They are very popular and can be found in agriculture fairs proving the economic implication of this plant in the population's income [14].

Strawberry tree honey is another not so popular product, characterized by its strong and bitter taste which is very different from the taste people normally relate with honey. Strawberry tree honey is a known product of Mediterranean regions, such as Spain and Portugal [15].

Since ancient years, there are records of the use of several parts of this plant (roots, leaves, fruit and flowers) in folk medicine and phytotherapy to treat cardiovascular, dermatological, gastrointestinal, and urological disorders as well as diabetic and inflammatory ailments [16].

The fruit itself has already some alcoholic content, so its fermentation and subsequent distillation results in products such as firewater and spirit beverages. In Portugal, the fruits plantation is majorly aimed at the production of firewater, namely *Aguardente de Medronho* [4, 10, 12]. In other countries, like Italy and Greece, the fruit is also distilled which results in the beverages *Corbezzolo* and *Koumaro* [17].

Strawberry tree fruit aqueous extracts have recently been applied to yogurts, burgers and Frankfurters (German sausages) [18-21]. This extracts improved the product's antioxidant activity, therefore increasing their stability [19]. The studies suggest that the fruit can be a good alternative to chemical food additives [18, 19].

2. *Arbutus unedo* L. Chemical Composition

2.1. *Arbutus unedo* L. Fruit's Chemical Composition

Strawberry tree fruits have been subjected to a vast amount of studies regarding their physic-chemical properties. One should notice that the fruit's chemical composition can vary according to the region where it was planted, the year, its genetic characteristics as well as the exposition to sunlight, wind and the amount of water available to the fruit during its ripening [22-24].

The general chemical composition of strawberry tree fruit available in literature is given in Table 1. This fruit has a moisture content that ranges between 46.82-71.89 g/100g of fresh weight, although this can vary according to light exposure, water content of the soil [22, 23, 25]. So, the dry matter of this fruit will be around 28.11-53.18%, this is considered high for a berry fruit, for example, strawberries and raspberries have a dry matter of only 10.2% and 15.5% respectively (% of edible portion) [26]. So we can consider the *A. unedo* fruit as a fruit with a low water content (which can be related with its high fiber content).

The energetic value presented by these fruits ranges between 61.48 and 135.93 kcal per 100 g of fresh fruit [23]. This fruit is considered to be acidic, as its pH is about 4.6 [27], however this value is high for a berry fruit, as berry fruits usually have a pH around 3 and *A. unedo* fruit has a pH near the one of bananas (4.7) [26].

Besides moisture, sugars are the main component of *Arbutus* fruit, representing from 14 to 32% of the fruits fresh weight, a value thought to be high for a fruit. All other berry fruits have lower sugar content; this fruit's sugar concentration is very near the one of bananas, a fruit known for having an high sugar content [26]. From the total sugars, Ruiz-Rodrígues *et a*L. claim that fructose is the main sugar present in this fruit (15% of fresh weight), followed by glucose (7% of fresh weight) and sucrose (only 0.4%) [23], maltose is also present on small amounts (1.1% of the fruit's dry weight) [28]. Comparing strawberry tree fruit to strawberries, black currant, red currant, raspberries and grapes, this fruit has a very high content of fructose (these berries have about 2-7% fructose) [26]. This fact can also explain why these berries have such a sweet taste since fructose is the sweetest of all non-synthetic carbohydrates [26]. Even the glucose content (can reach up to 6.49 g/100g f.w) is considered high when compared to other fruits; for example grapes, a very sweet fruit, have a glucose content of 7.2g/100g of edible portion [26]. According to

Alarcão-e-Silva *et al*., carbohydrates can reach up to 50% of the total weight of dry fruits when the fruit is ripe [22]. In this study, the authors concluded that the sugar content of the berries increases with the maturation state, a factor that explains how mature state can affect the fruit's chemical composition. When the fruit is unripe, saccharose is the major sugar (87.7 ± 0.6 grams per kg of dry fruit) available in the *Arbutus* fruit [22]. This can possibly be explained because of the enzymatic hydrolysis that occurs in the fruit's carbohydrates and pigments during ripening [26].

Protein is also present in a considerable amount in strawberry tree fruit, ranging between 0.581–1.187 g per 100 g of fresh fruit, followed by ashes (0.685-1.058 g per 100 g of fresh fruit) and fat (up to 0.779 g per 100 g of fresh fruit) [23]. The ash content is very similar to all other fruits according to literature [26]. Having about 0.8% fat, this fruit has a lipid content a bit higher than the values normally reported for fruits (0.1-0.5 % f.w.) [26].

The *Arbutus* fruit is a very good source of minerals, as its mineral content can reach up to 2.82 % (w/w) of the fruit's fresh weight [27]. The study performed by Ruiz-Rodríguez *et al*., showed that these fruits are especially rich in potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) [23]. The first one is present in high amounts, 79.72 –323.14 mg/100 g of fresh weight, especially if we consider that minimum daily requirement for this mineral is estimated to be 782 mg [26]. Calcium content ranges between are 40.54–104.12 mg/100g f.w. and magnesium and sodium are present in 9.56–45.85 and 4.33–9.94 mg/100g f.w. respectably. As we can see in Table 1, there are other minerals but in much smaller amounts.

Table 1: General physic-chemical characteristics of *Arbutus unedo* L. fresh ripped fruits.

Properties		***Values***	***Reference(s)***
Moisture (g/100g)		46.82-71.89	[23, 25]
Ash (g/100g)		0.685-1.058	[23]
pH		4.6±0.10	[27]
Energy (kcal/100 g)		61.48–135.93	[23]
Macronutrients	*Lipids (g/100)*	0.299–0.779	[23]
	Proteins(g/100g)	0.581–1.187	[23]
	Carbohydrates (g/100g)	14.11-31.55	[23]
	- *Glucose*	2.34–6.49	[23]
	- *Fructose*	3.64–14.54	[23]
	- *Sucrose*	Traces–0.483	[23]
	- *Maltose (% dry weight)*	1.11±0.06	[28]
Micronutrients	*Minerals (%(w/w))*	0.7-2.82	[23, 27]
	Na (mg/100g)	4.33–9.94	[23]
	K (mg/100g)	79.72–323.14	[23]
	Mg (mg/100g)	9.56–45.85	[23]
	Ca (mg/100g)	40.54–104.12	[23]
	Fe (mg/100g)	0.354–1.856	[23]
	Cu (mg/100g)	0.073–0.208	[23]
	Mn (mg/100g)	0.038–0.178	[23]
	Zn (mg/100g)	0.188–0.762	[23]
	Vitamins(mg/100g):		
	- *Vitamins C (total)*	122.0–262.7	[23]
	- *Carotenoids (total)*	0.064±0.014	[29]
	- *β-Carotene*	0.219-0.890	[23]
	- *Lycopene*	0-0.262	[23]
	- *Total Tocopherols (mg/100 g dry weight)*	23.46 ± 0.26	[25]
Soluble solids (% Brix)		21.05 ± 0.07	[24]
Total phenols mg GAE/100g		590-1973.6	[23, 24]
Total dietary fibres (g/100 g)	*Total:*	10.04–22.27	[23]
	Soluble dietary fibres	2.17–4.19	[23, 24]
	Insoluble dietary fibres	7.86–18.55	[23]
Organic acids (mg/100g)	*Oxalic acid*	48.44–146.75	[23]
	Malic acid	203.3–299.6	[23]
	Fumaric acid	0.489–1.114	[23]
Sterols (mg/100g)	*Total:*	6–30	[30]
	- *β-sitosterol*	6–30	
	- *Campesterol*	1	
	- *Stigmasterol*	Traces	
	- *Cholestan-3-one*	Traces	

The amount of vitamins and carotenoids in *Arbutus* fruit are higher than the amounts present in other fruits. Strawberries for example have a vitamin C content of 64 mg/100g f.w. and orange, known in fold medicine for having a high vitamin C content only presents a concentration of this vitamin of about 50 mg/100g f.w. [26]. Once again this parameter can vary according to the fruit's ripening stage [22]. The total vitamin C content

was determined by Ruiz-Rodríguez *et al*., who concluded that the composition of this fruit contains up to 262.7 mg/100g of f.w. of total vitamin C [23], while a higher amount was found by Alarcão-e-Silva *et al*., whose samples contained 542 ± 11 mg of ascorbic acid per 100 g of dry matter of the fruit (when it is ripe) [22]. When compared to the quantity present in other fruits, like peaches, apples and cherries [26, 29] the amount of vitamin C is considered low. Strawberries, for example, have an ascorbic acid content of about 60 mg per 100 g of edible portion, a much higher value; on the other hand, pears only have about 1-4% [26]. The total vitamin E present in strawberry tree fruit is about 23.46 ± 0.26 mg/100 g dry weight of total tocopherols, being α-tocopherol the most important form of vitamin E present, with 21.98 ± 0.18 mg/100 g dry weight [25]. Carotenoids are present in the fruit too, especially β–carotene, which is present in the amount of 0.219-0.890 mg per 100g of *Arbutus* fruit fresh weight [23]. Total carotenoids were quantified by Pallauf *et al*., stating that the fruit has an amount of 0.0647±0.014 mg per 100 g edible portion [29]. Alarcão-e-Silva *et al*. refer the changes in the amount of β-carotene with fruit ripening, ranging between 38.1 ± 3.7 mg/100 g of dry matter (unripe) and 70.9±5.2 mg/100 g of dry matter (mature) [22]. Lycopene is also present, but in very small amounts, 0-0.262 mg/100g f.w. [23]; it can be responsible for the fruit's bright red colour.

Regarding dietary fibre content, *Arbutus unedo* L. fruits can have a total amount of fiber content that varies between 10.04–22.27 grams per 100g of fresh fruit [23]. This value is higher than the one of shell-nut fruits (which ranges between 2-13.5% of fresh edible portion) and it is considered very high for a fruit [26]. The dietary fibre of the fruit can be divided into soluble and insoluble fibre. Soluble fibre is present in lower amounts (2.17–4.19 g/100g f.w. [23, 24]) than insoluble fibre (7.86–18.55 g/100g f.w. [23]). ºBrix of *A. unedo* fruits is 21.05 % [24]. (°Brix is a measure of the soluble solids in solution and it can be used as an indicator of sugar concentration and maturity [26, 31].)

The organic acids identified in the study of Ruiz-Rodríguez *et al*. were oxalic acid (48.44–146.75 mg/100g f.w.), malic acid (203.3–299.6 mg/100g f.w.) and fumaric acid (0.489–1.114 mg/100g f.w.) [23]. Alarcão-e-Silva *et al*. reported that, again, these values depend on ripening state, and claimed that quinic acid is the major organic acid present in the fruit (about 7.35g/100g of dry weight when the fruit is unripe and 5.99g when the fruit is ripe) [22]. L-Malic and citric acids are considered the major organic acids of fruits, whereas Malic acid is predominant in pomme and stone fruits, while citric acid is

most abundant in berries. So *A. unedo* fruit has an organic acid profile similar to the one of pomme fruits [26, 31].

Sterols were also present in strawberry tree fruits collected from *Penacova* in central Portugal. Several have been found, namely β-sitosterol, campesterol, stigmasterol and cholestan-3-one [30]. The sterol found in higher amount was sitosterol, which represented alone almost all of the sterol content of the fruit. As the normal dietary intake of phytosterols ranges from 200–400 mg/day, one can say that *Arbutus unedo* L. fruits are not particularly rich in this components [26].

The phenolic fraction of strawberry tree fruit is of great interest, mainly because of the health promoting effects attributed to these compounds. The total phenolic content (TPC) of the fruit can vary between 590-1973 mg of Galic Acid Equivalents (GAE) per 100g of fresh fruit [22, 23]. However, these values can vary according to ripening stage, area, study and method of analyses used reason why the TPC can vary so much. The highest reported TPC was the one reported by Ruíz-Rodríguez *et al*. of 1973.6 mg GAE/100g [23]. In the *Arbutus unedo* L. berries, various classes of phenolic compounds have been identified, mainly in the flavonoid class. The phenolic fraction contained in the fruits of *Arbutus unedo* L. is very diverse and can include several chemical classes, such as tannins, flavonoids, anthocyanins, ellagic acid derivatives and gallic acid derivatives [28, 29, 32, 33]. The determination of total concentration of anthocyanins was also performed by Pallauf *et al*. reaching a value of 3.77 mg per 100 g of fresh fruit [29]. Proanthocyanidins are another chemical group within the flavonoids present in the berries, being present in 27.46 mg per 100 g of edible fruit [29]. Another class of phenolic compounds present in *A. unedo* fruits are the ellagic acid derivatives, existing in the concentration of 1.54 mg per 100 g of fresh fruit [29]. Flavonols are also present in the fruits and were once more quantified by Pallauf *et al*. The total amount of flavonols is 1.14 mg per 100 g of edible fruit [29]. According to Pallauf *et al*., the main Flavan-3-ol present in *Arbutus unedo* L. fruits is catechin followed by gallocatechin (3.64 and 4.16 mg per 100 g of fresh weigh respectably) [29]. The phenolic acids available in the *Arbutus unedo* fruits were studied in the reports of Ayaz *et al*., and Guimarães *et al*. [28, 33]. Gallic acid is the main phenolic acid present in the fruits (10.7 mg per 100 g of dry fruit) [28]. Some tannins were also identified in *Arbutus unedo* fruits, the tannin found in the highest concentration was galloylquinic acid (8.65 mg per 100 g of dry weight) [33].

2.2. *Arbutus unedo* L. Leaves Chemical Composition

Studies about the leaves' chemical composition regarding other chemical families rather than polyphenols are still missing. Many of these studies are more focused on antioxidants than on other families of compounds. The composition of the fruits is relatively well-known when compared to leaves (Table 2).

Table 2: General chemical composition of *Arbutus unedo* L. leaves.

Compound(s)		***Values***	***Reference(s)***
Vitamins (% of dried weight)	α-Tocopherol	0.0132	[34]
Essential oil (% of essential oil)	(E)-2-decenal α-Terpineol Hexadecanoic acid (E)-2-undecenal	12.0 8.8 5.1 4.8	[35]
Terpenoids	α-Amyrin acetate Betulinic acid Lupeol	Not quantified	[36]
Phenolic Compounds (mg /100 g f.w.)	Total (mg GAE/g d.w.)	53.3–113.6	[8]
	Arbutin Ethyl gallate *p*-hydroxybenzoyl-arbutin Galloyl arbutin Catechin Gallocatechin	62.7 44.0 2.5 11.4 54.6 35.5	[37]
	Quercitrin (mg/g of dry leaf) Isoquercitrin (mg/g of dry leaf) Hyperoside (mg/g of dry leaf) Rutin Chlorogenic acid (mg/g of dry leaf)	1.21-2.20 0.07-0.33 0.11-0.35 Traces 0.61-1.46	[38]

In terms of Vitamins, only vitamin E was detected and studied in *Arbutus unedo* L. leaves. Kivcak *et al*. studied *Arbutus* leaves in Turkey and identified the presence of α-Tocopherol in the leaves by TLC [34]. The authors also found out that this component can vary according to the time of the year. In March, the leaves have a higher amount of this molecule. Maximum content of tocopherol was determined by colorimetry as 0.01328% of dried weigh. The same authors also studied the leaves in terms of essential oil composition. Leaves collected in *Izmir-Ciceklikköy* (Turkey) had in their composition (E)-2-decenal, α-terpineol, hexadecanoic acid, and (E)-2-undecenal [35]. Finally, Gaspar *et al*. have studied

the *Arbutus unedo* leaves in terms of terpenoids. The terpenoids identified were α-amyrin acetate, betulinic acid and lupeol [36].

Perhaps the best studied family of compounds in the *A. unedo* leaves are the phenolic compounds. The TPC of A. unedo leaves can vary between 53.3 and 116.3 mg GAE/g of dried leaves according to Malheiro *et al*. [8].

There is a large variety of phenolic compounds that have been identified in the leaves of *Arbutus unedo* L. in different studies. These include compounds such as tannins, flavonoids, phenolic glycosides and hydroquinones [7, 8, 37, 38]. Identification and quantification of these polyphenols has been done in the works of Fiorentino *et al*. and Maleš *et al*. [7, 37, 38]. Fiorentino *et al*. identified twelve compounds, and according to this work, the polyphenols present in higher concentration were arbutin (62.7 mg per 100g of fresh leaves), catechin (54.6 mg/100g fresh leaves) and ethyl gallate (44.0 mg/100g fresh leaves) [37]. In the two works of Maleš *et al*. [7, 38] polyphenols were identified by TLC and quantified, using leaves collected in different times of the year. In this work five compounds were identified and quantified, with their concentration varying according to the time of the year: quercitrin , isoquercitrin, hyperoside, rutin (traces that were not possible to quantify) and chlorogenic acid [38]. One of the main conclusions was that the concentration of phenols varies during the months. For example, leaves should be collected in January to obtain the highest concentrations of hyperoside and quercitrin, in June, July, and October for chlorogenic acid, and for the fraction of quercitrin and isoquercitrin in November. Hydroquinones were also identified in *A. unedo* leaves by Fiorentino *et al*. [37]. These hydroquinones belong to the arbutin class. *p*-Hydroxybenzoyl-arbutin and galloyl arbutin are available in the leaves of *A. unedo* in the concentrations of 2.5 and 11.4 mg per 100 g of fresh leaves respectively [37]. In the same work, two flavan-3-ols were identified: catechin (54.6 mg/100g of fresh leaves) and gallocatechin (35.5 mg/100g of fresh leaves).

3. *Arbutus unedo* L. aqueous extracts: properties and biological effects

All throughout the Mediterranean and North African countries, *Arbutus unedo* L. has long been used in the region's folk medicine. The entire plant is reported to have medical properties. Almost all parts of the plant are used in infusions, decoctions etc. The health promoting characteristics attributed to *Arbutus unedo* are related with the treatment

of gastrointestinal and urological problems, hypertension and cardiac diseases, diabetes and as anti-inflammatory and anti-microbiological effects.

The leaves are widely used in traditional medicine and have a lot of health benefits. They are considered to be strong antioxidants, to have vasorelaxant effects, to have antimicrobial activity, to improve cardiovascular health, to help modulate inflammatory processes and to have some anti-tumour properties [16]. Some studies are now trying to relate these effects with its compounds (namely phenolic compounds present is the *A. unedo* leaves). However, once more, very few studies have been performed and/or have effectively proved these effects which have been reported by ancient medical practices.

3.1. Chemical composition of aqueous extracts of fruits, leaves and roots

In a study from 2011, Mendes *et al*. compared the antihemolytic and radical scavenging activities of strawberry tree leaf and fruit aqueous extracts [39]. The total phenolic content of leaf and fruit aqueous extracts was also measured. Leaf aqueous extract showed a higher phenolic content (170.3 mg GAE/g extract) than fruit aqueous extract (16.7 mg GAE/g extract). In this same study by Mendes *et al*., the authors compared the amount of phenolic compounds present in the *Arbutus unedo* L. fruit and leaf aqueous extract which were previously identified by LC–DAD/ESI-MS analysis. Table 3 lists the phenolic compounds identified in this study. It is possible to conclude that the leaves aqueous extract have a greater amount of compounds when compared to the fruits aqueous extract [39]. To note that this study only identified the compounds, not quantified them, so more assessments in this matter are needed.

Table 3: Comparing the phenolic compounds present in the *Arbutus unedo* L. fruit and leaf aqueous extract [39].

Compounds identified	***Fruit***	***Leaf***
Gallic acid glucoside	**x**	**x**
Gallocatechin dimer		**x**
Galloylquinic acid	**x**	**x**
Gallic acid	**x**	**x**
Galloyl shikimic acid	**x**	**x**
Prodelfinidin dimer		**x**
Procyanidin dimer	**x**	**x**
Catechin monomer	**x**	**x**
Digalloylquinic acid	**x**	**x**
Strictinin ellagitannin	**x**	**x**
Galloyl derivative	**x**	**x**
Procyanidin gallate		**x**
Myricetin glucoside		**x**
Quercetin hexose galloyl derivative		**x**
Procyanidin gallate		**x**
Myricetin glucoside		**x**
Myricetin rhamoside	**x**	**x**
Quercetin glucoside	**x**	**x**
Quercetin derivative (arabinoside/xyloside)		**x**
Kampherol galloylglucoside		**x**
Ellagic acid arabinoside/xyloside		**x**
Ellagic acid rhamnoside	**x**	**x**
Ellagic acid	**x**	**x**
Quercetin hexoside derivative		**x**
Kampherol xyloside		**x**
Kaempferol rhamnoside		**x**
Kampherol galloylxyloside		**x**
Kaempferol hexoside derivative		**x**
Quercetin derivative (Rhamnoside/Hexoside)		**x**
Kampherol derivative (Rhamnoside/Hexoside)		**x**
Quinic acid derivative	**x**	
Digalloyl shikimic acid	**x**	
Ellagitannin derivative	**x**	
Trigalloyl shikimic acid	**x**	
Gallotannin	**x**	

The *Arbutus unedo* L. roots' composition has not been largely studied and little is still known. Dib *et al*. have done two studies about this matter from roots collected in the *Terni* forest (Tlemcen, Algeria) [40, 41]. On a first study, they have isolated several phenolic compounds such as (+)-catechin, (+)-catechin gallate from the roots of *A. unedo,* using a water/methanol/acetone mixture. In the same study, other compounds were identified by GC-MS: 4-hydroxy phenyl acetic acid; gallic acid; protocatechic acid; benzoic acid, 4-(acetyloxy)-3-methoxy-,methyl ester; gentisic acid; caffeic acid; catechin; phthalylglycine and bis(2-ethylhexyl) phthalate [40]. On the second study, they have made a phytochemical screening of the roots and quantitative analysis. In the water extract, they

have detected several types of compounds such as quinones, anthocyanins, anthraquinones, flavonoids and tannins [41]. After the quantitative analysis they determined that *A. unedo* roots had a anthocyanin content of 3.65 mg/g f.w., total flavonoid content of 0.56 mg/ g f.w. and a flavones and flavonols content of 0.17mg/g f.w. [41].

Once more these studies focus more on antioxidants than on other families of compounds.

3.2. *Arbutus unedo* L. aqueous extracts' bioactive properties

Testing the bioactivity of extracts *in vivo* after the laboratory chemical assays is very important for research. In the work of Fortalezas *et al*. the antioxidant properties of the strawberry tree fruit were assessed *in vitro* in a neurodegeneration cell model [42]. Although the hydroalcoolic extract revealed a high phenolic content in the *in vitro* test (16.46 mg GAE/ g of dry weight), the extract caused no effect on cells viability. So this study was a good example of the importance of the need to evaluate the biological function of phenolic compounds *in vivo*, or even in a cell model in order to validate their *in vitro* antioxidant activity. More *in vivo* studies of the extracts of *Arbutus unedo* L. are thereby needed, because sometimes the *in vivo* effects are a lot different than the ones that were expected.

In another study from 2009, conducted by Andrade *et al*., *Arbutus unedo* L. ethanolic and acetone extracts showed to have an high content of phenolic compounds (254.50 and 328.58 mg GAE/g plant extract). However, *A. unedo* extracts caused the decrease of cell viability (mainly because they were able to decrease mitochondrial dehydrogenases activity), regardless of the solvent used for the extraction [19]. This works suggests that there can be a relationship between cytotoxicity and polyphenolic fractions of vegetal extracts, because a high amount of phenols may be linked to a pro-oxidant effect.

One of the main conclusions of Guimarães *et al*. was that bioactivity of the strawberry tree fruits can be more related to the phenolic compounds present in each extract than to their concentration [11]. Two different enriched phenolic extracts were prepared in order to evaluate and compare their bioactivity: non-anthocyanin phenolic compounds enriched extract and anthocyanins enriched extract. Various fruit extracts were prepared. Their phenolic content was measured and the antitumor activity and hepatotoxicity were evaluated. *A. unedo* fruit extracts revealed to have a higher bioactivity

and the highest antioxidant activity in all the *in vitro* assays (which the authors relate to the presence of galloyl derivatives and to the presence of flavan-3-ols). *A. unedo* also had the best antitumor inhibition effect. This way, this study related biological effects to different compounds. So phenolic extract, in case of *Arbutus unedo* L., was more bioactive that anthocyanin extract [11].

In the study by Dib *et. al* moderate antibacterial activity was reported for water extract and phenolic extract of *A. unedo* against *E. coli* and *Staphylococcus aureus* [41]. This activity was attributed to the phenolic compounds detected in both water and phenolic extracts such as quinones, anthraquinones, flavonoids, tannins and anthocyanins.

Arbutus unedo L. antiaggregant effects in human platelets was also evaluated by El Haouari *et al*. [43]. Extracts from *Arbutus unedo* were obtained by extraction with water, diethyl ether and ethyl acetate. Platelets were treated with increasing concentrations of crude aqueous (0.015-1.5 mg/mL), ethyl acetate or diethyl ether extracts. All the extracts reduced platelet aggregation evoked by thrombin, had strong ROS scavenging activity, reduced store-operated Ca^{2+} entry induced by thrombin and also reduced basal and thrombin-stimulated tyrosine phosphorylation. All these parameters make *Arbutus unedo L*. extracts good antiaggregants which is likely to valorise *A. unedo* as a good treatment or prevention treatment to cardiovascular diseases.

The aim of the study by Afkir *et al*. was to evaluate if the treatment with *Arbutus unedo* L. leaf and root aqueous extracts reduced hypertension, by inhibiting nitric oxide synthase [44]. This study used an *in vivo* approach as rats were treated with 250 mg per kg of each extract each day. The vascular reactivity of the rat's aorta was measured *ex vivo*. In conclusion, the treatment of rats with *A. unedo* aqueous extract regressed hypertension development, prevented the myocardial hypertrophy; ameliorated vascular reactivity, and renal functional parameters. Authors relate these findings with the presence of polyphenols in the extracts that modulate the NOS activity. The authors also reported that the effect was greater and more efficient with root than leaf extract.

As far as hypertension is concerned, this plant is used in traditional medicine against this disease. Typically, an infusion of roots or leaves can be made. Some more studies were done regarding the plant's anti platelet aggregation activity. In various studies, the leaf and root aqueous extract showed: an inhibition of thrombin-induced platelet aggregation, which the authors related to the tannins present in the leaves [45];

provoked an *in vitro* inhibition of aggregation of rat platelets (effect that could be attributed to a number of phytochemicals present in the roots) [46]. This type of extract had also a vasorelaxant activity in rat isolated aorta (as the tannins and catechin gallate present in the leaf methanolic extract showed a strong vasorelaxant activity) [47].

Regarding inflammation, Mariotto *et al*. performed a study using an animal model of acute inflammation and a cellular model using human alveolar epithelial cells and the human monocytic leukemia cells [48]. Rats were treated with carrageenan in order to induce lung inflammation. Rat cells and human alveolar epithelial cells were also cultivated. Carrageenan has administrated to rats in order to induce lung inflammation. *Arbutus unedo* leaves aqueous extract were administered to both groups (in a dose correspondent to 20 mg GAE/kg). The aim of the study was to evaluate if the administration of this extract affected the following: STAT1/3 activation, TNF, IL-1 and IL-6 production in pleural exudate, lung iNOS, COX-2 and ICAM-1 expression, neutrophil infiltration, the nitration of cellular proteins by peroxynitrite, lipid peroxidation, prostaglandin E2 levels, nitrite/nitrate levels and the amount of lung injury. In summary, this report lead to the conclusion that *Arbutus unedo* L. aqueous extract reduced the production of IL-6 in the human cell lines which strongly inhibits the activation of STAT3. This way it prevented the formation of other pro-inflammatory cytokines such as TNF-α and IL-1. In the carrageenan-induced rats there was a decreased recruitment of neutrophils and a down regulation of the expression of iNOS and COX-2 which finally led to the decrease of inflammation and of tissue injury. The study concluded that inflammation was significantly attenuated by the treatment with *A. unedo* extract. Once again, authors relate this finding with flavonoids that are known to be present in this plant [48].

3.2.1. Studies on Antihyperglycaemic Activity

Since the fruit and leaves can be easily found and consumed by the population [4, 14], they can easily be combined in the treatment of type 2 Diabetes or/and can be used to reduce the risk of this disease. The roots of *A. unedo* are claimed to have antidiabetic effects [49, 50]. However, no antidiabetic studies have been done about the antidiabetic properties of fruits and leaves of *Arbutus unedo* until this date [16, 49].

Regarding the antidiabetic effects of *Arbutus unedo* L., only two studies are known. Bnouham *et al*. have studied the hypoglycaemia effect of *Arbutus unedo* root

aqueous extracts in neonatal streptozotocin-induced diabetic rats under chronic treatment [49, 50]. These studies have been conducted in an animal model with STZ-diabetic rats. They showed that *Arbutus unedo* decreases blood glucose levels. According to Bnouham *et al*. the doses 400mg of root aqueous extract per litre and 500mg of root aqueous extract per kg of body weight exhibit plasma glucose lowering effect, in Wistar rats [49, 50]. The *in vitro* study of glucose utilization was also tested and the data suggest that the combination of insulin and extract (insulin at 2 UI/mL and aqueous extract of *A. unedo* at 1 mg/mL) potentiates its activity and enhances the utilization of glucose [49].

Taking in consideration this two works by Bnouham *et al*., physiological characteristics of Wistar rats (body weight of about 300g and daily water intake of 20-50 ml a day [51]), one can estimate that the doses given were 20mg of extract each day [49] and 150mg of extract each day [50].

So, according to these works, a dose between 20 and 150mg of root aqueous extract will present and antidiabetic effect in rats. These doses can serve as reference for further works with other parts of the *Arbutus* plant.

4. Diabetes Mellitus: some considerations and study model

4.1. Pathology and biochemical changes

Diabetes Mellitus is a disease caused by anomalies in glucose homeostasis and can be divided into two types: a non-insulin dependent (type 2) and another caused by deficient insulin production (type 1) [52]. Chronic hyperglycaemia is a characteristic of diabetes due to poor glucose regulation caused by decreased insulin secretion and/or by decreased insulin activity. Insulin is a hormone produced by the pancreatic β-cells whose main function is to maintain blood glucose homeostasis by increasing glucose uptake by cells, increasing glycogen production and glycolysis and signalizing cells to increase protein and fatty acids synthesis [52]. This hormone can also act indirectly in fatty acids homeostasis as it increases fatty acids and triglyceride synthesis in liver and fat tissues and decreases lipolysis in adipocytes which is responsible for the body fat increased storage [52].

Diabetes type 2 is a very common health problem in developed countries, particularly in the USA and in Europe. The World Health Organization claims that 347

million people worldwide have diabetes and will be the 7th leading cause of death in 2030 [53].

Type 2 diabetes mellitus is a chronic disease that is associated with obesity and excess of sugar and fat in the diet. In type 2 diabetes there is deficient production of insulin or a decrease uptake of glucose by the cells [52]. There is a progressive development of hyperglycaemia and the insulin resistance (mainly in muscle and liver tissues) due to increased body fat. This type of diabetes can remain "silent" during year as initially pancreas is able to produce more insulin in order to try to overcome the body's insulin resistance. However, as this is the result of a diet rich in sugar and poor glycaemic control, the pancreas starts to shut down since there is a progressive failure of the β-cell output, resulting first in glucose intolerance and then ultimately in type 2 diabetes. Glucose metabolic pathways and organ systems homeostasis are affected resulting in accelerated lipolysis, hyperglucagonemia and increased glucose reabsorption in kidneys. The increased gluconeogenesis results in both renal and hepatic glucose release. In type 2 diabetes patients there is an accumulation of glycogen in the kidney, which leads to the increased renal glucose release [52, 54]. Other effects of diabetes is a high percentage of glycated haemoglobin as a high concentration of glucose in the blood can lead to its non-enzymatic linkage to lysine residues of a variety of proteins, making this parameter a direct reflection of a mean blood glucose levels [52]. Protein glycosylation can also take place in cellular membrane proteins leading to disrupted cellular signalling Microalbuminuria is a characteristic of diabetes and it is defined as the albumin expression rate being a signal of early renal damage [52].

Chronic hyperglycaemia caused by diabetes is associated with long term cellular damage and multiple organ failure which can lead to retinopathy, nephropathy, neuropathy heart disease and atherosclerosis [52].

4.2. Insulin Cascade

A series of metabolic responses in cells are induced by insulin. The insulin pathway is illustrated in figure 3. Firstly, insulin binds to its receptor inducing the auto phosphorylation of a number of tyrosine residues. Another group of proteins, the insulin receptor substrates (IRS) have a phosphotyrosine-binding (PTB) domain which recognises the tyrosine residues and are recruited to the receptor at the cell membrane. The IRS

molecules are this way phosphorylated at their numerous tyrosine residues by the insulin receptor. Some of these phosphorylated residues are then recognised by the SH2 (Src homology 2) domain of the p85 regulatory subunit of PI 3-kinase. PI 3-kinase has itself a catalytic subunit, the p110, which phosphorylates another protein at the plasma membrane of the cell, the PtdIbs(4,5)P2 generating a second messenger, the PtdIns(3,4,5)P3. This, on the other hand, stimulates insulin-dependent processes. When insulin binds to its receptor another protein is also phosphorylated, the Cbl. The Cbl forms a complex with the adaptor protein CAP which is recruited to a region of the plasma membrane denominated RAFT. In the plasma membrane Clb also interacts with the adaptor protein Crk which is associated with factor C3G. Members of the GTP-binding protein family, TC10 are activated by C3G, throughout an unknown mechanism this GTP-binding proteins activate effector molecules to promote GLUT4 translocation [55-57].

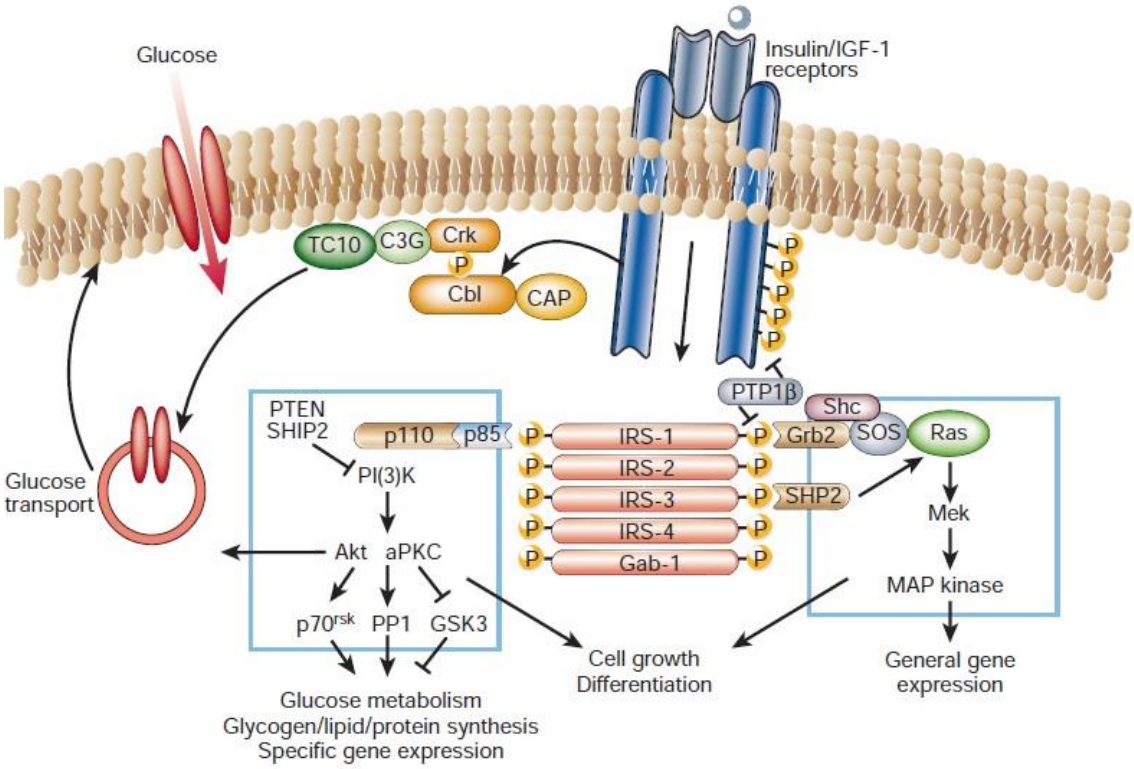

Figure 3: The insulin pathway [56].

Insulin receptor substrate family proteins (IRS) are a family of proteins that transmit signals from insulin to intracellular pathways. One of the pathways they mediate is the PI(3)K/Akt pathway. P110 is involved in the phosphorylation of the protein PI(3)K (phosphatidylinositol 3-kinase) which activates a serine-threonine kinase known as Akt or

protein kinase B (PK-B). Akt can be recruited to the cell membrane where is activated. After it can have multiple functions. It can phosphorylate multiple substrates who act on gluconeogenesis and glycogenolysis pathways, it can inhibit glycogen synthase kinase-3 (GSK-3) inducing glycogen synthesis, it can induce protein synthesis, regulate cellular metabolism and gene expression. Akt is also known to play a role in GLUT4 translocation [55-57]. The different IRS proteins seem to serve different functions at the cellular level, probably having different distribution throughout the tissues. For example, studies showed that rats lacking IRS-2 gene have a diabetic phenotype [56].

4.3. Glucose Transporters

GLUT are a class of genes that encode a family of plasma membrane proteins named Glucose Transporters (GLUTs) whose function is to mediate the transport of glucose over the cellular membrane. Various types of GLUTs exist, namely GLUT-2 and GLUT-4. GLUT-2 is expressed in liver cells and pancreatic β-cells as well as kidney tubular cells. On the other hand, GLUT-4 is mainly expressed in fat tissues although it can also be present in other types of striated muscular tissues. GLUT-2 isoform mediates the bidimensional transport of glucose in the hepatocyte and is thought to be involved in the glucose sensing mechanism of the pancreatic cells. The GLUT4 isoform changes in response to insulin and is responsible for most of the insulin-stimulated uptake of glucose [55].

4.4. Type 2 Diabetes effects on microelement homeostasis

Several studies report that diabetes can disturb mineral homeostasis, namely copper, iron and zinc [58-67]. Zinc is a trace element required to multiple cell regulatory reactions and has a role in diabetes development and glucose metabolism. It plays a role in insulin production secretion and storage, as Zn transporter (ZnT8) regulates insulin secretion from the pancreatic β-cells. Besides, zinc deficiency may potentiate insulin resistance thereby affecting the glucose uptake [59, 64]. Zinc metabolism is disturbed by high glucose levels in diabetes mellitus. In diabetic patients there is also an increased loss of zinc in urine due to polyuria [59]. Zinc tissular levels can be also affected as rats used as a model of type 2 diabetes presented lower zinc levels in kidneys, testis and fat, but higher

zinc levels in spleen, pancreas and prostate, which indicate a redistribution during the diabetic state [59].

Copper is another trace mineral that also plays a role in various enzymes catalytic centres. It also protects the body against oxidative damage and has a direct influence in lipid metabolism. Disrupted tissular copper levels are thought to negatively affect some metabolic pathways related with diabetes [64]. Higher serum concentration of Cu was found in type 2 diabetic patients and an association between these high values and diabetes complications was established [58]. There is also a relationship between an high-fat diet (in Zargar study, obese patients with diabetes), diabetes and a slight elevation of copper levels [68].

Iron is another essential mineral that plays multiple functions in physiology, mostly associated with redox reactions and transporting oxygen by haemoglobin. Iron is stored in the form of ferritin wich is a biomarker of iron status. Recently it was found that serum ferritin levels are elevated in diabetic patients [64, 66]. Higher levels of ferritin indicate increased tissular iron deposition [66]. It is also known that diabetes can be a risk factor for hemochromatosis which elevates the serum iron levels. A recent study a demonstrated that administration of iron can induce diabetes in animals, establishing a positive association between elevated iron stores and type 2 diabetes [66]. Iron can also interfere with proper insulin metabolism in liver and pancreas as elevated iron levels may lead to its deposition in the pancreatic ß-cells. This results in impaired insulin secretion and high iron hepatic stores can cause insulin resistance by preventing insulin to bind with liver's receptors [66].

4.5. Diabetes and inflammation

Both type 1 and type 2 diabetes are associated with increased inflammation as this state is thought to mediate the development of insulin resistance and pancreatic β-cell dysfunction. Diabetes causing oxidative stress is related with nephropathy and retinopathy [69]. Numerous inflammatory response associated molecules are increased in diabetic patients. Diabetic patients were also found to have decreased immunity as the expression of molecules as IL-2R and CR3 appeared to be downregulated [70]. These molecules play a key role in inflammatory response as being responsible for lymphocyte and monocyte proliferation. High fat feeding or over nutrition can be directly involved in an increased

inflammatory state. High fat diets can lead to increased body fat and adipose tissue that secrets adipokines such as TNF-α and IL-6 [71]. Increased body fat leads to increased production and release of these molecules which will cause the activation of the innate immune system.

Inflammatory cytokines such as TNF-α have been reported to be significantly increased in diabetic rats [72]. Tumour necrosis factor (TNF-α) is a cytokine released in the inflammatory process. Recently, it was found out that TNF-α also plays a role in the early events of insulin transmembrane signalling [73]. During the insulin pathway, both the insulin receptor j3-subunit and IRS-1 (its major cytosolic substrate) are phosphorylated by an insulin-induced tyrosine cascade [56, 57]. High concentrations of TNF-α decrease the insulin-stimulated auto phosphorylation of the insulin receptor and the phosphorylation of IRS-1 [73]. This can explain how insulin resistance can be related with a pro inflammatory state, as high concentrations of TNF-α occur in inflammatory response [71].

Interleukin 6 (IL-6) is another pro inflammatory cytokine whose expression is enhanced in the plasma and adipose tissue of obese insulin-resistant subjects [70]. It is produced in many inflammatory cells, adipocytes, muscular cells and pancreatic β-cells [72]. It can be responsible for inducing insulin resistance in adipocyte tissue and liver, and may act along with other pro inflammatory cytokines to produce β-cell damage. It can also have a modulatory effect in fat tissue and genetic variances in the genes encoding to this protein being related with the development of diabetes [122]. High concentrations of this cytokine are related to an inflammatory state.

4.6. *In vivo* animal model of type 2 diabetes

Rats are the most commonly used vertebrate species; they are popular because of their availability, size, low cost, ease of handling, and fast reproduction rate [74]. One of the most used animals in the animal experimentation is the Wistar rat, commonly known as the albino rat.

The Wistar rat is a breed from the albino rats and was developed by the Wistar Institute to be used in research as a model organism. These rats are characterized by having white pelage and red eyes, a wide head with long ears, and a tail that has less in length than the length of the animal's body. Because they share 99% of their genes with

humans, they are widely considered to be the prime model of inherited human disease [74].

In laboratory rats, diabetes can be induced by the injection of streptozotocin, so this disease can be studied in an animal model. This chemical is toxic and has the ability to destroy the β-cells of the pancreas, which are responsible for producing insulin. In medical research it is used to induce diabetes in order to produce an animal model for diabetes. A single large dose is used to induce type 1 diabetes and multiple low doses are used to induce type 2 diabetes [75].

Other studies suggest that a combination of high-fat feeding (were 40% of calories come from fat) and low dose of STZ injection/ multiple low doses of STZ injection can be effectively used in order to mimic type 2 diabetes in rats [76, 77]. This rat model will mimic the physical and metabolic characteristic of type 2 diabetic humans: increased body fat, hyperglycaemia, high fatty acid profile although being non-insulin dependent.

Feeding rats with a high-fat diet can promote the development of hyperglycaemia and insulin resistance. High dose injections of STZ (single shot of 80 mg/kg), can critically damage pancreas resulting in compromised β-cell functioning which leads to deficient or no insulin secretion. This secretion can be so low that it resembles more type 1 diabetes. On the other hand, multiple low-dose injections of STZ (pe. 30mg/kg at weekly intervals for 2 weeks) do not have such arch effect and induce a gradual decrease of insulin secretion, which is exactly what happens in humans with a natural progression of type 2 diabetes [78]. Thus, high-fat diet and multiple low doses of streptozotocin causes high blood glucose levels and low insulin concentrations symptoms that are consistent with the features of type 2 diabetes in humans [76, 77]. By developing a stable type 2 diabetic rat model it can be possible to securely evaluate the antidiabetic potential of plant materials administered orally.

Rodent semi-purified diets can be obtained following the AIN-93M recommendations [79]. High-fat diets are obtained from basal AIN-93M purified diets being are prepared using the same method and recommendations as the AIN-93M diet. With the only different being that 40% of the diet's caloric intake comes from fat, they are obtained by the replacement of wheat starch by lard (fat 15% and soybean oil 10%). These diets can be obtained by the mixing of common ingredients such as casein (14%), soybean oil (10%), wheat starch (46.5%), lard (10%), sucrose (10%), potato starch (5%). Vitamin

mix AIN-93M (1%) and mineral mix AIN-93M (3.5%) are also added in order to assure the animal's correct intake of these essential nutrients [79].

5. Framework and aims of this master thesis

Arbutus unedo L. is a shrub widely distributed in the Mediterranean regions, namely in Portugal, where it can be found mostly in the southern region. There is not much information available on this species. The fruit's chemical characteristics are relatively well-known. As far as the leaves are concerned, not much is known about their chemical properties (or at least they are not so well studied as the fruits). A vast amount of biological effects were attributed to *A. unedo*, as there are many epidemiological studies about the shrub's applicability in the treatment of gastrointestinal and urological problems, hypertension and cardiac diseases, diabetes and how this plant has anti-inflammatory, anti-microbiological and lipid oxidation inhibitory properties. Works regarding the biological activities of *Arbutus unedo* L., most of the times use the entire plant or only roots and leaves as well as selected types of extracts.

Diabetes is an epidemic disease that affects deeply the body homeostasis. Besides high blood glucose, high concentration of lipids and insulin resistance it can affect numerous other parameters. It has consequences in other body organs namely in terms of mineral homeostasis. Zinc, copper and calcium homeostasis has been reported to be disturbed in various body tissues. As diabetes can itself cause an inflammatory status, the expression and release of inflammatory factors and cytokines is also affected. This inflammatory status is also thought to disrupt insulin signalizing cascade and glucose transporters translocation.

Even though *Arbutus unedo* L. is used in folk medicine against diabetes, the only work that has been done on this topic only uses roots' aqueous extract. A study on the antidiabetic effects of *Arbutus unedo* L. leaves and fruits is of great interest. In this way, it will be possible to value *A. unedo* and to let the population know more about this endemic species. This can also help in the valorisation of strawberry tree fruit and can be the starting point of the development of new food products and supplements that use this fruit to promote human health.

So, the specific aims of this study are to:

- Establish optimal extraction parameters to obtain *Arbutus unedo* L. fruits aqueous extracts;
- Characterize extracts from *Arbutus unedo* L. berries and leaves in terms of the total phenolic content;
- Evaluate, *in vivo*, on an animal model of type 2 diabetes, the antidiabetic effects of *Arbutus unedo* L. aqueous extracts, in selected serum and hematological parameters.
- Evaluate, *in vivo*, on an animal model of type 2 diabetes, the effects of *Arbutus unedo* L. aqueous extracts on tissular mineral homeostasis
- Evaluate, *in vivo*, on an animal model of type 2 diabetes, how *Arbutus unedo* L. aqueous extracts affects the expression of key genes related with inflammation and glucose metabolism, namely encoding genes for TNF-α, IRS-2, IL-6, GLUT-2 and GLUT-4.

CHAPTER II - Material and Methods

1. Samples

The leaves and fruits of *Arbutus unedo* L. were collected in the 24th of October 2014, in *S. Pedro de Alva* (*Penacova*, centre region of Portugal). Plant materials were randomly collected from adult wild plants that grew freely in the woods. Only fruits at the ripe stage (with red yellowish colour, not being fully ripped) and healthy fresh adult leaves were selected. The samples were immediately frozen and freeze dried prior to extraction.

2. Preparation of extracts from *Arbutus unedo* L. fruits and leaves

2.1. Selection of optimal extraction parameters to obtain extracts from *Arbutus unedo* L. fruits

The procedure was performed according to Malheiro *et al*. with some adaptations and testing different conditions [8, 39, 42, 49, 50, 80].

Briefly, the fruits were crushed and powdered and 5g of powdered fruit were extracted with different solvents, during different extracting periods at selected temperatures as showed in table 4. The extracts were filtered under vacuum (glass Büchner funnel with nº 2 pore), evaporated under vacuum (rotary evaporator) and the resulting concentrated extracts were finally lyophilized. For each condition, three samples were obtained.

Table 4: Extraction optimal parameters tested to obtain extracts from *Arbutus unedo* L. fruits.

Assay	*Extraction time*	*Solvents*	*Temperature of extraction*	*Mass/Volume Ratio*	*Reference(s)*
1	45min	Water	100ºC	5g/250mL	[8, 39]
2	45min	Water	90ºC	5g/250mL	[8, 39]
3	45min	Water	80ºC	5g/250mL	[8, 39]
4	60min	Water	90ºC	5g/250mL	[49, 50]
5	120min	Water	90ºC	5g/250mL	[49, 50]
6	45min	Water	90ºC	5g/125mL	[42]
7	45min	Water	90ºC	5g/62.5mL	[42]
8	45min	Ethanol 50%	63ºC	5g/250mL	[80]

On assay 8, the selected temperature was 63ºC, due to the fact that the extraction was made with Ethanol 50%. This temperature was used in a previous work using the same solvent to obtain a plant aqueous extract, suitable to test *in vivo* [80].

2.2. Extraction conditions used to obtain the *Arbutus unedo* L. leaf extract

The procedure was performed according to the procedure described by Malheiro *et al*. [8]. Briefly, the dried leaves were powdered with a food processor and 5g of powdered leaves were extracted with 250 mL of water at 100ºC for 45 min. The extracts were filtered under vacuum (glass Büchner funnel with nº 2 pore), evaporated under vacuum (rotary evaporator) and the resulting concentrated extracts were finally lyophilized.

2.3. Total Phenolic Determination

Total phenolic Concentration (TPC) was determined using the Folin-Ciocalteu method as described by Singleton [81]. The method consists in the addition of 500 μL of distilled water to 125 μL of the diluted sample. For each extract, a mother sample was prepared by dilution of 50mg of extract in 10mL of solvent (methanol:water). For leaves extract, 1:2, 1:4 and 1:16 solutions were prepared. In order to fit the calibration curve, in this analysis, only the 1:16 (0.3125 mg in 10 mL of solvent) dilution was used. To this mixture, 125 μL of Folin-Ciocalteu reagent was added and the mixture was kept in the dark for 5 minutes. After, 1.25 mL of Na_2CO_3 in the concentration of 75 g/L and 1 mL of distilled water are added. The solutions were kept in the dark again for 60 min and then the absorbance was measured at λ=760 nm in a 6405 UV/Vis Jenway Spectrometer. The blank was prepared by replacing the volume of the sample by distilled water.

Previously, a calibration curve was performed using gallic acid as standard. For this calibration curve, various solutions of gallic acid were prepared in concentrations of 61.995, 123.99, 186, 248 and 310 mg/L. The calibration curve was given by the A_{760} against the concentration of gallic acid. All the measurements were made in triplicate, using three aliquots of each extract and the average value was calculated in each case. The A_{760} of the blank was subtracted to each sample A_{760}. The results were expressed as mg of GAE per 100 g of extract.

The optimal extraction parameters to obtain the fruit extract were established by combining the best extraction yield with the highest total phenolic concentration.

3. Evaluation of the impact of *Arbutus unedo* L. extracts intake in a rat model of type 2 diabetes

In order to evaluate the *in vivo* properties of *Arbutus unedo* L., a trial was performed. The doses given orally were: 1.25 and 0.5 g/kg b.w. per day of fruit and leaf extracts respectively.

3.1. Diets

Arbutus unedo L. fruit's extract were found to be a very hydroscopic material. Thereby, in order to mix it with the diet the extract was previously mixed in 16.7% of maltodextrin. Briefly, 339 g of extract were mixed with 67.80g of Maltodextrin in warm (70ºC) water, according to the ratio of 2g of Maltodextrin: 10g of crude (sticky) extract: 5mL of distilled water. The samples were then freeze dried at 50ºC.

Four types of diets were prepared for this experiment: the control diet which was prepared according to AIN- 93M rodent diets recommendations [79], a high-fat diet on which 40% of the energy was provided by fat and high-fat diet mixed with each of the plant extracts. The control diets were prepared by mixing casein (14%), soybean oil (10%), wheat starch (56.5%), sucrose (10%), potato starch (5%), vitamin mix AIN-93M (1%) and mineral mix AIN-93M (3.5%). High-fat diets were prepared using the same method and recommendations as the AIN-93M diet, the only difference being the replacement of wheat starch by lard (fat), thereby being composed finally by casein (14%), soybean oil (10%), wheat starch (46.5%), lard (10%), sucrose (10%), potato starch (5%), vitamin mix AIN-93M (1%) and mineral mix AIN-93M (3.5%). In order to obtain the plant enriched diets, normal high-fat AIN-93M diets were prepared the only difference being the replacement of 12% and 7% of wheat starch by 12% and 7% of fruit extract and leaves extract in case of High-Fat Fruit Extract diet and High-Fat Leaf Extract diet respectively. The amount of extracts were different in order to fit the doses of 1.25g/kg b.w. of fruit extract and 0.5g/kg b.w. of leaves extract, as determined by the total amount of available materials. The diets were prepared weekly and stored at 4ºC in plastic tupperwares until use. Figure 4 illustrates the consistency of the high-fat diet.

Table 5 summarizes the composition of the experimental diets used in both trials of this experiment. Table 6 gives the composition of both mineral and vitamin mix.

Table 5: Composition of Control, High-Fat and High-Fat plant extract diets.

Ingredient (g/kg)	*Diets*			
	C	HF	HF+FE	HF+LE
Casein	140	140	140	140
Sunflower oil	100	100	100	100
Lard	0	100	100	100
Wheat starch	565	465	453	458
Sucrose	100	100	100	100
Potato starch	50	50	50	50
Mineral Mix	35	35	35	35
Vitamin Mix	10	10	10	10
Plant Extract	-	-	12	7

Abbreviations: HF- High Fat; HF+FE-high fat with fruit extract (1.25g/kg); HF-LE- high-fat with leaf extract (0.5g/kg)

Figure 4: Two male Wistar rats used in the trial fed with the AIN- 93M derived high-fat diet. Note consistency of the diet.

Table 6: Composition of AIN-93M vitamin and mineral mix, adapted from Reeves *et al*. [79].

Vitamin	***g/kg mix***	***Mineral***	***g/kg mix***
Nicotinic acid	3	Calcium	5000
Ca Pantothenate	1.6	Phosphorus	1992
Pyridoxine-HCL	0.7	Potassium	3600
Thiamin-HCL	0.6	Sulphur	300
Riboflavin	0.6	Sodium	1019
Folic acid	0.2	Chloride	1571
D-Biotin	0.02	Magnesium	507
Vitamin B-12	2.5	Iron	35
Vitamin E	15	Zinc	30
Vitamin A	0.8	Manganese	10
Vitamin D3	0.250	Copper	6
Vitamin K	0.075	Iodine	0.2
		Molybdenum	0.15
		Selenium	0.15
		Silicon	5
		Chromium	1
		Fluoride	1
		Nickel	0.5
		Boron	0.5
		Lithium	0.1
		Vanadium	0.1

3.2. Animals, diets and experimental protocol

Male adult Wistar rats (n = 28, 11 weeks old) were purchased from the Licensed Laboratory of the Animal Breeding Centre (Poznan, Poland). After arrival at the animal care facility, rats were kept under controlled temperature (21 ± 2ºC) and humidity (55-60%) with a 12h/12h day/night cycle throughout the experiment. After a 5 day adaptation period, animals were divided into 4 groups (of 7 rats each, initial mean body weight = 330 g), and kept in metal-free individual cages with 2 rats per cage (Figure 5). The control

group was fed the AIN-93 M standard diet, while 3 groups received high-fat diet (HF) for 2 weeks.

Figure 5: Allocation of the Wistar rats throughout the experiment (kept in metal-free individual cages containing 2 rats each).

The AIN-93M and HF diets were prepared according to the AIN-93M recommendations, with slight modifications [79]. AIN-93 (control group) was composed by casein (14%), sunflower oil (10%), wheat starch (56.5%), sucrose (10%), potato starch (5%), vitamin mix AIN-93 M (1%) and mineral mix AIN-93 M (3.5%). While, the HF diets (40% calories from fat) were obtained from the basal AIN-93 diet, by replacement of wheat starch with fat (lard 15% and sunflower oil 10%), being thereby composed by casein (14%), sunflower oil (10%), wheat starch (46.5%), lard (10%), sucrose (10%), potato starch (5%), vitamin mix AIN-93M (1%) and mineral mix AIN-93M (3.5%). Diets were prepared weekly and stored in sealed containers at 4 ± 1 °C. Food intake was measured daily and body mass every 7 days.

After 2 weeks of HF diet feeding, rats were subjected to multiple intraperitoneal injection of STZ freshly dissolved in 0.1 M-citrate buffer (pH 4.4), given in 3 subsequent doses: 20, 10 and 25 mg/kg body weight, in weekly intervals, while rats of the control group were injected in the same manner, but with the carrier alone (citrate buffer). The approach with multiple doses of STZ has been shown to be more efficient in producing diabetes in rat [78]. Finally, presence of diabetes (DB) in rats was confirmed by measuring

fasting blood glucose concentration in blood samples (> 11 mmol/l) withdrawn from the tail tip after 48 h using a glucometer (iXell®, Genexo, Warsaw, Poland).

Thus, the four experimental groups were, as follows: (1) no-DB/not treated n=5 (fed control diet); (2) DB/not treated (fed HF diet) n=8; (3) DB/FE, 1.25g/kg b.w./day fed HF diet (HF + FE) n=6; (4) DB/LE, 0.5g/kg fed HF diet (HF + LE) n=6. The doses were based on the available amount the materials.

After 4 weeks of feeding and overnight fast, the animals were anesthetized with CO_2 inhalation and dissected to collect blood and the internal organs. Blood samples were drawn from the heart aorta into Vacutest tubes with plasma coagulant (Medlab-Products, Raszyn, Poland), coagulated at room temperature for 20 min, and centrifuged at 4000 rpm. Internal organs (liver, kidneys, heart, spleen, pancreas, testes) and both femoral bones were removed for further analysis. Organs were washed in saline (0.9% NaCl), weighed and stored at -20°C until analysis. Serum samples were separated and kept in aliquots at −70 °C for biochemical assays.

All animal procedures and the protocol were conducted according to EU Directive 2010/63/EU for animal experiments and approved by the Local Animal Bioethics Committee in Poznan, Poland (No 3/2015). All necessary efforts were made to minimize the number of animals used and their suffering.

3.3. Analytical methods

3.3.1 Blood indices

Blood (whole and serum) analyses were conducted in a certified laboratory (Laboratorium Medvczne Synevo, Poznan).

Analysis for the insulin levels, HOMA-IR and gene expression took place in Uniwersytet Przyrodniczy w Poznaniu although they were not executed entirely by me. Another investigator working on this *in vivo* experiment kindly performed the analysis and supplied the results.

a) Blood hematological indices

Blood haematological indices, as given below, were determined by appropriate analytical methods, as follows. Blood haemoglobin (Hb) level was determined by the Drabkin cyanohemoglobin method [82]. Red Blood Cell Count (RBC), Haematocrit

(HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), White Blood Cell Count (WBC), Platelets (PLT), Platelet Distribution Width (PDW), Mean Platelet Volume (MPV), Platelet Large-Cell Ratio (P-LCR) and Red Cell Distribution Width Based On Standard Deviation (RDW-SD) were all were obtained using the CELLDYN-1700 analytical haematology system.

b) Blood biochemical indices

Blood biochemical indices, as given below, were determined by appropriate analytical methods, as follows: serum glucose concentration by the hexokinase method [83]; total cholesterol, HDL cholesterol and TAG concentrations were determined using enzymatic methods while LDL cholesterol concentration was given by the calculation method [84-86]; urea concentration by the urease and glutamine dehydrogenase method [87].

The activity of the enzymes ALT, AST and ALP was measured using kinetic method with NADH and TRIS buffer [88]. Serum creatinine concentration was also determined by a kinetic method using picric acid [87] while the total protein concentration was measured by a colorimetric method using Cu^{2+} ions [86].

The C-reactive protein was also measured by a immunoturbidimetric method performed using a COBAS INTEGRA analyzer (Roche Diagnostics), were the C-reactive protein agglutinates with latex particles coated with monoclonal antiCRP antibodies and the resulting aggregates are quantified turbidimetrically [89].

The Serum Fe concentration was determined by the Guanidine/Iron-Zine method were by the action of guanidine cloridrate the Fe(III) is released from transferrin and reduced to Fe(II) which will kelate with IronZine and form a reddish complex that can be quantified by absorbance [90, 91].

Insulin was measured by a specific RIA kit for rats (Linco) according to the procedure by Cacho *et al*. [92].

Finally, the Insulin resistance was characterized by the homeostasis model assessment (HOMA) indices using the equation given below [92, 93]:

$$HOMA - IR = \frac{((\text{Fasting Glucose [mmol dm}^{-3}] \times \text{Fasting Insulin [mIU dm}^{-3}])}{22.5}$$

3.3.2. Microelements (Fe, Zn, Cu) status in tissues

Firstly, a small slice of each tissue has collected into an Eppendorf and kept at 105ºC for 2 days in order to determine the dry mass of the tissue. After, 1g of the different tissues were weighted and collected into Teflon vessels (MARS-5) and digested in 65% (w/w) of HNO_3 (GR ISO). The vessels were placed inside the microwave oven (Microwave Digestion System (MARS 5, CEM)) according to each tissue's selected program. The clear mineralized samples were transferred quantitatively to a volumetric 25mL polypropylene flask and the volume was filled with high-grade deionized water. The mineralized was then transferred to clean (25-50 mL) polypropylene bottles.

Thereafter, the concentrations of Fe, Zn and Cu in the mineral solutions were measured by the flame atomic-absorption spectrometry (F-AAS) method (AAS-3 spectrometer, Zeiss, with BC, Germany). The accuracy of quantitative determinations of Fe, Zn and Cu was assured by simultaneous analyses of the certified reference material (Pig Kidney BCR® No. 186, Brussels), with the recovery of: 98.6%, 97.5% and 98.4%, respectively.

3.3.3. Gene Expression by Relative real-time PCR

Total RNA from rats' liver, muscle and fat tissue was isolated using the tripure isolation kit according to the manufacturer's protocol (Roche diagnostic, Germany). The quantity and quality of RNA was determined by 260/230 nm and 280/260 nm absorbance ratios using a NanoDrop 2000 spectrometer (Thermo Scientific, USA). After RNA isolation, a total of 1 μg RNA was used to perform the reverse transcription (RT). RT was executed using a Transcriptor First Strand cDNA Synthesis Kit (Roche diagnostic, Germany), using a combination of anchored-oligo(dT)18 and random hexamer priming, being performed at 25 ºC for 10 min, followed by 1h at 50°C and finally the inactivation of RT enzyme was performed for 5 minutes at 85°C. cDNA quantity and quality were also assessed using a NanoDrop 2000 spectrometer (Thermo Scientific, USA). cDNA was stored at 4ºC until analysis (up to 2 weeks).

Real-time PCR was performed with gene-specific intron spanning primers. Each real-time PCR reaction (n=4) containing Light-Cycler Fast Start DNA Master SYBR Green I and 3 μL of diluted cDNA in a total volume of 10μl was carried out using QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific, USA). Real

time PCR was performed under the following conditions; 45 cycles during 10 minutes at 95°C for pre-incubation, then 10 seconds at 95°C for denaturation and 7 seconds at 60°C for annealing and extension, further, the melting curve consisted on 15 s at 95ºC and 60ºC, 1min. The specificity of PCR products was monitored on the basis of melting curve analysis.

Genes' expression was measured by a comparative CT method (ΔΔCT) real time PCR GAPHD expression was used as an internal control. Before using the ΔΔCT method for quantitation, a validation experiment was performed for primer efficiency investigation.

3.3.4. Statistical analyses

All the results are presented as means ± standard deviation. Normality of distribution of data was verified by the Shapiro–Wilk test. Significance of differences of means was calculated using, one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's HSD) Means were considered statistically different at $p < 0.05$. All calculations were made using the software GraphPad Prism version 6 for Windows (trial version, GraphPadSoftware, San Diego California, USA).

The graphics presented in "Chapter III – Results" illustrate results that differ statistically (with exception to figure 29). The graphics presented in Appendix 2 illustrate the results that do not differ statistically (with exception to figure 59)

CHAPTER III- Results

1. Extraction yields obtained for *Arbutus unedo* L. fruit aqueous extract (with different extraction conditions) and *Arbutus unedo* L. leaf aqueous extract

By testing the different extraction conditions in order to obtain the *A. unedo* fruit extract, different extraction yields were obtained. The obtained yields, as well as the extraction yield for the different tested conditions, are showed in Table 7.

Table 7: Yield, extraction yield (Mean ± SD) of *Arbutus unedo* L. fruit extracts obtained for each of the different conditions tested.

Condition tested	***Extraction method***	***Yield (g)***	***Extraction yield (% w/w)***
Temperature - 45min - 5g/250mL - water	100ºC	3.10	58.5 ± 6.7
		2.55	
		3.17	
	90ºC	3.93	78.4 ± 1.0
		3.96	
		3.98	
	80ºC	3.81	73.5 ± 5.1
		3.90	
		3.45	
Time - 90º C - 5g/250mL - water	45 min	3.93	78.4 ± 1.0
		3.96	
		3.98	
	60min	3.12	57.1 ± 3.9
		2.79	
		2.76	
	120min	3.09	59.5 ± 2.4
		2.89	
		3.06	
Mass/Volume Ratio - 90º C - 45 min - water	5g/250mL	3.93	78.4 ± 1.0
		3.96	
		3.98	
	5g/125 mL	3.09	56.0 ± 5.3
		2.87	
		2.54	
	5g/62,5 mL	3.12	60.4 ± 3.4
		2.88	
		3.14	
Solvents - 90º C - 63º C***** - 45 min - 5g/250mL	Water	3.93	78.4 ± 1.0
		3.96	
		3.98	
	Ethanol 50%*****	3.36	61.6 ± 7.6
		2.66	
		3.28	

* for the extraction with Ethanol 50%, the extraction temperature was 63ºC.

There was no opportunity to test different extraction conditions in order to obtain the *Arbutus unedo* L. leaves aqueous extract. Consequently, the extract was obtained by

following the procedure developed by Malheiro *et al*. [8]. The extractions yields obtained by these conditions are shown in Table 8.

Table 8: Yields and extraction yield (Mean ± SD) of *Arbutus unedo* L. leaf aqueous extracts.

Extracting Conditions	***Yield (g)***	***Extraction yield (% w/w)***
100ºC, 45min, 5g of leaves for 250mL of water	1.602 1.613 1.623	31.80 ± 0.23

2. Total Phenolic Determination

The total amount of phenols was also measured according to the Folin-Ciocalteu method [81], expressed in GAE. Same procedure was performed in leaves aqueous extract and in the fruits aqueous extracts obtain by different extraction conditions.

After, the graphic of the A_{760} against the concentration of gallic acid was plotted and the calibration curve was obtained. Table 16 of Appendix 1 presents the A_{760} obtained for different concentrations of gallic acid (in triplicate). This calibration curve is illustrated in figure 6; analysing this figure, we can see that the calibration curve presents a correlation of 0.9902 and linearity in the concentration ranges of gallic acid.

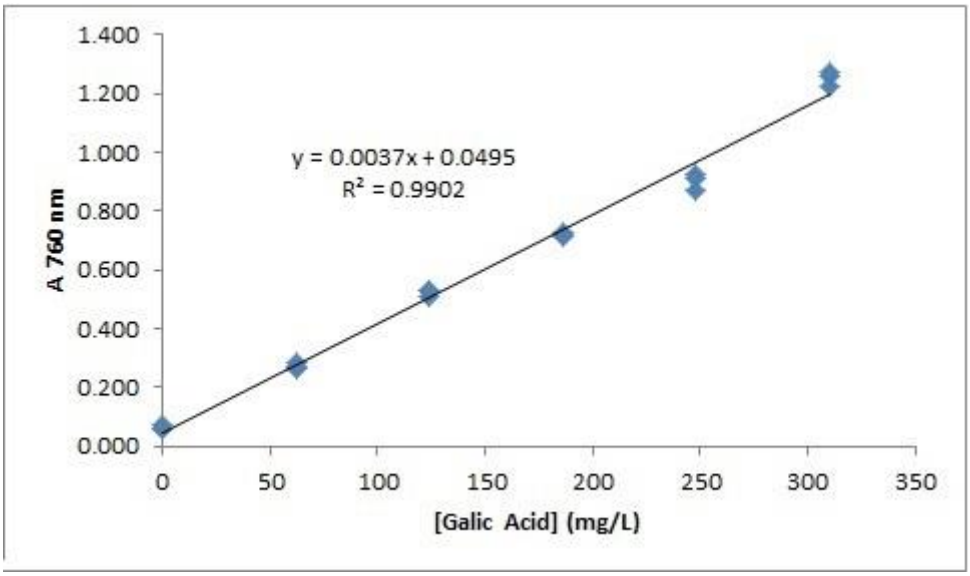

Figure 6: Calibration curve of gallic acid

The results obtain for each extract are shown in table 9.

Table 9: Total phenolic content of *Arbutus unedo* L. fruits and leaves aqueous extracts (Mean ± SD).

	Assay	***Extraction conditions***	***Total phenols (mg GAE/g of extract) ****
Fruit	1	100ºC 45 min 5g/250mL water	2.41 ± 0.03
	2	90ºC 45 min 5g/250mL water	2.52 ± 0.07
	3	80ºC 45 min 5g/250mL water	2.12 ± 0.06
	4	90ºC 60min 5g/250mL water	1.94 ± 0.10
	5	90ºC 120min 5g/250mL water	2.12 ± 0.17
	6	90ºC 45 min 5g/125mL water	2.35 ± 0.17
	7	90ºC 45 min 5g/62.5mL water	1.74 ± 0.04
	8	63ºC 45 min 5g/250mL Ethanol 50%	2.80 ± 0.25
Leaf	9	100ºC 45 min 5g/250mL water	24.45 ± 1.31

*n=3 replicates from each extract

According to table 10, the best extraction conditions to obtain the highest TPC were: **5 grams of fruit powder in 250 mL of Ethanol (50% v/v) at 63ºC during 45 minutes;** and **5 grams of fruit powder in 250 mL of water at 90ºC during 45 minutes.**

3. Evaluation of the impact of *Arbutus unedo* L. extracts intake in a rat model of type 2 diabetes

The effect of feeding rats with the high-fat diets, followed by a STZ injection and treatment with high-fat diet containing *Arbutus unedo* L. leaf aqueous extract (AU_L) and fruit aqueous extract (AU_F) in overall growth and organ indices in rats is given in Table 10. It can be seen that there is no significant difference in food intake in the four experimental groups. There was no influence of the tested extracts in this parameter (graphic in Appendix 2).

Table 10: Effect of high-fat diets/STZ injection and *Arbutus unedo* L. leaf and fruit aqueous extract in overall growth and organ indices in rats (mean ± SD)

Index	**Experimental group**			
	No-Diabetes/Control	Diabetes/No-treatment	AU_L	AU_F
Food intake (g day $^{-1}$)	20.09 ± 1.46	20.89 ± 1.76	20.27 ± 2.57	20.36 ± 3.40
Body mass gain (g)	39.4 ± 4.39^{b}	-0.5 ± 15.68^{a}	7.83 ± 19.99^{a}	5.17 ± 28.24^{a}
Relative body mass gain (%)	9.78 ± 1.11^{b}	-0.28 ± 3.75^{a}	$1.89 \pm 4.93^{a,b}$	$1.68 \pm 8.04^{a,b}$
Body mass/ body length g cm $^{-1}$)	16.7 ± 1.3^{b}	$14.7 \pm 1.3^{a,b}$	13.7 ± 1.3^{a}	$14.6 \pm 2.1^{a,b}$
Liver (% b.m.)	3.04 ± 0.24	3.38 ± 0.32	3.24 ± 0.10	3.64 ± 0.54
Kidneys (% b.m.)	0.60 ± 0.04^{a}	0.85 ± 0.11^{b}	0.80 ± 0.11^{b}	0.829 ± 0.202^{b}
Spleen (% b.m.)	0.15 ± 0.03	0.15 ± 0.02	0.14 ± 0.01	0.16 ± 0.03
Heart (% b.m.)	0.28 ± 0.02	0.29 ± 0.03	0.29 ± 0.03	0.30 ± 0.06
Testes (% b.m.)	0.93 ± 0.04	0.97 ± 0.07	0.98 ± 0.08	0.77 ± 0.42
Pancreas (% b.m.)	0.24 ± 0.04	0.26 ± 0.02	0.25 ± 0.02	0.25 ± 0.07
Left Femur bone (% b.m.)	0.21 ± 0.02	0.24 ± 0.02	0.25 ± 0.03	0.24 ± 0.05
Right Femur bone (% b.m.)	0.22 ± 0.01	0.25 ± 0.01	0.25 ± 0.02	0.24 ± 0.05

Abbreviations: b.m. – body mass, AU_L – high-fat diet with *Arbutus unedo* L. aqueous Leaf Extract (0.5 g/kg)/STZ injected, AU_F – high-fat diet with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected. Means on same line without common superscript differ significantly ($p < 0.05$).

When comparing the experimental groups in terms of body mass gain, it can be seen that the high-fat feeding along with the STZ injection resulted in a significant ($p < 0.05$) decrease on the body mass gain and relative body mass gain (expressed as % of the initial body mass) when compared with the control (Figure 7 and 8). This effect is mostly pronounced in the high-fat diabetic control group. The groups that were fed with *A. unedo* aqueous extracts in the high-fat diet had an increased body mass gain when compared with the diabetic group although this was not considered statistically significant ($p > 0.05$).

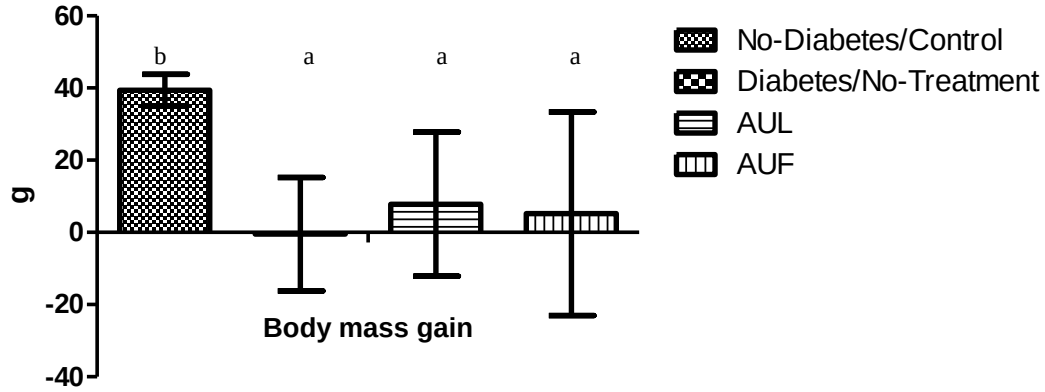

Figure 7: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **body mass gain (g)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

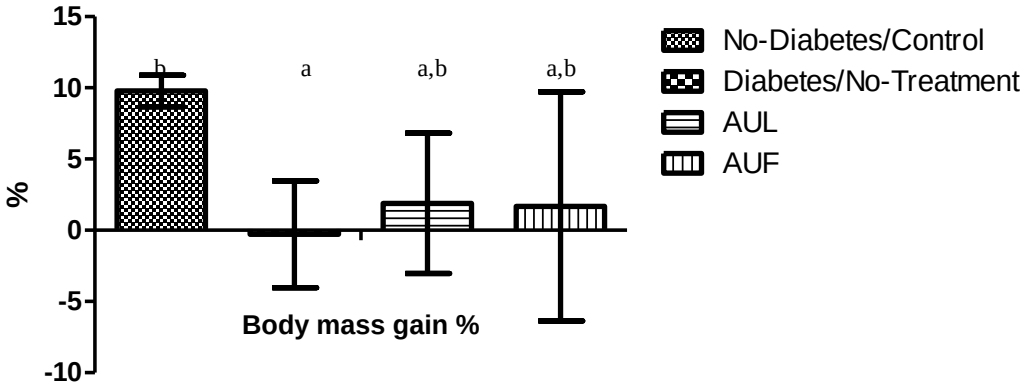

Figure 8: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **relative body mass gain (%)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Other growth indices were also determined. The high-fat feeding combined with the STZ injection appeared to slightly affect the body mass/ body length ration of the animals which is consistent with the decreased body mass gain presented during the experiment (Figure 9). This effect was greater in the AU_L group as this group had a slightly higher body mass gain. The high-fat diets also had a significant effect ($p < 0.05$) on the rat's relative kidney mass (expressed as % of body weight) as the diabetic control group along with the *A. unedo* supplemented groups had an increased kidney mass (Figure 10). Other tissues as liver, spleen, heart, testes, pancreas and femur bones were not affected by the high-fat diets and STZ injection in terms of relative mass (graphics in Appendix 2).

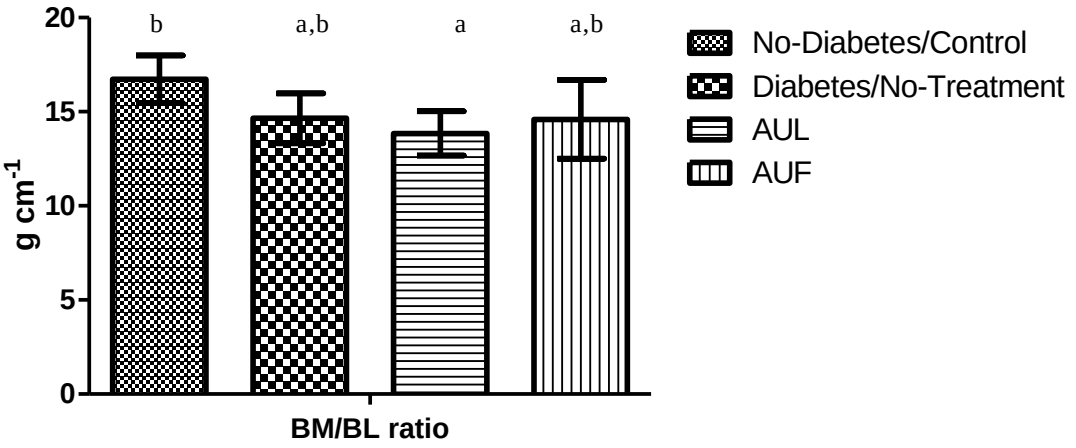

Figure 9: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **body mass/ body length ratio (g cm $^{-1}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

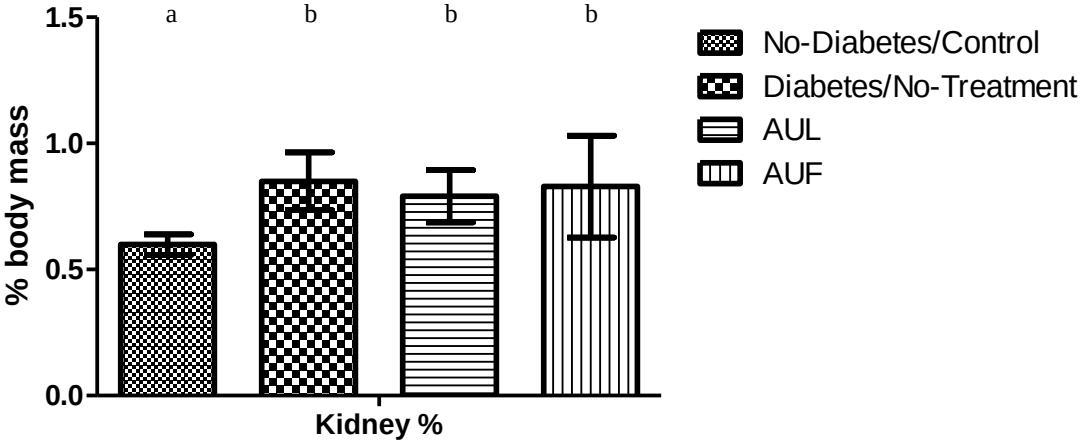

Figure 10: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **kidney weight (% of rat's body mass)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

The effect of high-fat diets, STZ injection and *Arbutus unedo* L. leaf and fruit aqueous extract in blood biochemistry parameters of rat's serum was also one of the aims of this work. Table 11 summarizes how these conditions affected serum carbohydrates levels, serum lipid indices and toxicity biomarkers. Rats which were injected with STZ and fed with a high-fat diet presented a significant ($p < 0.05$) elevated blood glucose concentration, serum urea concentration and serum creatinine concentration (Figure 11, 12

and 13). In the *A. unedo* supplemented groups, the creatinine concentration was not as high as in the diabetic not treated group. Total cholesterol, triacylglycerols levels, HDL cholesterol and LDL cholesterol levels were not significantly affected (Appendix 2). The increased glucose concentration is a metabolic change directly related with diabetes mellitus. These diabetic not-treated groups of rats also had an increased insulin resistance (HOMA-IR) index ($p < 0.05$). The diabetic rats also presented higher insulin levels but this change was not as high as initially expected and these levels are similar to the ones of the control group.

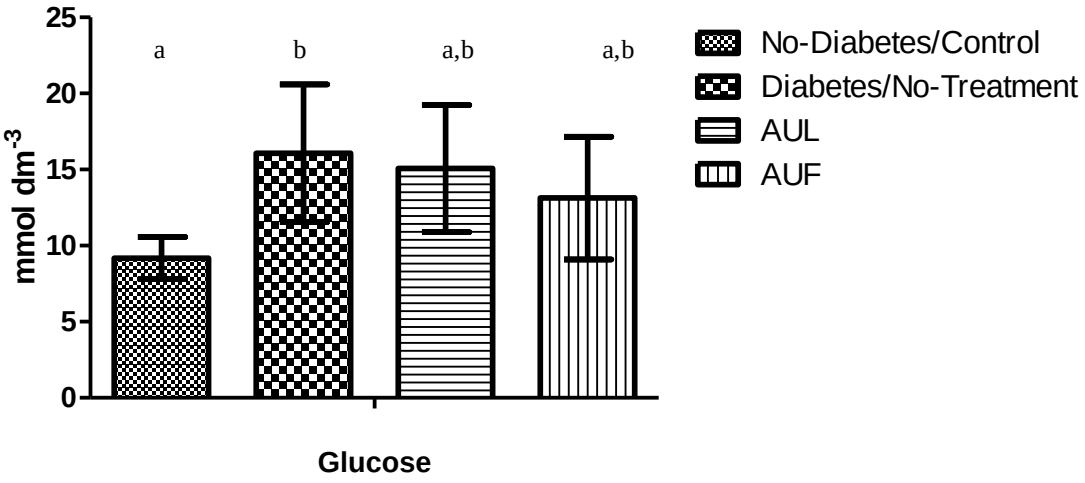

Figure 11: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum glucose levels (mmol dm $^{-3}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Table 11: Effect of high-fat diets, STZ injection and *Arbutus unedo* L. leaf and fruit aqueous extracts in blood biochemistry parameters of rats' serum (mean ± SD).

Blood Parameter	**Experimental group**			
	No-Diabetes/Control	Diabetes/No-treatment	AU_L	AU_F
Glucose concentration (mmol dm $^{-3}$)	9.18 ± 1.38^{a}	16.08 ± 4.52^{b}	15.07 ± 4.18^{a,b}	13.13 ± 4.04^{a,b}
Total cholesterol concentration (mg dL $^{-1}$)	99.34 ± 15.01	83.34 ± 17.04	87.68 ± 12.44	97.98 ± 16.14
HDL cholesterol concentration (mg dL $^{-1}$)	72.80 ± 3.19	64.69 ± 13.32	79.07 ± 19.46	75.92 ± 11.41
LDL cholesterol concentration (mg dL $^{-1}$)	12.43 ± 3.03	8.66 ± 3.79	8.85 ± 5.74	8.42 ± 3.73
Triacylglycerols concentration (mg dL $^{-1}$)	74.63 ± 38.12	91.51 ± 60.11	57.34 ± 25.80	66.98 ± 20.65
HOMA-IR index	13.38 ± 3.09^{a}	37.71 ± 5.76^{c}	16.87 ± 2.66^{a,b}	22.75 ± 6.271^{b}
Insulin (mU/mL)	33.93 ± 9.36^{a}	42.53 ± 18.87^{a,b}	27.59 ± 6.96^{a}	63.93 ± 13.80^{b}
Urea (mg dL $^{-1}$)	29.28 ± 2.67^{a}	65.95 ± 15.70^{b}	66.23 ± 21.69^{b}	48.53 ± 12.96^{b}
Creatinine (mg dL $^{-1}$)	0.39 ± 0.06^{a}	0.55 ± 0.09^{b}	0.54 ± 0.12^{a,b}	0.43 ± 0.08^{a,b}
ALT (U L $^{-1}$)	26.00 ± 10.32^{a}	53.00 ± 26.38^{a,b}	74.50 ± 32.87^{b}	48.20 ± 14.79^{a,b}
AST (U L $^{-1}$)	78.00 ± 32.60	97.43 ± 18.21	112.20 ± 34.37	132.67 ± 91.81
ALP (U L $^{-1}$)	60.20 ± 8.73^{a}	153.14 ± 45.32^{b}	124.60 ± 52.31^{a,b}	127.80 ± 9.52^{b}
CRP (mg L $^{-1}$)	0.11 ± 0.04	0.11 ± 0.03	0.084 ± 0.03	0.132 ± 0.026
Total Fe concentration (µg dL $^{-1}$)	116.76 ± 14.75	143.11 ± 21.25	136.77 ± 15.50	128.80 ± 8.64
Total protein concentration (g dL $^{-1}$)	6.86 ± 0.40	6.36 ± 0.52	6.28 ± 0.42	6.55 ± 0.47

Abbreviations: ALT - Alanine Aminotransferase, AST - Aspartate Aminotransferase, ALP - Alkaline phosphatase, CRP - C-reactive protein, AU_L – high-fat with *Arbutus unedo* L. aqueous Leaf Extract (0.5 g/kg)/STZ injected, AU_F – high-fat with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected. Means on same line without common superscript differ significantly ($p < 0.05$).

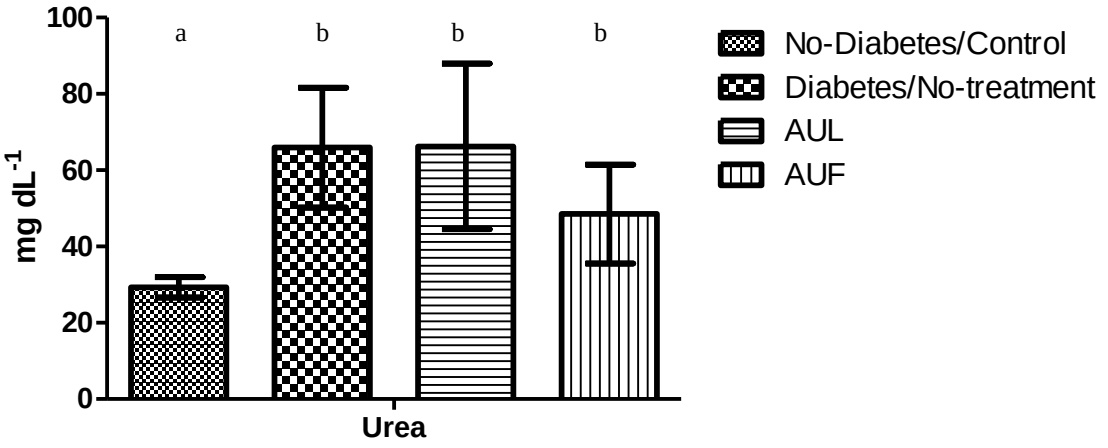

Figure 12: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum urea levels (mg dL $^{-1}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

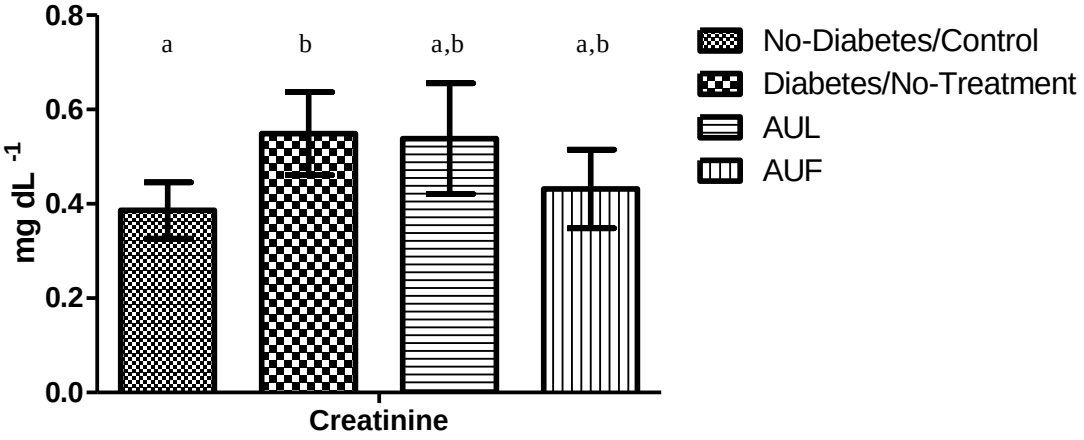

Figure 13: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum creatinine levels (mg dL $^{-1}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Other toxicity biomarkers were also analysed in the experimental groups' sera. STZ-injected rats feed with the high-fat diets presented a significant ($p < 0.05$) increased phosphatase concentration (Figure 14). The enzyme ALT as slightly increased in the

diabetic not treated group and was presented in higher concentrations in the serum of *A. unedo* supplemented groups, especially the leaf aqueous extract group (Figure 15). Other markers as creatinine and serum urea concentration were also increased in these groups ($p < 0.05$). The administration of high-fat diets and STZ injection had no consequences in the AST, C-reactive protein, serum Fe levels and blood protein concentration levels (Appendix 2).

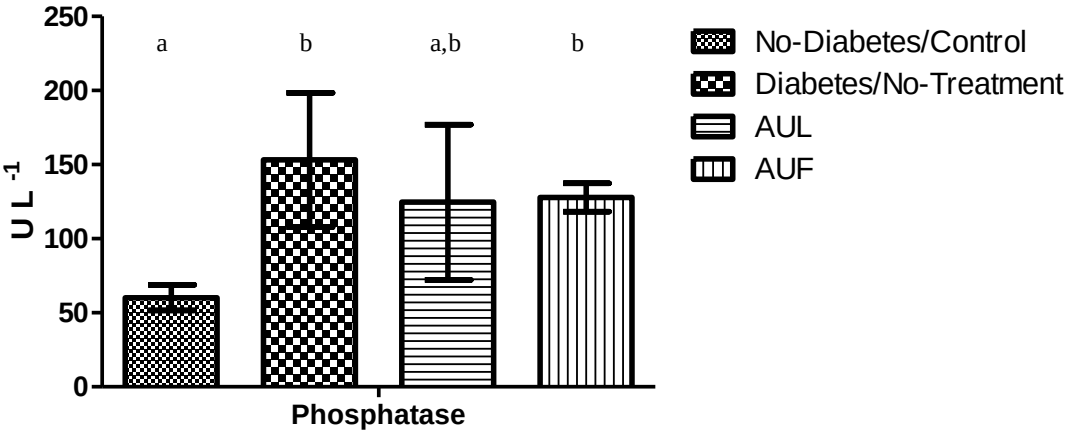

Figure 14: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum Phosphatase levels (U L $^{-1}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

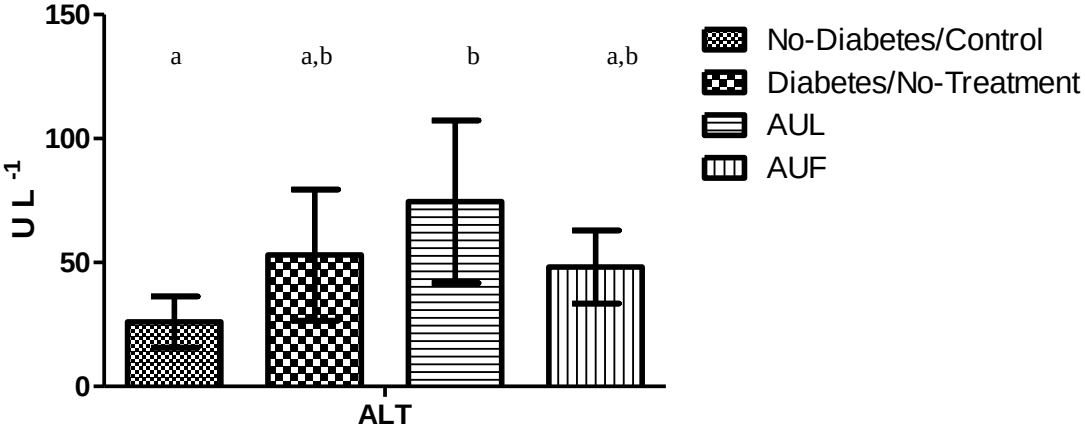

Figure 15: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum ALT levels (U L $^{-1}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Adding *Arbutus unedo* L. fruit and leaf aqueous extract (in the doses of 0.5 g/kg b.w and 1.25 g/ kg b.w. respectly) to the high-fat diets had some effects in blood biochemistry and toxicity biomarkers. Two groups of rats were fed with the high-fat diet supplemented with these extracts and some metabolic changes were detected. When compared to the diabetic control group, the *Arbutus unedo* L. fruit and leaf aqueous extracts supplemented groups presented a tendency towards reducing blood glucose concentration to the levels presented by the non-diabetic control group. Nevertheless the insulin levels were increased in the *Arbutus unedo* L. fruit aqueous extract supplemented group (Figure 16), being normalized in the leaf extract supplemented group ($p < 0.05$). Both *Arbutus unedo* L. aqueous extract supplemented groups had an improved HOMA-IR index, as the insulin resistance decreased in these groups with special emphasis in leaf aqueous extract group which HOMA-IR index is almost the same as the control group (Figure 17).

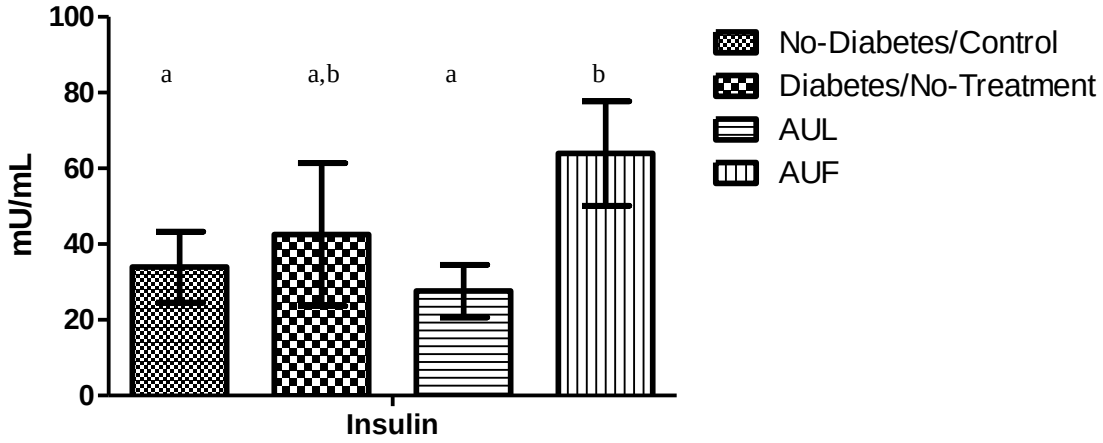

Figure 16: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **insulin levels (mU mL $^{-1}$)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

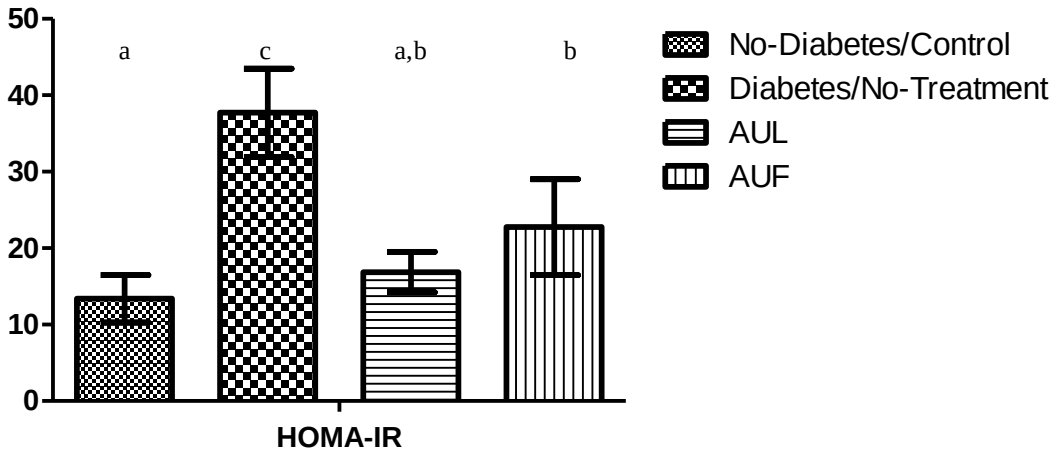

Figure 17: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **HOMA-IR index**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Urea levels (Figure 12) were higher in all the groups injected with STZ, the supplementation with the *A. unedo* aqueous extracts showed no effect ($p < 0.05$). Creatinine levels were also higher in the STZ injected groups when compared with the control; however in the *Arbutus unedo* L. fruit and leaf aqueous extract supplemented groups there was a tendency towards decreasing creatinine levels to controllevels. The inflammation marker CRP remained unchanged in all groups (data on Appendix 2). Surprisingly ALT serum concentration was higher in the group fed with the *Arbutus unedo* L. aqueous extract, an effect considerably higher in the leaf extract supplemented group ($p < 0.05$). Phosphatase levels were much higher in diabetic group than in control group. This effect was not improved in the group supplemented with *Arbutus unedo* L. fruit aqueous extracts as the serum levels of phosphatase remained high. This time the most beneficial effect was presented by the leaf extract group were phosphatase levels slightly decreased (to levels presented by the control group).

The haematogram of the animals was also analysed mainly to reinsure that the animals remained healthy during all the experiment. The results can be seen in table 12.

There were no significant changes in almost all of the tested blood morphology indexes (Appendix 2). On the STZ- injected/high-fat diets fed groups the platelets levels (PLT) were lower (Figure 18) and red cell distribution width (RDW) has higher (Figure 19 and Figure 59 of Appendix 2). *A. unedo* extracts appear to have attenuated these effects as

there was a tendency to normalize this parameter's levels to levels presented by the control group. Procalcitonin (PCT) is a peptide which is produced as a response to an inflammatory stimulus, namely bacterial infections [94]. On all diabetic groups (except AU_F) this value was reduced when compared to the control (Figure 20).

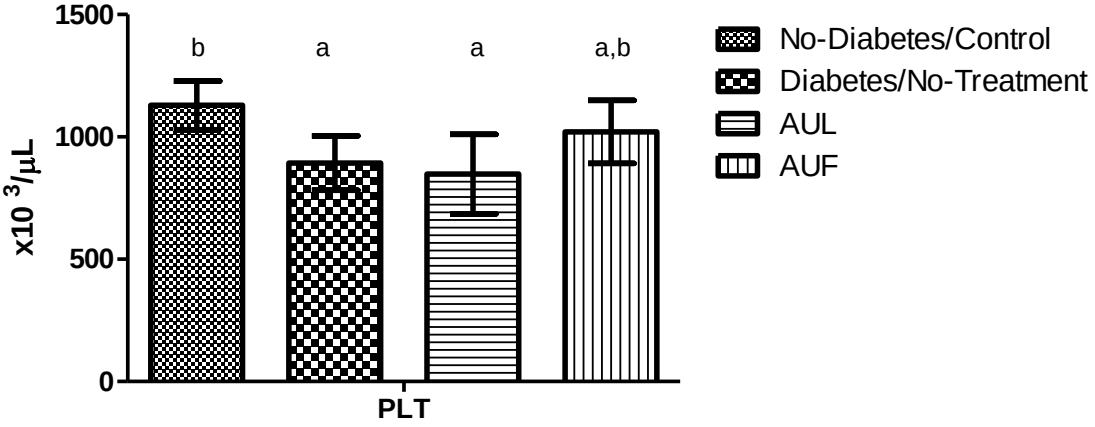

Figure 18: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **platelets levels**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

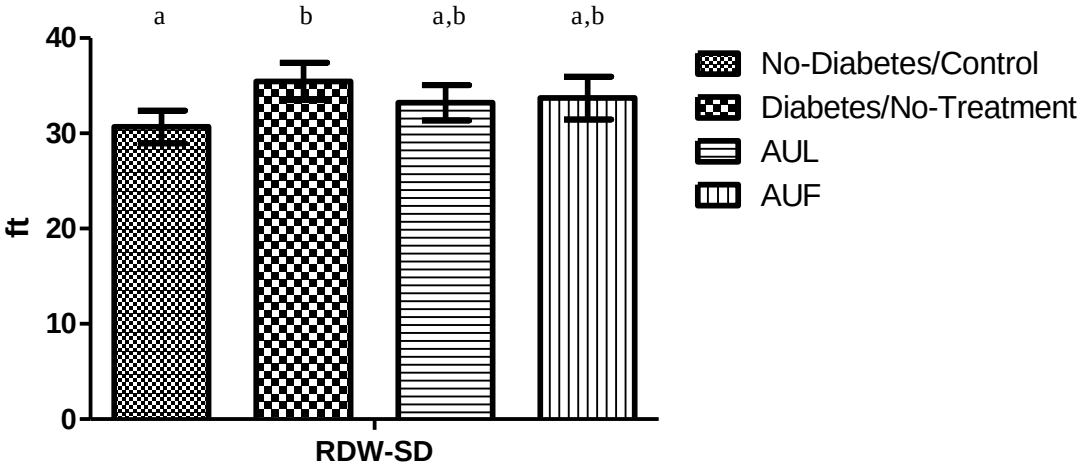

Figure 19: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **red blood cell distribution width (with standard derivation)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Table 12: Effect of high-fat diets/STZ injection and *Arbutus unedo* L. leaf and fruit aqueous extract on blood morphology and hematology in rats (mean ± SD).

Blood Index	**Experimental group**			
	No-Diabetes/Control	Diabetes/No-treatment	AU_L	AU_F
WBC (x10 ^{3}/µL)	4.51 ± 1.04	2.80 ± 0.98	3.67 ± 1.05	4.60 ± 1.96
RBC (x10 ^{6}/µL)	9.42 ± 0.17	9.77 ± 0.46	9.84 ± 0.46	9.77 ± 0.32
HGB (g/dL)	15.24 ± 0.38	15.95 ± 0.56	15.97 ± 0.99	15.83 ± 0.56
HCT (%)	44.64 ± 1.58	46.14 ± 2.28	45.63 ± 2.67	45.63 ± 2.48
MCV (fl)	47.39 ± 1.32	47.30 ± 2.77	46.38 ± 0.96	46.73 ± 3.11
MCH (pg)	16.18 ± 0.23	16.40 ± 0.87	16.22 ± 0.40	16.22 ± 0.46
MCHC (g/dL)	34.14 ± 0.55	34.71 ± 1.04	34.97 ± 0.70	34.73 ± 1.50
PLT (x10 ^{3}/µL)	1128.80 ± 99.15^b	893.14 ± 111.67^a	848.00 ± 162.17^a	1020.80 ± 128.32^{a,b}
RDW-SD (fl)	30.68 ± 1.72^a	35.45 ± 1.96^b	33.20 ± 1.84^{a,b}	33.70 ± 2.24^{a,b}
RDW-CV (%)	21.74 ± 0.95^a	24.24 ± 1.20^b	23.55 ± 0.98^b	23.73 ± 0.89^b
PDW (fl)	9.10 ± 0.44	9.54 ± 0.24	9.57 ± 0.24	9.83 ± 0.83
MPV (fl)	7.92 ± 0.2	8.11 ± 0.29	7.98 ± 0.16	8.22 ± 0.39
P-LCR (%)	10.70 ± 1.60	11.70 ± 1.60	11.17 ± 0.94	11.86 ± 1.92
PCT (%)	0.89 ± 0.08^b	0.75 ± 0.06^{a,b}	0.59 ± 0.09^a	0.83 ± 0.09^b
NEUT(x10 ^{3}/µL)	0.35 ± 0.10	0.25 ± 0.09	0.29 ± 0.08	0.31 ± 0.03
LYMPH (x10 ^{3}/µL)	3.72 ± 1.01^b	1.92 ± 0.53^a	2.75 ± 0.59^{a,b}	2.49 ± 0.93^{a,b}
MONO(x10 ^{3}/µL)	0.50 ± 0.11^b	0.19 ± 0.08^a	0.21 ± 0.07^a	0.22 ± 0.10^a
EO (x10 ^{3}/µL)	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
BASO (x10 ^{3}/µL)	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
NEUT (%)	6.35 ± 4.17	11.15 ± 4.14	9.06 ± 3.13	11.38 ± 4.36
LYMPH (%)	77.62 ± 11.57	79.97 ± 6.29	82.78 ± 4.80	81.68 ± 4.22
MONO (%)	9.98 ± 3.06	6.44 ± 2.21	6.60 ± 2.27	6.10 ± 1.08
EO (%)	0.94 ± 0.34	1.64 ± 0.78	1.42 ± 0.57	0.85 ± 0.21
BASO (%)	0.92 ± 1.79	0.70 ± 1.10	0.14 ± 0.31	0.00 ± 0.00

Abbreviations: AU_L – high-fat with *Arbutus unedo* L. aqueous Leaf Extract (0.5 g/kg)/STZ injected, AU_F – high-fat with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected. WBC - White Blood Cell Count, RBC - Red Blood Cell Count, HGB – haemoglobin, HCT - Hematocrit , MCV - Mean Corpuscular Volume, MCH - Mean Corpuscular Haemoglobin , MCHC - Mean Corpuscular Haemoglobin Concentration , PLT - Platelets, RDW-SD - Red Cell Distribution Width Based on Standard Deviation, RDW-CV - Red Cell Distribution Width Based on Coefficient of Variation, PDW - Platelet Distribution Width, MPV - Mean Platelet Volume , P-LCR - Platelet Large-Cell Ratio, PCT – Procalcitonin, NEUT – Neutrophils, LYMPH – Lymphocytes, MONO – Monocytes, EO – Eosinophils and BASO – Basophils. Means on same line without common superscript differ significantly ($p < 0.05$).

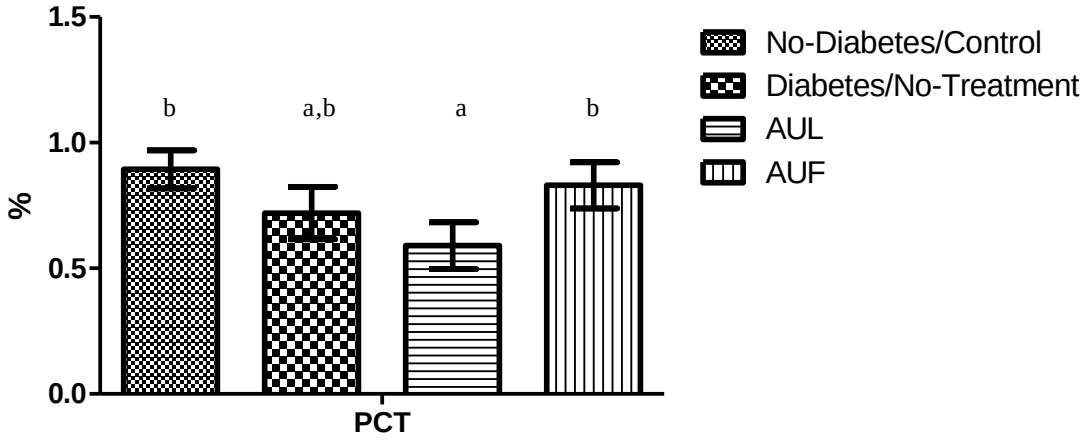

Figure 20: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **procalcitonin percentage**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

The high-fat feeding and STZ injection also appeared to decrease the amount of lymphocytes and monocytes, as all the diabetic groups had decreased levels of this white globes (Figure 21 and 22). In the case of lymphocytes, once more the treatment with the *A. unedo* plant materials appears to slightly increase the lymphocyte levels (to levels showed by the control group).

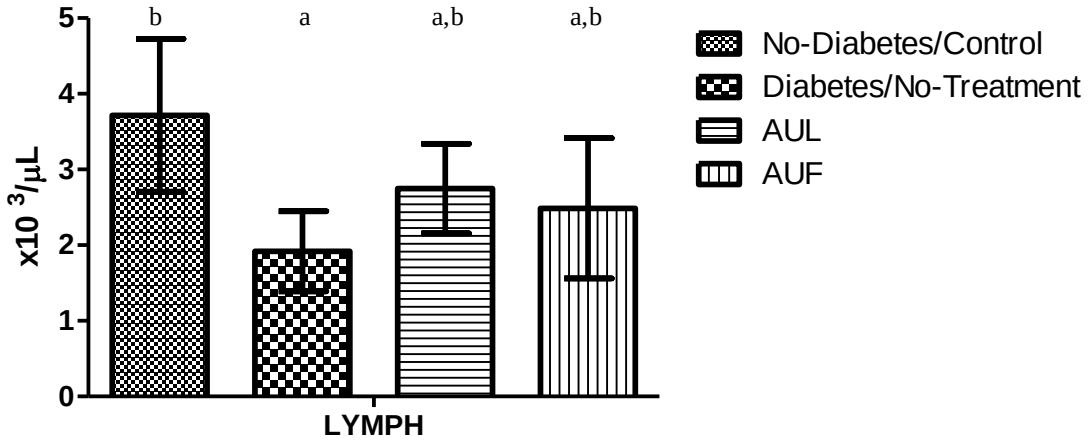

Figure 21: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **lymphocytes levels**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

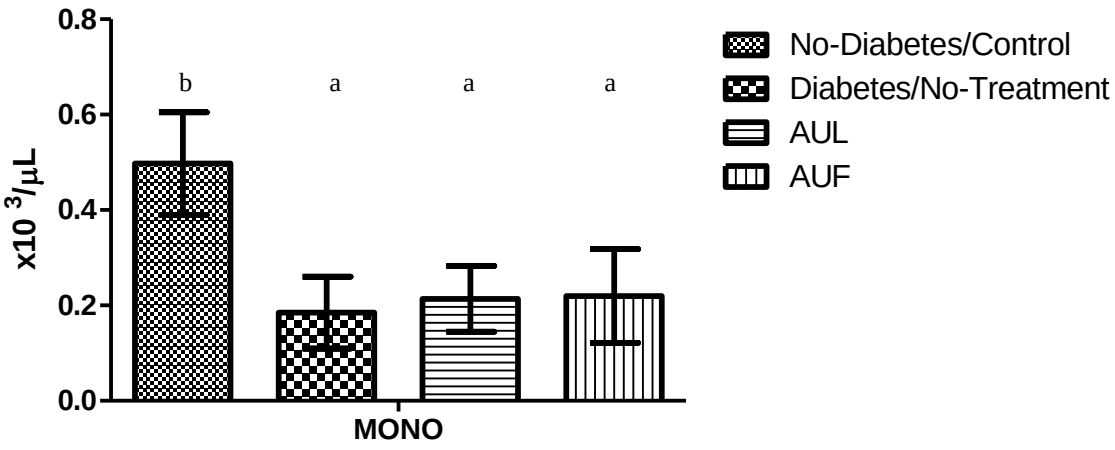

Figure 22: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **monocytes levels**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

The tissular copper, zinc and iron levels in the rats' organs was also measured in order to clarify in STZ injection, high-fat feeding and *Arbutus unedo* L. extracts had some effects on microelement homeostasis. These results are presented in table 13. In this experiment, there was an elevation of the kidney copper levels, kidney zinc levels and kidney iron levels in all diabetic groups treated and not-treated. The *Arbutus unedo* L. aqueous extracts, HF feeding and STZ injection had no impact in the microelements' hepatic levels.

In renal tissue, there was an elevation of Cu levels in the diabetic not-treated group (Figure 23). This effect was not attenuated by the supplementation with *A. unedo* leaf or fruit aqueous extract.

Table 13: Effect of high-fat diet/STZ injection and *Arbutus unedo* L. aqueous leaf and fruit extract on the tissular Cu, Zn and Fe levels in rats organs (mean ± SD).

Index	**Experimental group**			
	No-Diabetes/Control	Diabetes/No-treatment	AU_L	AU_F
Cu (μ g^{-1} dry mass)				
Liver	20.08 ± 1.11	18.30 ± 2.53	17.9 ± 12.29	17.89 ± 3.30
Kidney	26.87 ± 5.60 [a]	54.29± 15.82 [b]	67.86 ± 19.39 [b]	55.42 ± 16.85 [b]
Zn (μ g^{-1} dry mass)				
Liver	144.69 ± 16.78	141.48 ± 9.47	149.31 ± 48.08	159.25 ± 18.10
Kidney	94.63 ± 4.06 [a]	117.0 ± 10.8 [b]	122.47 ± 16.53 [b]	112.82 ± 16.11[a,b]
Fe (μ g^{-1} dry mass)				
Liver	417.98 ± 54.08	487.06 ± 108.45	424.72 ± 93.28	473.96 ± 101.14
Kidney	287.7 ± 10.72[a]	334.0 ± 23.89[a,b]	382.0 ± 50.76[b]	360.00 ± 62.39[a,b]

Abbreviations: AU_L – high-fat with *Arbutus unedo* L. aqueous Leaf Extract (0.5 g/kg)/STZ injected, AU_F – high-fat with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected, Cu – Copper, Zn – Zinc, Fe – Iron. Means on same line without common superscript differ significantly ($p < 0.05$).

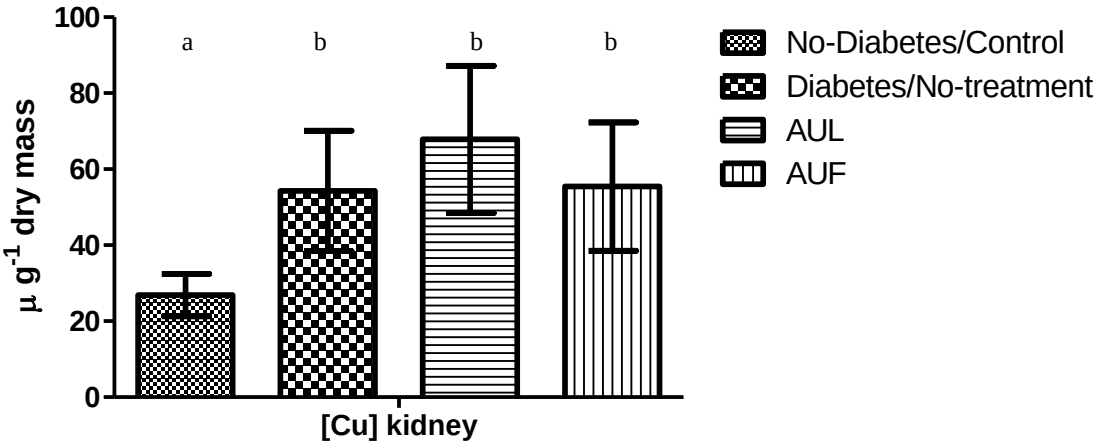

Figure 23: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **copper concentration in kidney tissue (μg $^{-1}$ dry mass)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

The kidney zinc concentrations were affected by high-fat feeding and STZ injection (Figure 24). However in this case, no beneficial effects can be attributed to *Arbutus unedo* L. leaf aqueous extract, as no significant change between the diabetic

control groups and the AU_L treated group was noticed ($p > 0.05$). However, in the case of the AU_F group, a slight decrease in zinc renal levels towards concentrations presented by the control group was noticed.

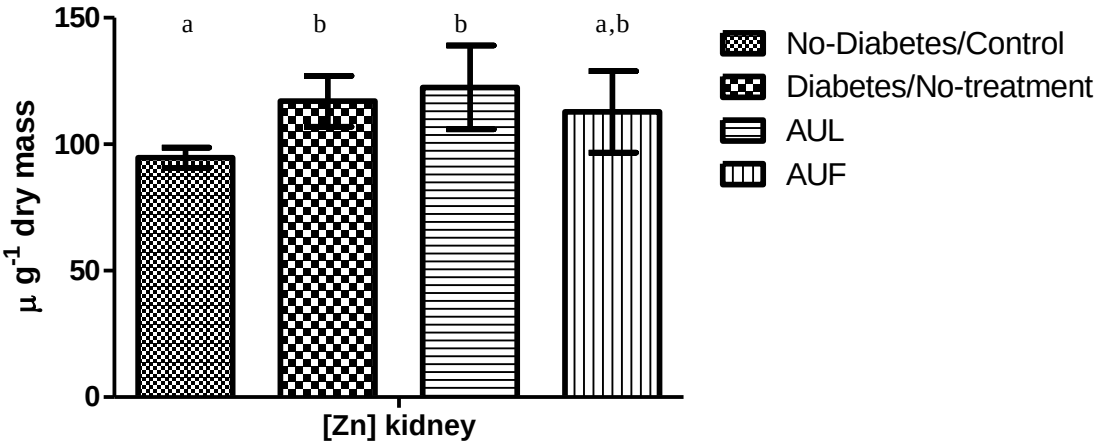

Figure 24: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **zinc concentration in kidney tissue (µg $^{-1}$ dry mass)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

The combination of STZ injection and high-fat feeding appeared to have slightly increased the renal iron levels in all the diabetic groups (Figure 25). This increase was even greater in the group supplemented with *Arbutus unedo* L. leaf aqueous extract ($p < 0.05$).

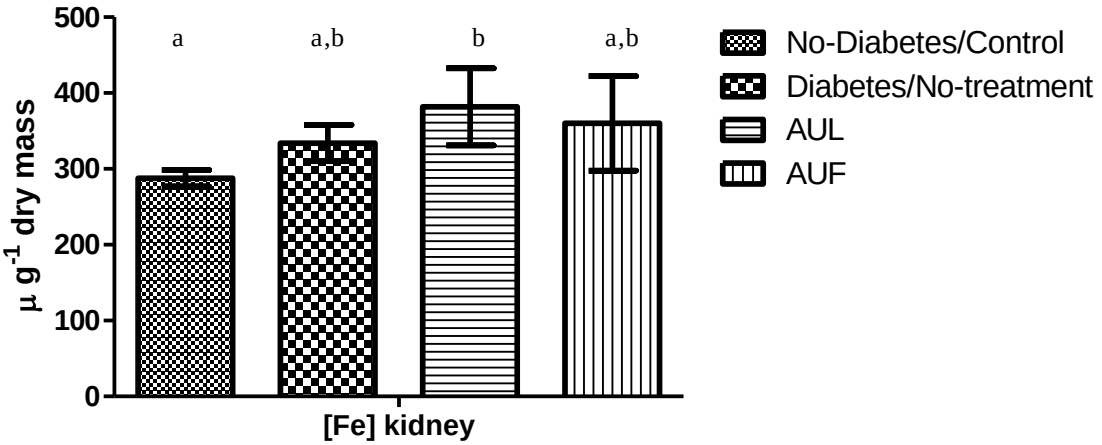

Figure 25: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **iron concentration in kidney tissue (µg $^{-1}$ dry mass)**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Interestingly, no significant changes in copper, zinc or iron homeostasis in hepatic tissues was noticed in all experimental groups (data shown in Appendix 2).

Lastly, the effect of the aqueous extracts from *Arbutus unedo* L. fruits and leaves on the expression of certain genes was also studied. These genes play a role in the glucose metabolism and/or in inflammation response, the results are shown in Table 14. It was analysed the expression of these genes on hepatic, muscular and fat tissues.

TNF-α or tumour necrosis factor is a cytokine produced by macrophages in response to an inflammatory process and is present mainly in the acute phase reaction [95]. When comparing the diabetic control group with the control group an increased expression of this gene in the hepatic and muscular tissues can be noticed; in fat tissue no significant changes occurred ($p < 0.05$). High-fat feeding combined with STZ injection increased then TNF-α gene expression.

In liver tissue, *A. unedo* aqueous extracts showed to have an effect on the expression of the gene that encodes this protein (Figure 26). In both leaf extract and fruit extract supplemented groups there was a significant decreased expression of this gene ($p < 0.05$). On muscular tissue there was also a significant decreased expression of TNF-α gene on the group fed with *Arbutus unedo* L. fruit extract; in the case of leaf extract the effect was not as strong (Figure 27).

Table 14: Effect of high-fat diet/STZ injection and *Arbutus unedo* L. aqueous leaf and fruit extract on the expression of different genes (mean ± SD).

Gene Expression (ΔΔCT)	**Experimental group**			
	No-Diabetes/Control	Diabetes/No-treatment	AU_L	AU_F
TNF-α				
Liver	1.0 ± 0.27[a]	34.94 ± 43.41[b]	3.41 ± 3.48[a]	3.93 ± 3.39[a]
Muscle	1.71 ± 0.99[a,b]	16.42 ± 8.230[b]	11.76 ± 9.24[a,b]	0.61 ± 0.43[a]
Fat tissue	1.63 ± 1.13	1.65 ± 0.58	1.40 ± 0.25	0.98 ± 0.26
IRS-2				
Liver	1.09 ± 0.28	1.28 ± 0.17	1.24 ± 0.16	1.28 ± 0.04
Muscle	1.51 ± 0.45	1.68 ± 0.48	1.56 ± 0.16	1.88 ± 0.22
Fat tissue	0.74 ± 0.20[a]	1.14 ± 0.35[b]	1.28 ± 0.42[a,b]	1.22 ± 0.61[a,b]
IL-6				
Liver	0.65 ± 0.13	1.27 ± 0.28	1.137 ± 0.38	1.01 ± 0.55
GLUT-4				
Liver	0.66 ± 0.25	1.64 ± 1.33	0.79 ± 0.15	0.96 ± 0.31
Muscle	0.98 ± 0.03[a]	3.04 ± 1.50[b]	1.68 ± 1.07[a,b]	3.61 ± 3.35[a,b]
Fat tissue	6.47 ± 1.68[b]	2.67 ± 1.97[a]	1.38 ± 0.20[a]	1.14 ± 0.70[a]
GLUT-2				
Liver	0.52 ± 0.45	1.50 ± 1.69	1.26 ± 0.57	0.74 ± 0.52
Muscle	82.56 ± 100.03[a]	17.96 ± 14.09[a]	134.78 ± 92.88[a,b]	282.47 ± 82.89[b]

Abbreviations: TNF-α – Tumour Necrosis Factor α, IRS-2 - Insulin Receptor Substrate 2, IL-6 – Interleukin 6, GLUT-4 – Glucose Transporter 4, GLUT-2 – Glucose Transporter 2, AU_L– high-fat with *Arbutus unedo L*. aqueous Leaf Extract (0.5 g/kg)/STZ injected, AU_F– high-fat with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected. Means on same line without common superscript differ significantly ($p < 0.05$).

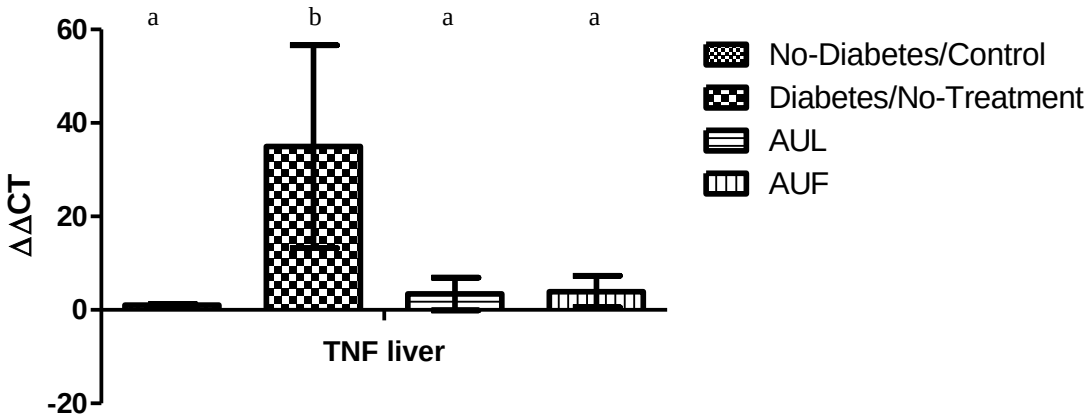

Figure 26: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of TNF alpha gene in hepatic tissue**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

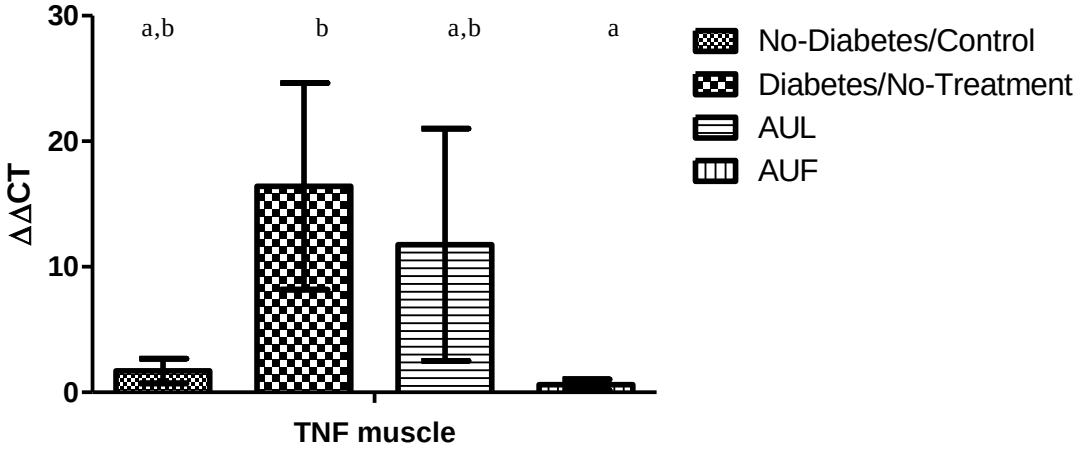

Figure 27: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of TNF alpha gene in muscular tissue**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

The IRS-2 gene encodes a protein named insulin receptor substrate 2. There was no significant change in the expression of this gene in hepatic and muscular tissues in all experimental groups (Appendix 2). In fat tissue the high-fat diabetic groups showed an increased expression of IRS gene in relation to control group however there was a slight difference between the untreated diabetic group and the *A. unedo* treated diabetic groups (Figure 28).

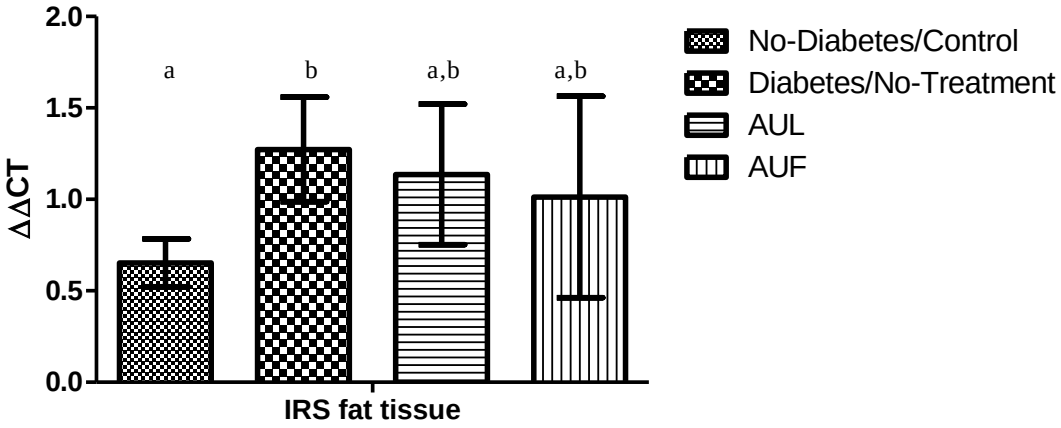

Figure 28: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of IRS gene in fat tissue**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Interleukin 6 (IL-6) is an interleukin that acts as a pro-inflammatory cytokine as well as an anti-inflammatory myokine and is encoded by the IL6 gene [95]. In the rats´ muscular tissue there was no expression of this gene (data not showed) and in the fat tissue the data collected was not enough to have significant relevance. In hepatic tissue, when comparing control, diabetic and AU_L groups, no difference in the expression of IL-6 can be noticed. On the diabetic group treated with *A. unedo* fruits aqueous extract, however, there is an increase in the expression of Interleukin 6 (Figure 29). Within this group of rats there were many differences in the expression of this gene, reason why the SD is so high. For this reason this increased expression is not statistically significant ($p > 0.05$).

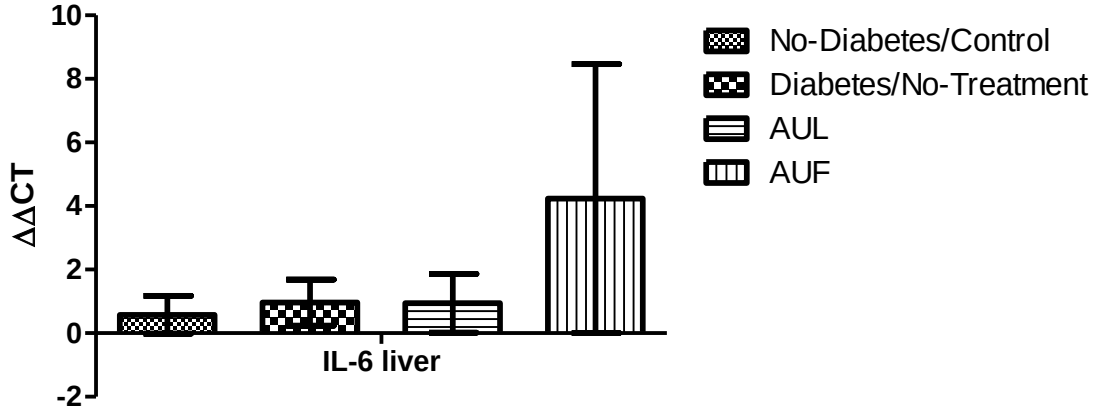

Figure 29: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of IL-6 gene in hepatic tissue**.

When comparing the expression of GLUT-4 in muscle and fat tissues some differences were noticed between the experimental groups. On the hepatic tissue, there were no significant changes (Appendix 2). In muscular tissue there was a significant increased expression of GLUT-4 gene in the high-fat fed STZ-injected diabetic groups when compared with the control group (Figure 30). The treatment with *Arbutus unedo* L. aqueous extracts had some effect in regulating the gene's expression to levels presented by the control group although they were very low. In the rat´s fat tissue there was a significant change in the expression of GLUT-4 gene between control group and diabetic groups. Nevertheless, the supplementation with *A. unedo* extracts had no effect in regulating the expression of this gene (Figure 31).

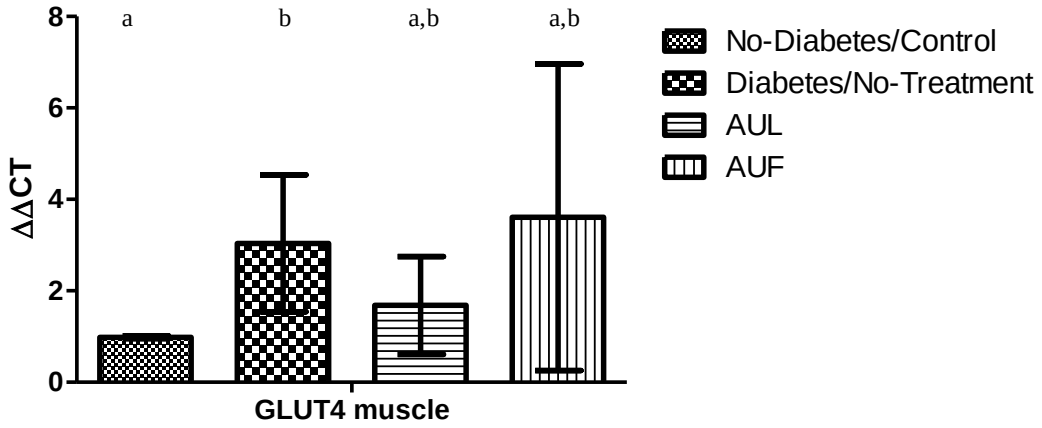

Figure 30: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of GLUT-4 gene in muscular tissue**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

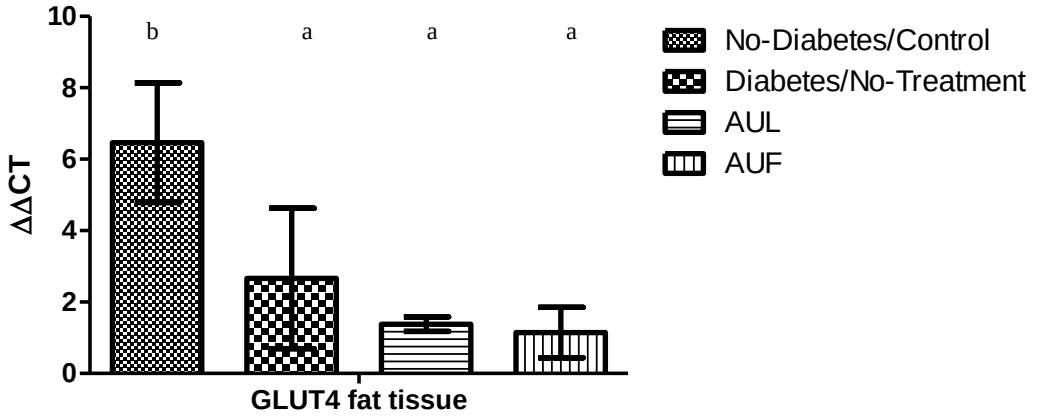

Figure 31: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of GLUT-4 gene in fat tissue**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

GLUT-2 expression was only measured in hepatic and muscular tissues as this glucose transporter is not specific of fat tissue. On the rats' liver no significant changes between the control and diabetic treated and not treated groups was noticed (Appendix 2). Bigger changes in the expression of the GLUT-2 gene occurred in the muscular tissue (Figure 32). The control group and the diabetic not treated group had the same level of expression of this gene. In both *Arbutus unedo* L. treated groups there was an increased expression of this gene in relation to the diabetic and control groups though. The group supplemented with the *A. unedo* leaf extract has a slightly higher range of expression

compared to the control and diabetic groups. Nevertheless the group fed with the fruit extract has a significant higher expression of GLUT-2.

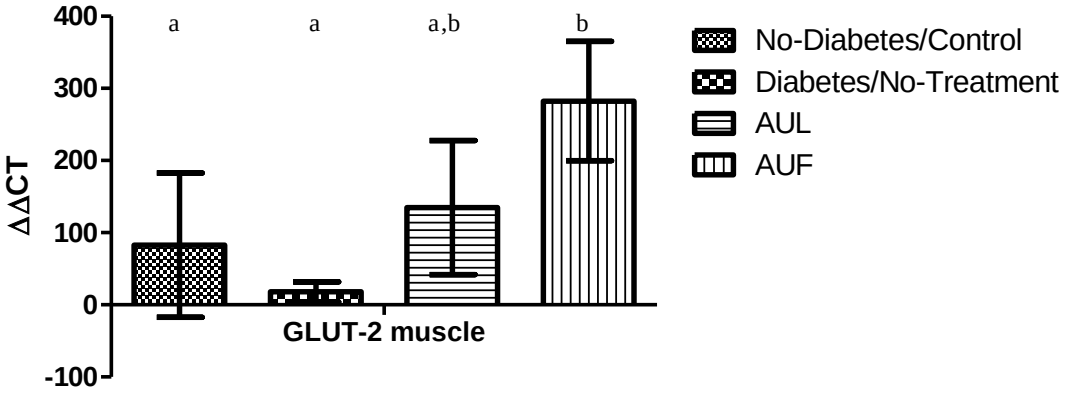

Figure 32: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of GLUT-2 gene in muscular tissue**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Table 15 sums up all the significant effects of the treatment of high-fat STZ-injected diabetic rats with *Arbutus unedo* L. leaf and fruit extracts. Whether or not some of these effects are beneficial will be discussed in the next chapter.

It is possible to see that these extracts had so beneficial effects on some of the biological indices tested. A mild therapeutic potential of *Arbutus unedo* L. extracts has been evidenced in this work on selected blood, haematological, tissular indices as well as in gene expression. In this trial, the doses tested were relatively low due to limited availability of the materials.

Table 15: Significant effects of supplementation with *Arbutus unedo* L. leaf and fruit aqueous extracts in HF/STZ diabetic rats (mean ± SD).

Significant effects of Arbutus unedo L. ***supplementation***	***Experimental group***			
	No-Diabetes/Control	Diabetes/No-treatment	AU_L	AU_F
Blood biochemistry				
Glucose concentration (mmol dm $^{-3}$)	9.18 ± 1.38^{a}	16.08 ± 4.52^{b}	15.07 ± 4.18^{a,b}	13.13 ± 4.04^{a,b}
HOMA-IR index	13.38 ± 3.09^{a}	37.71 ± 5.76^{c}	16.87 ± 2.66^{a,b}	22.75 ± 6.271^{b}
Insulin (Um/mL)	33.93 ± 9.36^{a}	42.53 ± 18.87^{a,b}	27.59 ± 6.96^{a}	63.93 ± 13.80^{b}
Creatinine (mg dL $^{-1}$)	0.39 ± 0.06^{a}	0.55 ± 0.09^{b}	0.54 ± 0.12^{a,b}	0.43 ± 0.08^{a,b}
ALP (U L $^{-1}$)	60.20 ± 8.73^{a}	153.14 ± 45.32^{b}	124.60 ± 52.31^{a,b}	127.80 ± 9.52^{b}
ALT (U L $^{-1}$)	26.00 ± 10.32^{a}	53.00 ± 26.38^{a,b}	74.50 ± 32.87^{b}	48.20 ± 14.79^{a,b}
Blood morphology and haematology				
PLT (x10 ^{3}/µL)	1128.80 ± 99.15^{b}	893.14 ± 111.67^{a}	848.00 ± 162.17^{a}	1020.80 ± 128.32^{a,b}
RDW-SD (fl)	30.68 ± 1.72^{a}	35.45 ± 1.96^{b}	33.20 ± 1.84^{a,b}	33.70 ± 2.24^{a,b}
PCT (%)	0.89 ± 0.08^{b}	0.75 ± 0.06^{a,b}	0.59 ± 0.09^{a}	0.83 ± 0.09^{b}
LYMPH (x10 ^{3}/µL)	3.72 ± 1.01^{b}	1.92 ± 0.53^{a}	2.75 ± 0.59$^{a.b}$	2.49 ± 0.93^{a,b}
Tissular mineral levels				
Zn kidney	94.63 ± 4.06 ^{a}	117.0 ± 10.8 ^{b}	122.47 ± 16.53 ^{b}	112.82 ± 16.11 ^{a,b}
Gene expression				
TNF-α Liver	2.0 ± 0.27^{a}	34.94 ± 43.41^{b}	3.41 ± 3.48^{a}	3.93 ± 3.39^{a}
TNF-α Muscle	1.71 ± 0.99^{a,b}	16.42 ± 8.230^{b}	11.76 ± 9.24^{a,b}	0.61 ± 0.43^{a}
IRS-2 Fat tissue	0.74 ± 0.20^{a}	1.14 ± 0.35^{b}	1.28 ± 0.42^{a,b}	1.22 ± 0.61^{a,b}
GLUT-4 Muscle	0.98 ± 0.03^{a}	3.04 ± 1.50^{b}	1.68 ± 1.07^{a,b}	3.61 ± 3.35^{a,b}
GLUT-2 Muscle	82.56 ± 100.03^{a}	17.96 ± 14.09^{a}	134.78 ± 92.88^{a,b}	282.47 ± 82.89^{b}

Abbreviations: ALP – Alkaline Phosphatase, ALT- Alanine Aminotransferase, PLT – platelets, RDW-SD – Red Cell Distribution Width based on Standard Derivation, PCT- Procalcitonin, LYMPH – Lymphocytes, Zn – Zinc, TNF-α – Tumour Necrosis Factor α, IRS-2 – Insulin Receptor Substrate 2, GLUT – Glucose Transporters, AU_L– high-fat with *Arbutus unedo* L. aqueous Leaf Extract (0.5 g/kg)/STZ injected, AU_F– high-fat with *Arbutus unedo* L. aqueous Fruit Extract (1.25g/kg)/STZ injected. Means on same line without common superscript differ significantly ($p < 0.05$).

CHAPTER IV- Discussion

1. Preparation of extracts from *Arbutus unedo* L. fruits and leaves

1.1. Selection of optimal extraction parameters to obtain extracts from *Arbutus unedo* L. fruits

As mentioned previously, in order to obtain the *Arbutus unedo* L. fruit extract the optimal solvents, mass-volume ratio, extraction periods and extraction temperatures were first optimised.

The temperatures used for *A. unedo* fruit extract preparation showed different extractions yields which varied between 58.3 and 78.4% at 90ºC; the extraction yield obtained was higher (78.4%), so this temperature was selected to perform the next assays.

Two more extraction periods were tested, 60 minutes (57.1% extraction yield) and 120 minutes (59.5% extraction yield). None of them presented a better extraction yield than the one obtained in the 45 minute extraction, reason why the next assays were performed at 90ºC during 45 minutes.

In this work, it was also evaluated how the mass/volume ration affected the extraction capacity. Two more different rations were tested (5g/125mL and 5g/62.5mL) and none of them gave a better extraction yield that the ratio tested according to literature (5g/250mL) [39]. So this ratio was not altered in the next test.

Another solvent, Ethanol, was also tested. In order to not evaporate the solvent, a different temperature was used in this extraction (63ºC). Extraction yield varied between 78.4% in the aqueous extract and 61.6% in the hydro-ethanolic extract. Once more, the method reported in literature gave a better extraction yield.

The optimal solvents, mass-volume ratio, extraction periods and extraction temperatures were established as **5 grams of fruit powder in 250 mL of water at 90ºC during 45 minutes** as this conditions resulted in the best extraction yield.

The yield obtained for this extraction was 78.4% (w/w). In a research work done by Mendes *et al*. a similar procedure was performed to obtain *Arbutus unedo* L. fruit aqueous extract. However, the only experimental difference was that the extraction was performed at 100ºC [39]. The extraction yield obtained in their work was 39.3% which is a much lower value when compared to the one obtained in this work. In order to obtain the extraction yields, the freshly lyophilised extracts were weighted right after lyophilisation was concluded. After some time, the fruit extract used in this work acquired a “sticky” texture which suggested that some water was still present in the extract. So the

lyophilisation was not entirely efficient. This could have led to a somewhat over estimated extraction yield.

1.2. Preparation of extracts from *Arbutus unedo* L. leaves

Due to lack of time to perform the same methodology in order to establish the best extraction conditions of the leaves, the following procedure was used: **5 grams of leaf powder in 250 mL of water at 100ºC during 45minutes**. The extraction yield obtained was 31.8% , about the same value that was achieved by Mendes *et al*. who performed the same procedure [39] and obtained an extraction yield of 32%.

1.3. Total Phenolic Determination

The *A. unedo* fruit's aqueous extracts appeared to have TPC content ranging from 1.74 to 2.5 mg GAE/g of extract (table 9). The hydro-ethanolic fruit extract presented a slightly higher TPC of 2.8 mg GAE/g of extract, so the difference was not big. Leaves aqueous extract, on the other hand, showed a much higher value, of about 24.5 mg GAE/g of extract.

According to this, the best extraction conditions to obtain the highest TPC were: **5 grams of fruit powder in 250 mL of Ethanol (50% v/v) at 63ºC during 45minutes,** and **5 grams of fruit powder in 250 mL of water at 90ºC during 45minutes.** These last conditions also proved to be good in order to obtain the highest amount of phenolic compounds.

The total phenolic content measured in this work was different from the values obtained in previous works. According to Mendes *et al*. the leaves aqueous extract had a phenolic content of 170.3 mg GAE/g extract and fruit aqueous extract 16.7 mg GAE/g extract [39]. Mendes *et al*. used the exact same method to obtain the leaves aqueous extract; in the case of the fruit extract, the only difference was the extraction temperature. These values are much higher than the ones obtained in this thesis. Oliveira *et al*. have also performed a study on the TPC of leaf aqueous extracts from *Arbutus unedo* L. obtained using the same procedure [96]. According to this study the leaves aqueous extract had a TPC of 172.21mg GAE/g of extract, very similar to the content quantified by Mendes.

Another conclusion that can be drawn is that the leaves aqueous extract has a total phenolic concentration about 10 times higher than the fruits aqueous extract.

In conclusion, the optimal solvents, mass-volume ratio, extraction periods and extraction temperatures were established as **5 grams of fruit powder in 250 mL of water at 90ºC during 45 minutes** and these were used to obtain the *A. unedo* fruit aqueous extracts. These conditions resulted in the best extraction yield and TPC.

2. Evaluation of the impact of *Arbutus unedo* L. extracts intake in a rat model of type 2 diabetes

After the selection of optimal parameter of extraction, the *Arbutus unedo* L. aqueous extracts were obtained and tested *in vivo*. Aqueous extracts obtained from fruits and leaves of *Arbutus unedo* L. were administrated to male Wistar rats for four weeks in the doses 1.25g /kg b.w./day in case of leaf extracts and 0.5g /kg b.w./day in case of fruit extract.

Previously, it was estimated that *Arbutus unedo* L. fruit aqueous extract had c.a. 2.517 mg GAE per gram of extract and *Arbutus unedo* L. leaf aqueous extract had c.a. 24.450 mg GAE per gram of extract. In terms of Total Phenolic Content and considering the doses given orally at the animals, one can estimate that the group supplemented with the *Arbutus unedo* L. fruit aqueous extract ingested approximately 3.146 mg GAE / kg b.w./ day and the group supplemented with the *Arbutus unedo* L. leaf aqueous extract ingested approximately 12.225 mg GAE / kg b.w./ day. This means that the group supplemented with the leaf extracts ingested a dose of phenolic compounds about 4 times higher than the fruit extract treated group. However, this did not mean that the AU_L group experienced more beneficial effects than the AU_F group, as it will be described, in this chapter.

As previously reported, leaf aqueous extract has a much higher phenolic content than fruit extract 170.3 mg GAE/g extract vs. 16.7 mg GAE/g extract [39]. The composition of both extracts, as reported in this same work, is different as more phenolic compounds were identified in the leaf extract. The author related the different phenolic content of both extracts with the different phenolic composition. Different phenolic compounds exist in leaf and fruit; also, the same compound can exist in different concentrations (tables 1 and 2). Bioactive properties of *Arbutus unedo* L. aqueous extracts (for leaf, fruit or roots) have been associated with antioxidants and phenolic compounds by

several authors [11, 41, 44-48]. Besides this family of compounds not much is known about leaves and roots composition. Bioactivity of *A. unedo* leaf and fruit aqueous extract has been attributed to specific compounds such as galloyl derivatives [11], tannins [45], catechin gallate [47]. These compounds are present in the roots' extract as well [40].

The *Arbutus unedo* L. roots' composition has not been largely studied and once more, the existing studies focused only on phenolic compounds. The roots' aqueous extract of *A. unedo* proved to have anti diabetic properties [49, 50]. No relation with the composition of the extract was made (in terms of phenolics or any other family of compounds) as the chemical composition of the extract was not studied [49, 50]. Bioactivity of the roots' aqueous extract was attributed, in another work, with the presence of compounds such as quinones, anthocyanins, anthraquinones, flavonoids and tannins which are also present in leaves and fruits of *A. unedo* [41].

Concerning anti-diabetic potential, the studies available in the literature only took in consideration the plasma glucose lowering effect [49], or performed testes like Oral glucose Tolerance Test (OGTT) and Intravenous Glucose Tolerance Test (IVGTT) in order to evaluate this property [50]. However, diabetes is a metabolic disease, which affects a lot more biological parameters. Taking in consideration this two works by Bnouham *et al*., physiological characteristics of Wistar rats the doses administrated ranged between 66.7 mg/kg b.w. day [49] and 500 mg/kg b.w./day [50]. So the doses given in Bnouham's works were lower than the doses administrated in this thesis.

In order to have an animal model of type 2 diabetes, or non-insulin dependent diabetes, a combination of high-fat feeding and streptozotocin injection were applied to the animals [75, 97].

Concerning overall growth and organ mass indices in rats, *A. unedo* extracts HF diet and STZ injection had significant effects on body mass gain, and consequently in body mass/body length ratio, and kidney size. In the case of body mass gain, the experimental group made diabetic lost body weight during the experiment. There was no significant change between the three diabetic groups, whether untreated or treated with the *A. unedo* extracts. The groups supplemented with the *A. unedo* extracts had a slight increase in body mass gain, although this increase was not statistically significant when compared to the diabetic group. As STZ destroys the pancreas β-cells and has a toxic effect, it was expectable to see a slight decrease in the animals' body weight after the administration of

the agent [78]. Nevertheless, it was expectable that the rats lost weight after the diabetes induction, but gained it back while being feed with a high-fat diet. In further studies conducted in the same lab, following the same procedure this happened: the diabetic rats put on weight [80, 98-100]. In another study where a STZ injection was used to induce type 2 diabetes, the rats showed a reduction of their body weighs compared to the normal rats [101]. Kasetti *et al*. related this phenomenon to poor glycaemic control [101]. According to this author, one possible explanation is that as rats cells fail to obtain glucose from the blood due to lack of insulin, they start obtaining it via gluconeogenesis which consumes muscular tissue in order to obtain aminoacids needed for this pathway. This led to muscle wasting and weight loss in these diabetic rats. The fact that the rats lost weight in this study influenced other parameters such as relative body mass weight and body mass/body length ratio. All the diabetic groups also presented an increased relative kidney size (expressed as percentage of the rat's final body weight). Chronic hyperglycaemia is associated with a long list of organ damage and dysfunction leading to kidney failure. Kidney damage is called nephropathy. In an early stage of diabetes there is a kidney hyper function, associated with an increased glomerular size and microalbuminuria [52], so the kidneys size can be affected. No significant change was measured in the three diabetic groups (untreated and treated), suggesting that the injection of STZ combined with the high-fat diet had an effect on the size of the kidneys and that this was not caused or changed by the administration of the extracts.

In this study, the blood glucose concentration was higher in all three diabetic groups (untreated and treated). Numerous other studies using STZ to induce diabetes confirmed this agent effectiveness to destroy pancreas β-cells and induce hyperglycaemia [60, 61, 65, 72, 101-112]. Nevertheless, supplementary *A. unedo* leaf and fruit extracts had a weak effect in lowering blood glucose. Even though the glucose levels were not as low as the ones presented by the control group, a slight decrease in glucose levels was noticed in treated groups.

The hypoglycaemic potential of both *Arbutus unedo* L. extracts used in this trial seems to be rather weak. However this might be due to small doses administrated and/or short duration of treatment, applied in this experiment. In order to verify the hypothesis whether *A. unedo* fruit and leaves extracts have the hypoglycaemic potential, a follow up study using higher doses of the material and longer period of time is warranted.

The effect of the leaf and fruit aqueous extract was weak, especially when comparing with the results obtained with the root extract which was administrated in lower doses [49, 50]. One possible explanation is that the root material can have a higher dose of bioactive compounds. When comparing both compositions of fruit and roots some differences can be seen in terms of phenolic compounds (no data is available in the literature to compare other family of compounds). For example, in terms of anthocyanins roots have a content of 365mg/100g f.w. vs a 3.77mg/100g f.w. anthocyanin content of the fruits [29, 41]. Roots also have a flavones and flavonols content of 17mg/100g f.w. while fruits only have a flavonol content of 1.14 mg/100g of edible fruit [29, 41]. This can help explain why the root's aqueous extract had a much higher hypoglycaemic effect, even when administrated in a lower dose. For example, catechin, a flavan-3-ol, was identified in the three materials. In roots it was not quantified, but in fruits and leaves it exists in different concentrations. According to Pallauf *et al.,* catechin exists in the concentration of 3.64 mg per 100 g of fresh fruit [29]. According to Fiorentino *et al.* leaves have a catechin content of 54.6 mg per100g of fresh leaves [37]. The concentration of this compound is about 10 times higher in leaves, so in roots it can also exist in very different concentrations. As previously reported by Guimarães *et al*., bioactivity of extracts from strawberry tree fruits can be more related to the phenolic compounds present in each extract than to their concentration [11]. The beneficial effects of the extracts can be attributed to various types of compounds existing in different concentrations in the plant materials.

Supplementary *Arbutus unedo* L. leaf and fruit aqueous extract had some therapeutic/ preliminary positive effects in diabetic rats, which were presented in blood biochemistry parameters of rats' serum, such as the serum concentration of glucose, phosphatase levels, creatinine, HOMA-IR index and insulin activity. No significant effects on blood lipid levels, AST, CRP, serum Fe and serum total protein were shown (data on Appendix 2).

Diabetes is characterized by insulin resistance deficiency which leads to an unregulated carbohydrate metabolism. Insulin levels are lower in patients with diabetes which is a consequence of the malfunction of pancreas β-cells [52, 97]. Pancreas damage induced by STZ lowers circulating insulin levels [78]. The insulin levels of the group supplemented with *Arbutus unedo* L. leaf extract were similar as the ones presented by the

healthy control group while the fruit extract group presented a much more increased insulin concentration.

The HOMA-IR index, defined as the fasting blood glucose to insulin ratio, is a good biomarker of insulin sensitivity. The higher this index the greater the insulin resistance [92]. In diabetes, the body's insulin resistance is known to be greater. Same effect was reported in other studies using an STZ induction of diabetes in rats [102, 103, 107]. As expected, the HOMA-IR index was much higher in the high-fat diet/STZ control group than in the control group. The increased HOMA-IR index is a reinforcement of the diabetic metabolic profile of these diabetic groups. Both supplementary *Arbutus unedo* L. extracts significantly reduced HOMA-IR index in diabetic rats, and this effect was a bit higher for the leaf extract supplemented group. But this should be confirmed using equal doses of the materials in a follow up study.

Serum urea concentration was elevated in all the diabetic experimental groups and supplementary *Arbutus unedo* L. plant extracts did not change this concentration. Increased serum urea was also reported in the study of Kasetti *et al.* using STZ-diabetic rats [101]. Similarly, Jayanthy *et* al. used a high-fat feeding STZ induced diabetic rats and reported the increase in serum urea concentration in untreated animals [102]. Thereby this increase in the urea levels can be completely attributed to the diabetic state of the animals. Diabetes can lead to a condition named ketoacidosis which is diagnosed by an increased level of urea in the blood [52].

Another biomarker of diabetic ketoacidosis is an increased creatinine concentration in blood [52]. High creatinine levels can be caused by kidney dysfunction [52]. Jayanthy *et al*. studied the effect of high-fat diet feeding combined with STZ injection on this parameter and concluded that creatinine levels are higher in untreated diabetic rats [102]. The same happened in the STZ-diabetic rats used in the study of Kasetti [101]. The HF/STZ control group presented a high creatinine concentration. The groups supplemented with the *Arbutus unedo* L. plant extracts appeared to have a slightly decreased creatinine concentration, suggesting that there is a tendency in reducing these levels to the ones of the control group. In the diabetic groups, kidney mass was increased when compared to the control healthy rats that can be attributed to intense renal excretion of glucose and metabolites (leading to gradual kidney damage). Elevated concentration of serum urea and creatinine are good biomarkers of the hyperglycaemia which causes renal dysfunction.

The activity of aminotransferases (AST and ALT) in blood is used as an indicator of liver damage. ALT (Alanine transaminase) is an enzyme found most commonly in liver cells [52] and when elevated can indicate liver damage. When comparing the healthy control group and the diabetic control group a slight difference was found. In the study of Jayanthy *et al.* the ALT levels were increased in STZ-injected diabetic rats [102]. Similar trends were reported by other authors [72, 101, 105]. In this experiment, blood ALT was a bit elevated in the diabetic groups, but with high individual response within each group. Supplementary *Arbutus unedo* L. leaf extract increased blood ALT level, while the fruit extract had slightly weaker effect in diabetic rats.

ALP (alkaline phosphatase) is another enzyme produced in the liver. An increase of this enzyme activity in blood can be a signal of liver disease: cholestasis, cirrhosis or tumours [52]. In this study, the diabetic not-treated group had the high activity of this enzyme. The same effect was reported in other studies in untreated diabetic rats [101, 102]. This may be explained with the fact that hyperglycaemia increases lipolysis and triglyceride breakdown. On the other hand, this increases the fatty acid content present in blood which will ultimately lead to an increased storage of this fatty acids in the liver [52]. The elevation of this enzyme in the diabetic study groups is due to the diabetic state they were in. Supplementary, *Arbutus unedo* L. leaf aqueous extract appeared to have some effects as blood ALP concentration showed a tendency to be reduced to levels presented by the control group.

Blood morphology and haematology indices are routinely measured biomarkers taken into consideration in assessing health status. There were no significant changes in almost all of the tested blood morphology indexes with exception of platelets levels (PLT), red cell distribution width (RDW), Procalcitonin (PCT), lymphocytes and monocytes.

Platelets levels were lower in the diabetic not treated group and on the *A. unedo* leaf aqueous extract supplemented group. However, the supplementation of *A. unedo* fruit aqueous extract revealed a tendency in contradicting this effect as the platelets levels slightly increased. The effects of insulin on platelets are reported by various and the results are controversial and contradictory. However, one can say that there is a dysfunction in platelet signalisation and formation in diabetic patients [113]. This dysfunction appeared to be slightly attenuated by the supplementation of *Arbutus unedo L*. fruit extract. A further study with higher doses is once more needed.

The red cell distribution width (RDW) was higher in all the diabetic groups. A high RDW tells how the red blood cells vary in size. When analysing this parameter there is also a need to analyse the MCV (mean corpuscular). STZ injection, high-fat feeding or *A. unedo* extracts did not affect the MCV levels. If the RDW level is increased and the MCV level is normal, it can indicate that the levels of folic acid decreased. This is the beginning stages of iron deficiency anaemia [114]. On the other hand, a recent study conducted by Magri *et a*l. [115] found out that there can be an association between an elevated RDW and albuminuria in patients with a diabetic background as the diabetic state can be a cause of nephropathy. The authors proposed that a high RDW can be a marker for altered glomerular haemodynamics. Since diabetic nephropathy is associated with red cell fragmentation, high RDW can be related with diabetic renal disease [115]. *Arbutus unedo* L. extracts slightly improved the levels of RDW in diabetic rats, showing a tendency to decrease this levels to levels presented by the control group.

Procalcitonin (PCT) is a peptide produced as a response to an inflammatory stimulus, namely bacterial infections [94]. In this trial the diabetic untreated group as well as the *A. unedo* leaf extract supplemented groups had a lower level of PCT compared to the healthy control group. In the *A. unedo* fruit extract supplemented group however, the PCT levels were the same as the ones presented by the control group.

The high-fat feeding and STZ injection also decreased the amount of lymphocytes and monocytes. Similar effects were reported by Mojani *et al.* [97]. In a study with diabetic patients, Chang *et* al. [116] found that activated lymphocytes from patients with type 2 diabetes had a lower percentage of IL-2R in their membranes which resulted in insufficient proliferation. The glycosylation of membrane proteins caused by hyperglycaemia resulted in a failed signalling system of the lymphocytes and monocytes. The authors also concluded that the monocytes may not contribute to the insufficient lymphocyte proliferation presented in their study [116]. In the study conducted by Mojani *et al.* [97] diabetes affected lymphocytes proliferation. This suggests that the diabetic status of the animals can result in a reduced immunity. The treatment with the *A. unedo* aqueous extracts appears to attenuate these effects as the lymphocytes population were slightly higher in the groups fed with this extracts. Probably *A. unedo* may help enhance immune system but once more a longer study with higher doses could help clarifying these effects.

Diabetes can disturb the homeostasis of some microelements, namely zinc, copper and iron [64, 68]. In a diabetic type 1 animal model differences in tissular levels of these microelements were found [60, 112]. In order to clarify if STZ injection, high-fat feeding and *A. unedo* extracts had some effects on the tissular copper, zinc and iron levels in the rats, these parameters were also measured. Studies conducted in the same laboratory showed how STZ injection combined with high fat diets affected Cu, Zn and Fe homeostasis [98-100, 117]. However, the effect of *A. unedo* aqueous (or other type of) extracts on microelements status has not been studies yet.

In this study, hepatic homeostasis of Cu, Zn and Fe was not disturbed by feeding high-fat diet combined with STZ injection. However, increased renal levels of these elements were observed.

The high-fat feeding and the STZ injection themselves caused increased renal zinc concentration and supplementation with *Arbutus unedo* L. leaf aqueous extract did not contradict this effect. However, supplementation with *Arbutus unedo* L. fruit aqueous extracts slightly decreased the concentration of zinc in the rat's kidneys to similar concentrations as the ones presented by the control group. Rats used as a model of type 2 diabetes presented lower zinc levels in kidneys [59]. So the results in this thesis are rather contradictory. Nevertheless, in a further trial, Kazi *et al.* found out that high levels of Zn were present in urine of type 2 diabetic patients [118]. As kidneys are partly responsible for urine production, this can be an explanation for the high concentration of zinc in the rats' kidneys. Further analysis to rats' urine should be taken to clarify this.

Arbutus unedo L. aqueous extracts given to diabetic rats showed no effect on copper renal concentrations, as they remained high. In another study conducted in the same laboratory with the same HF/STZ approach, high-fat diet combined with STZ injection also resulted in increased renal copper concentration [65, 98]. Apparently, *A. unedo* has no effect in regulating this mineral renal homeostasis.

In a study by Takita *et* al., spontaneously diabetic rats (considered a model for type 2 diabetes), had a lower liver and serum Cu levels, while Fe levels were increased [119]. The authors concluded that metabolic changes associated with diabetes affects mineral balance in various organs.

The high-fat feeding and the STZ injection themselves caused a slight increase in the renal iron concentration, while supplementation with *Arbutus unedo* L. leaf aqueous

extract aggravated this effect. Fruit aqueous extract had no effect in regulating this mineral homeostasis. Elevated kidney iron levels in HF/STZ rats were also reported in other studies [98, 99]. The Fe body levels in spontaneously diabetic rats were found to be higher too. This suggest that iron metabolism is altered in hyperglycaemia and insulin resistance [119]. In this study, HF/STZ rats presented a significantly higher RDW level while the MCV level remained normal which may indicate the beginning stages of iron deficiency anaemia. This could suggest problems with red blood cells function. Thus, these alterations in the renal Fe content as well as changes in red blood cells parameters can suggest a state of anaemia which is probably related to diabetes. Nevertheless the rats' serum's Fe levels remained normal. Most authors relate an increased ferritin concentration with a risk to develop diabetes [64, 67]. In this study, it can be proposed that the high-fat feeding combined with STZ injection can alone cause iron metabolism disturbances (that can worsen the diabetic state by aggravating the insulin resistance). Surprisingly this effect was higher in the group treated with the *Arbutus unedo* L. leaf extract than in the diabetic not treated group. Perhaps the rats in this group were most susceptible to these homeostasis alterations than the ones belonging to control group. The *A. unedo* leaf extract caused no alteration in the toxicity biomarkers, so renal toxicity of this material can be excluded. The fruit aqueous extract treated group also showed an increased renal iron concentration, similar to the one present in the diabetic not treated group. Once more, further trials should be carried out in other to prove its therapeutic or non-therapeutic benefits. In order to draw further conclusions about how the rats' iron homeostasis was affected, more body tissues have to be analysed.

When combining this mineral homeostasis data with organ size and haematogram index, it can be noticed that the altered value are related with kidney health. In this study, the diabetic rats had increased kidney mass and altered haematogram parameters which combined can indicate renal disease and disturbed kidney mineral homeostasis. Diabetes can ultimately lead to renal dysfunction [52, 120] which has been reported in diabetes in human subjects. In this study, diabetes caused by high-fat feeding combined with STZ injection lead to renal problems. Some light therapeutic effects were registered in the groups supplemented with *Arbutus unedo* L. aqueous extract (mainly the fruit extract) as the groups treated with this extracts had slightly improved levels of RDW. Further studies

with higher doses and longer duration are required in order to establish if these extracts have kidney protective effects.

Lastly, the effect of the aqueous extracts from *Arbutus unedo* L. fruits and leaves on the expression of certain genes was also studied. To this date, the only studies concerning how extracts of *A. unedo* may influence gene expression only focus on genes involved in inflammatory processes [48, 121, 122]. Only one study *in vivo* was performed until now and the materials examined were cells subjected to acute inflammation or cancer cells.

The genes examined in this study play a role in the glucose metabolism and/or in inflammation response. The expression of these genes on hepatic, muscular and fat tissues was analysed.

There were significant changes in the expression of TNF-α on both hepatic and muscular tissues. Several studies using STZ induced type 2 diabetic rats have reported that these animals have an increased expression of TNF-α [72, 73, 104] in pancreas, liver, serum and fat tissue. As expected, the high-fat fed STZ injected type 2 diabetic control group showed an increased expression of the gene encoding to this cytokine. This expression was downregulated to normal values on hepatic tissue in the groups treated with both *A. unedo* leaf and fruit aqueous extract. In muscular tissue, the group treated with *A. unedo* fruit aqueous extract had normal expression of TNF-α gene, although the effect was not so high in the group treated with the leaf extract. In terms of regulation of TNF-α expression, the *Arbutus unedo* L. fruit and leaf extracts proved to have beneficial effects, in both hepatic and muscular tissues. Since high concentrations of TNF-α is associated with increased insulin resistance, the *A. unedo* extracts might have potential to attenuate inflammatory responses in type 2 diabetes. In a study by Mariotto *et al.*, rats were subjected to carrageenan induced pleural and lung inflammation. One of the groups was also administered orally with 20mg GAE/ kg b.w. of *Arbutus unedo* L. leaf aqueous extract 1h hour prior to carrageenan injection [48]. The authors concluded that this extract had anti-inflammatory properties [48]. In this thesis high-fat feeding and STZ injection increased the expression of TNF-α gene in liver and muscle tissues. Nevertheless, the treatment with both fruit and leaf aqueous extracts reduced the expression of this gene to normal levels. In muscular tissue, the effect was more potent for fruit extract. The doses tested, in terms of phenolic content were 3.146 mg GAE / kg b.w./ day for AU_F group and 12.225 mg GAE / kg b.w./ day for AU_L group, much lower than the dose administrated in

the study of Mariotto. Even though, these lower doses proved to be effective. This is the first study reporting the *A. unedo* extracts can affect the TNF-α expression in different tissues of type 2 diabetic rats.

Although in another study using STZ injection to induce non-insulin dependent diabetes in rats, the IL-6 levels were enhanced in pancreas, kidney and serum [123]. IL-6 is thought to induce insulin resistance in liver as it is associated with inhibition of hepatic insulin-dependent receptor [123]. No statistically significant changes occurred in the expression of this gene in all experimental groups in the different tissues.

Arya *et al.* using STZ diabetic rats revealed that pro-inflammatory cytokines have antagonistic properties to insulin as they interfere with IRS phosphorylation, leading to insulin resistance [123]. There is, thereby a link between oxidative stress, inflammation, inflammatory cytokines and type 2 diabetes, as in their study the elevated levels of TNF-α and IL-6 in the serum and tissues of STZ rats suggested an inflammatory state which lead to insulin resistance [123].

The link between diabetes, insulin pathway and the interference of pro inflammatory cytokine cannot be dismissed. So, the expression of the genes encoding for IRS-2 and transporters GLUT-2 and GLUT-4 were evaluated in order to clarify whether insulin resistance and diabetic state would affect their expression

The IRS-2 gene encodes a protein named Insulin Receptor Substrate 2, a cytoplasmic molecule that acts as a molecular adaptor in the tyrosine kinase cascade, thereby mediating the effect of insulin as well as other proteins. IRS-2 is tightly related with diabetes and previous studies that also used SZT-injected diabetic rats reported that the animals had decreased expression of this gene [105, 108]. Insulin resistance is thought to be directly linked with deficient IRS-2 production, and this decreased IRS-2 expression was also reported in STZ-rats that were fed with an high-fat diet [124]. The same effect was thought to occur in this thesis. No significant changes in the expression of this gene were seen in hepatic and muscular tissues. The only significant changes in the expression of this gene occurred in fat tissue. However, the results obtained in the fat tissue were even more contradictory with the diabetic not treated group having an increased expression of this gene. The supplementation with *Arbutus unedo* L. extracts showed some effects in this matter. The expression of IRS-2 gene in *A. unedo* supplemented groups was a bit lower

than in the diabetic group. To further clarify this matter, analysis of related proteins involved in the insulin signalizing cascade should be performed.

In this study, in the diabetic rats (not treated group), the expression of GLUT-4 encoding gene in muscle tissue was greater than in the control group. This is opposite to the data available in the literature [106-108, 110, 111]. In all these studies, GLUT-4 expression and/or release decreased in STZ diabetic rats' muscular tissue. In muscle tissue *A. unedo* had a slight effect in "regulating" this gene expression as the expression remained a bit lower. In fat tissue and hepatic tissue, no significant difference occurred between any of the groups.

As the high-fat feeding and STZ-injection had no effect in decreasing the expression of IRS-2 in hepatic, fat and muscular tissue, this can help explain why these results were contradictory. Decreased GLUT-4 translocation has been observed by many researchers in STZ induced diabetic rats that reflects insulin resistance related to decreased ILR-2 expression. In fat tissue, the expression of IRS-2 was greatly enhanced.

In the case of GLUT-2 gene expression, studies with STZ rats reported that the hepatic levels of GLUT-2 have decreased [109, 110]. In this experiment such changes were not detected, as there were no significant changes in GLUT-2 expression in liver tissue. In muscular tissue, diabetic not treated rats did not present an increased expression of this gene when compared with the control group rats. The treatment with *Arbutus unedo* L. fruit aqueous extract enhanced even more this expression. Leaf extract has increased the gene's expression a bit. However, no therapeutic effects can be attributed to these extracts before clarifying why the expression of GLUT-2 did not change in the diabetic group.

Arbutus unedo L. aqueous extracts enhanced the expression of both GLUTs in the analysed tissues. In case of diabetes, this can be considered as beneficial although further clarifications are needed. Beneficial effects were seen in the modulation of the expression of TNF-α in a rat diabetic model.

In conclusion, this was the first trial which tested the therapeutic potential of *Arbutus unedo* L. fruit and leaf aqueous extracts given orally in small doses in an animal model of type 2 diabetes. Until now, no other study has explored so many parameters (blood indices, mineral status and gene expression) and correlated them with diabetes. Positive anti-diabetic and anti-inflammatory aspects were found for both aqueous extracts. *Arbutus unedo* L. fruit aqueous extract presented a tendency towards lowering blood

glucose levels. More beneficial effects were seen for this extract in terms of attenuating the effects of diabetes in renal zinc homeostasis and blood morphology and haematological parameters and regulating TNF-α gene expression in muscular tissue.

The other type of extract tested, the leaf extract, appeared to be more efficient in regulating insulin levels, decreasing HOMA-IR index and also presented a tendency towards lowering blood glucose levels. It also had a slight effect in decreasing phosphatase concentration.

Both extracts proved to efficiently improve insulin sensitivity (HOMA-IR index), slightly decreasing serum creatinine concentration, slightly improved the RDW-SD and lymphocytes concentration and decreasing TNF-α expression on liver and muscle tissue.

Although few effects were observed, one should notice that the doses tested were small and that the trial took place for a short period of time. Nevertheless, further investigation is needed and this trial was the bottom line in investigating the beneficial effects of *Arbutus unedo* L.

CHAPTER V- Conclusion and Perspectives

The aim of this study was to create the laboratory conditions to obtain the *Arbutus unedo* L. extracts and further to evaluate their antidiabetic and anti-inflammatory potential in a diabetic model of rats.

After careful analysis of available literature about the bioactive properties of *A. unedo* extracts, the fruit and leaf aqueous extracts were selected as appropriate materials for an *in vivo* trail. The aqueous extracts had already been used in the study of antidiabetic and anti-inflammatory potential of *A. unedo* plant materials. As this study was an *in vivo* study, it was decided that the type of extracts should be the least harmful possible.

In the first stage of experiment, different extraction conditions were tested to obtain the materials regarding the best yield and phenolic content. In order to obtain fruit aqueous extract, the optimal conditions were determined as being 5 grams of fruit powder in 250 mL of water at 90ºC during 45 minutes. The extraction conditions used in order to obtain the leaf aqueous extract were not optimized. The procedure used to obtain the leaf extract was the same as reported in the literature 5 grams of powdered leaves in 250 mL of water at 100ºC during 45 minutes [39].

It was found that the extraction yield of fruits and leaf extracts were not similar, as the yield obtained for the fruit extract was 78.4 % (w/w) and the yield obtained for leaf extract was 31.8 % (w/w). The total phenolic content in the fruit extract was 2.517 mg GAE/g of extract and in the leaf extract was 24.450 mg GAE/ g of extract. So, the total phenolic content of the leaf extract was 10-fold of that in the fruit ones. This was taken into consideration when selecting the doses administrated on the *in vivo* trial.

In the second stage of experiment, both *Arbutus unedo* L. aqueous extracts were given orally to male Wistar rats made diabetic by feeding high-fat diet and STZ-injection.

Due to limited amount of materials, diabetic rats received in food variable doses of extracts, namely fruit extract -1.25g /kg b.w./day and leaf extract - 0.5g /kg b.w./day for 4 weeks. The working aim of the biophase was to evaluate the effects of these extracts on nutritional indices, blood haematological and biochemical indices, mineral status (Fe, Zn and Cu) and studying the expression of selected genes related with inflammatory response and glucose metabolism (TNF-α, IRS-2, IL-6, GLUT-2 and GLUT-4 encoding genes). The study of mineral status was made in hepatic and renal tissues and the evaluation of gene expression was made in fat, muscular and liver tissues.

It was found that *A. unedo* leaf and fruit aqueous extracts exerted moderate anti-diabetic, anti-inflammatory and protective effects on kidney function in diabetic rats. Although both extracts (in the given doses and treatment time) did not significantly decrease blood glucose concentration, they presented a preliminary positive antidiabetic potential. They clearly improved insulin sensitivity (HOMA-IR index), slightly improved the RDW-SD value, platelets levels and lymphocytes concentration; slightly decreased serum creatinine and phosphatase levels and significantly decreased TNF-α expression in liver and muscular tissues. In some of the analysed parameters, although the results may not be statistically different, there was a tendency towards normalizing them to healthy control levels. These preliminary results are important guide lines to follow up studies. When comparing the activity of *A. unedo* leaf and fruit extracts, some differences were found:

- *Arbutus unedo* L. leaf extract was more efficient in decreasing insulin levels and decreasing HOMA-IR index.
- *Arbutus unedo* L. fruit extract was more potent in regulating TNF-α gene expression in muscular tissue.

This is the first trial showing that *A. unedo* extracts have some therapeutic potential for improving blood glucose, insulin and insulin sensitivity, and anti-inflammatory response in a diabetic rat model. The only trials on *A. unedo* anti diabetic activity available until now have used roots aqueous extract [49, 50]. Although the doses tested in this thesis were higher, the hypoglycaemic effect was not as potent as the one noticed in the previous works. As roots, fruits and leaves from the strawberry tree have different compositions, this can be an explanation for this weak anti diabetic effect. As roots are not as well studied as the fruit and leaves this effect cannot be attributed to any specific compound.

Even if it did not have a hypoglycaemic effect as strong as expected, the *A. unedo* leaf and fruit extracts cause some alterations in mineral homeostasis and gene expression. It has been reported in the literature that diabetes affects more parameters than only glucose and lipid metabolism. The high-fat feeding and STZ injection had disturbed zinc homeostasis in kidneys causing an increase in this mineral's concentration. Although this effect was not reported in further studies *A. unedo* fruit extract slightly decreased this mineral concentration. In this case, one can say that the effect of diabetes was attenuated by the supplementation with this extract. In terms of the expression of inflammatory

related genes and improving immunity, positive effects were also shown. As reported in literature [48], *A. unedo* downregulates the expression of TNF-α in inflammatory response. The same was observed in this thesis in muscle and liver tissues. Besides, this was the first time this gene's analysis was made in a type 2 diabetes model.

This study is the bottom line in this subject as no other had correlated and investigated so many diabetes related effects. The results demonstrate that there is a potential therapeutically application for this plant. Further investigation is needed in order to clarify the beneficial effects of *Arbutus unedo* L. Although the strawberry tree is spread all over the Portuguese and south Europe territory, is not very well known by the community. These preliminary results can lead to a valorisation of this plant and to improve the existing knowledge.

In terms of futures perspectives, further study is necessary to confirm these effects. More refined experimental models must be used, involving identification of major bioactive compounds, dose-response relationship, and longer duration of treatment. It would also be interesting to further investigate how these extracts affect selected gene expression. It would be also interesting to test other types of extracts or to administrate the freeze dried fruits and leaves of *Arbutus unedo* L. directly on the high-fat diets. In any extracting process, a fraction of the material's compounds is lost. So, giving the whole fruit to the animals will make it possible to study all the fruit's potential.

In order to explain the bioactive properties of the extracts, the extracts' composition in terms of phenolic compounds ought to be analysed. Other bioactive compounds should be also analysed as the extract has a "mix" of compounds that are likely to act simultaneously in order to present these therapeutic effects.

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APPENDIXES

Appendix 1- TPC measurement by Folin-Ciocalteau method

Table 16: Absorbance at 760 nm measured for each gallic acid solution used to plot the method's calibration curve.

[Gallic Acid] (mg/L)	$A_{760\ nm}$	*Mean* $A_{760\ nm}$	*SD*
61.995	0.262	0.274	0.0125
	0.287		
	0.274		
123.99	0.513	0.523	0.00907
	0.530		
	0.527		
186	0.729	0.723	0.00603
	0.717		
	0.724		
248	0.869	0.901	0.0289
	0.925		
	0.910		
310	1.224	1.251	0.0249
	1.256		
	1.273		
0	0.057	0.062	0.00839
	0.072		
	0.058		

Appendix -2 *In vivo* effects of the *Arbutus unedo* L. fruit and leaf aqueous extract in high-fat feed STZ-diabetic rats.

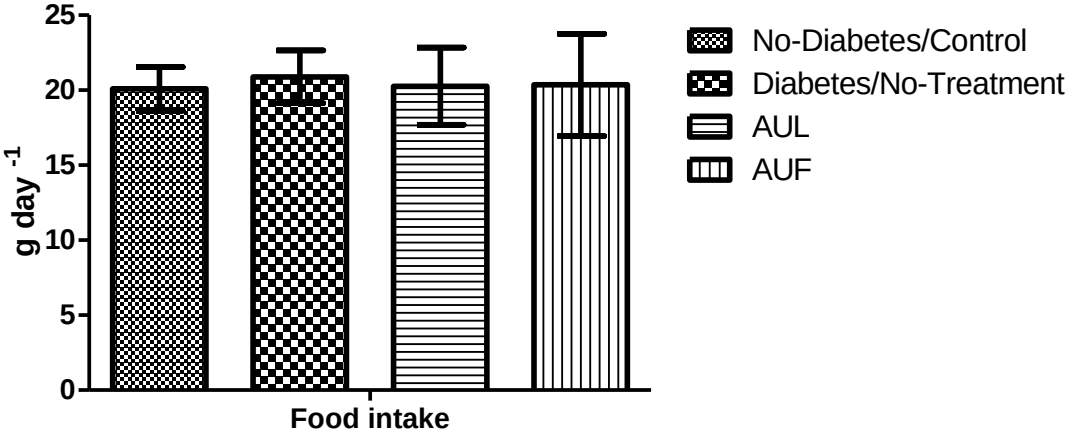

Figure 33: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **food intake (g per day)**.

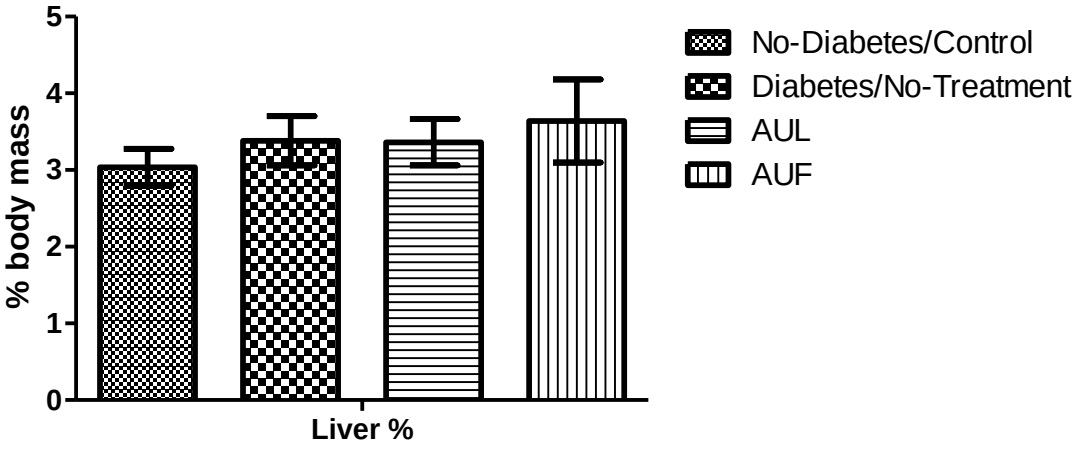

Figure 34: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **liver weight (% of rat's body mass)**.

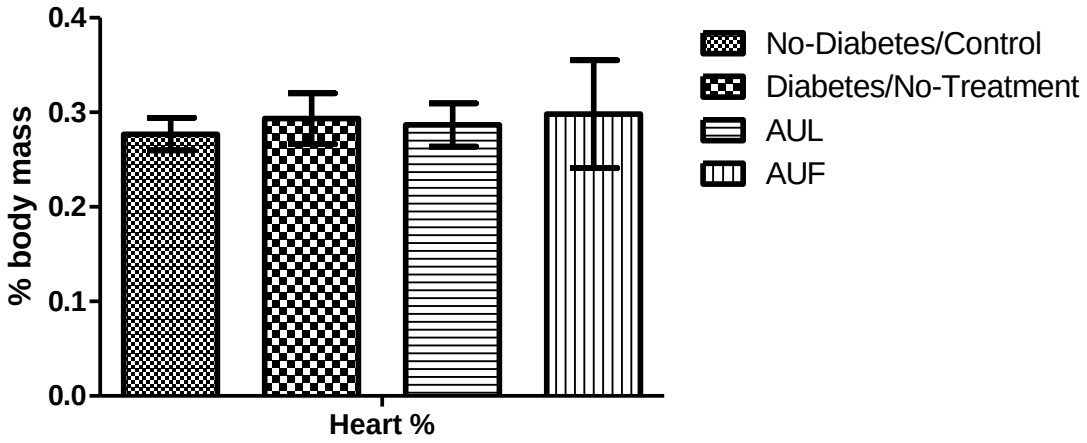

Figure 35: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **heart weight (% of rat's body mass)**.

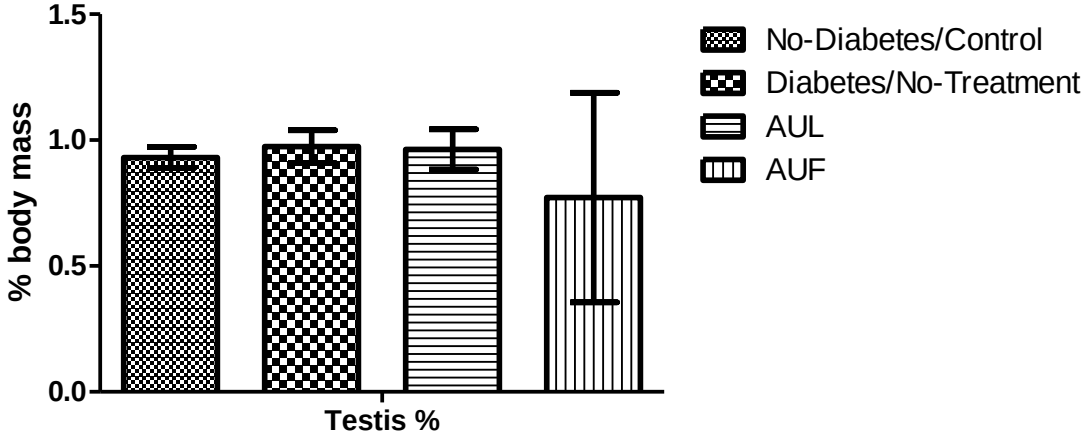

Figure 36: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **testis weight (% of rat's body mass)**.

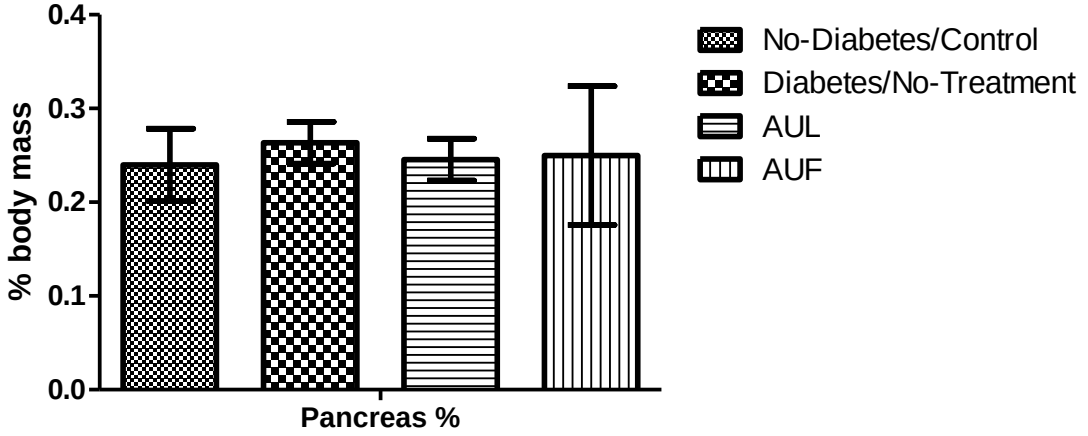

Figure 37: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **pancreas weight (% of rat's body mass)**.

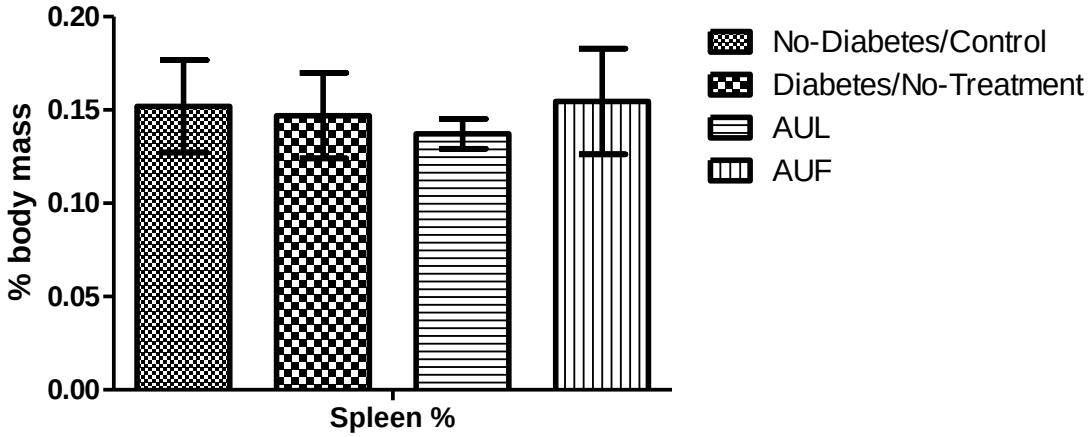

Figure 38: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **spleen weight (% of rat's body mass)**.

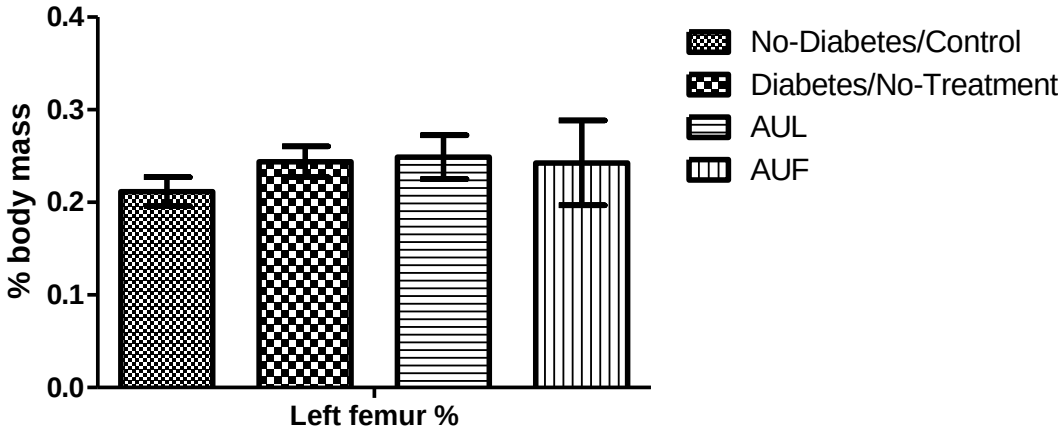

Figure 39: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **left femur weight (% of rat's body mass)**.

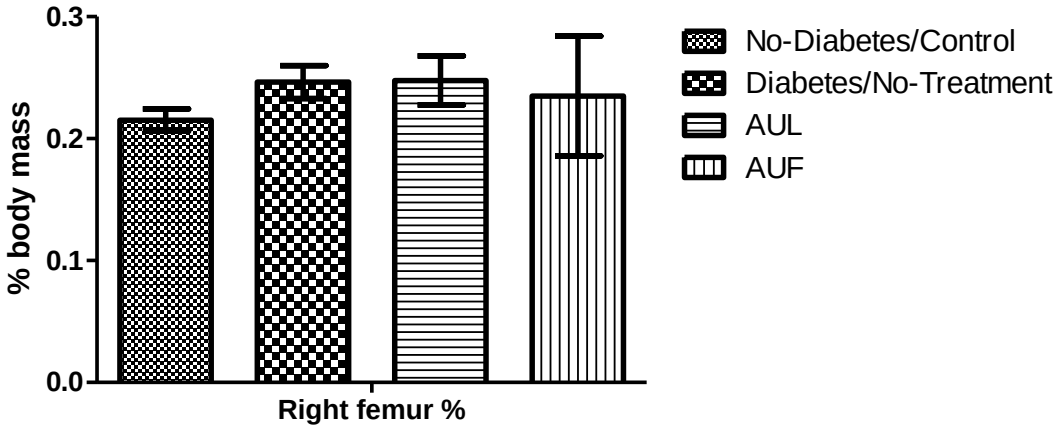

Figure 40: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **right femur weight (% of rat's body mass)**.

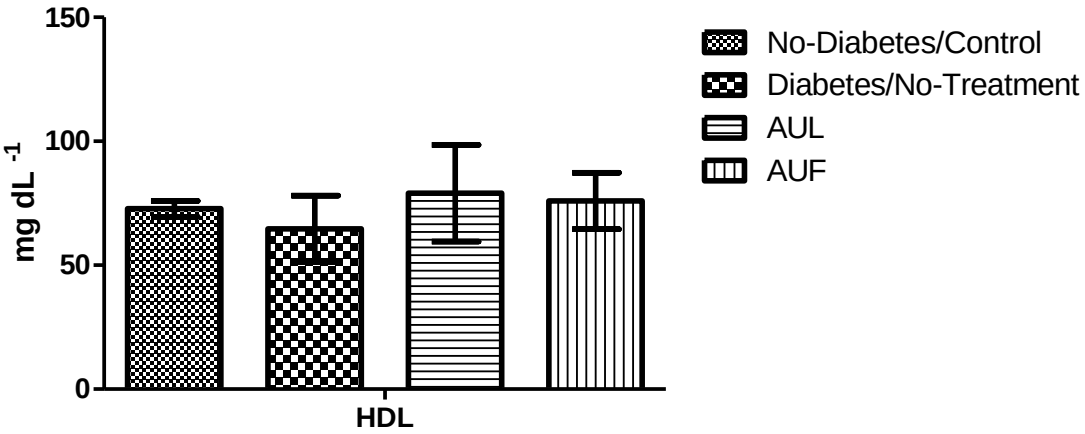

Figure 41: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum HDL levels (mg dL $^{-1}$)**.

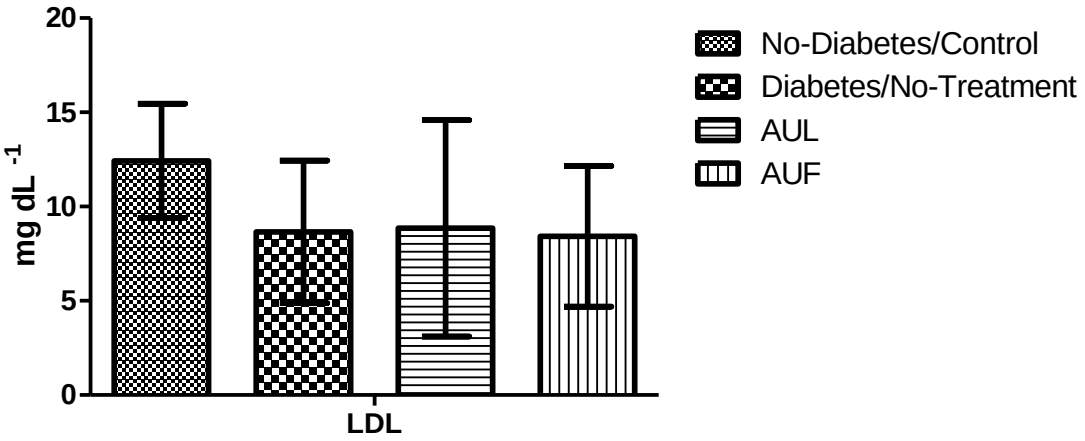

Figure 42: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum LDL levels (mg dL $^{-1}$)**.

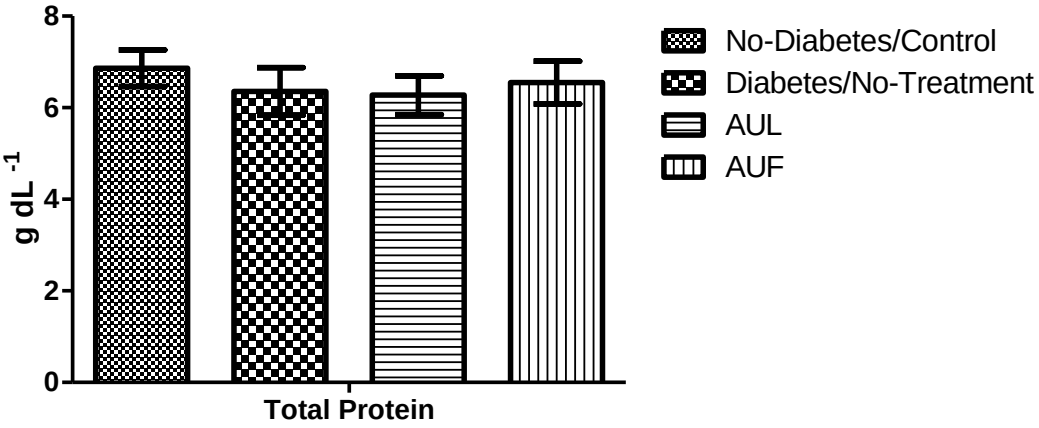

Figure 43: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum total protein levels (g dL $^{-1}$)**.

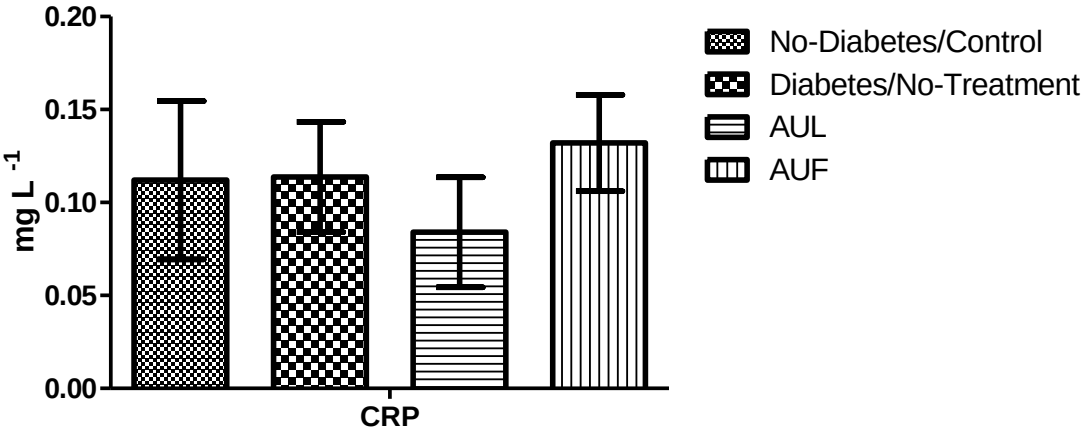

Figure 44: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum CRP levels (mg L $^{-1}$)**.

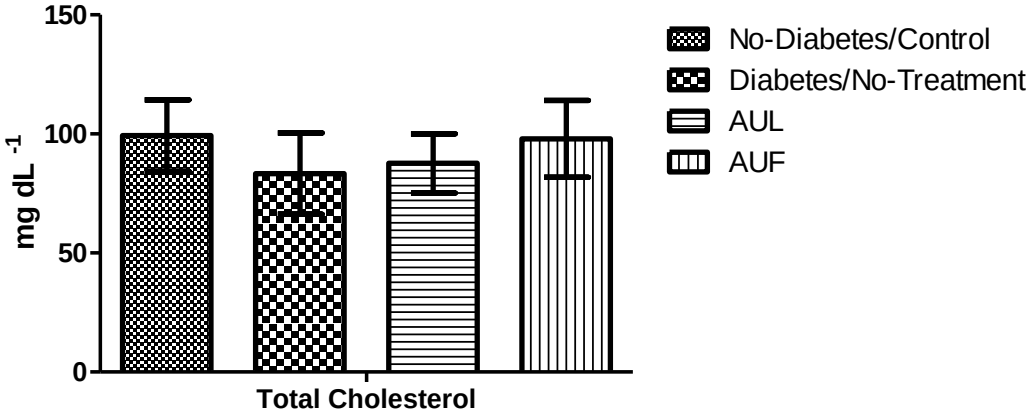

Figure 45: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum total Cholesterol levels (mg dL $^{-1}$)**.

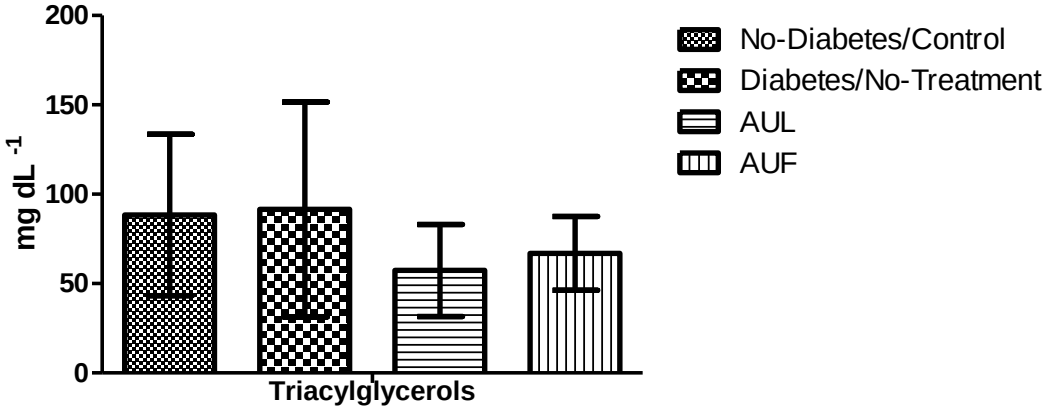

Figure 46: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum Triacylglycerol levels (mg dL $^{-1}$)**.

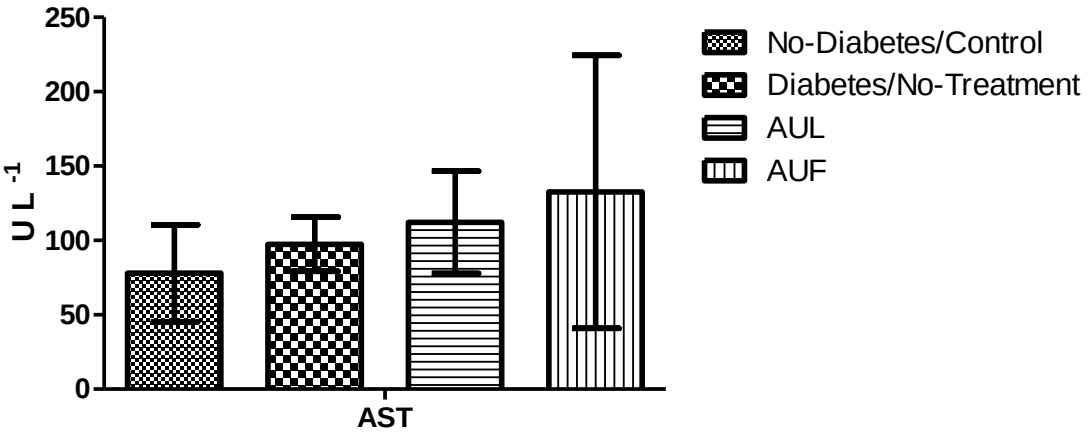

Figure 47: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **serum AST (U L $^{-1}$)**.

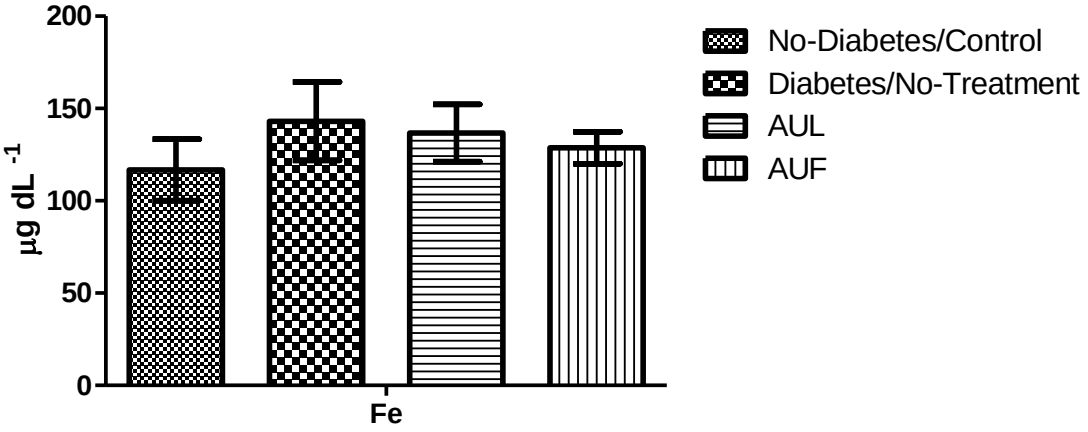

Figure 48: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in serum **iron levels (µg dL $^{-1}$)**.

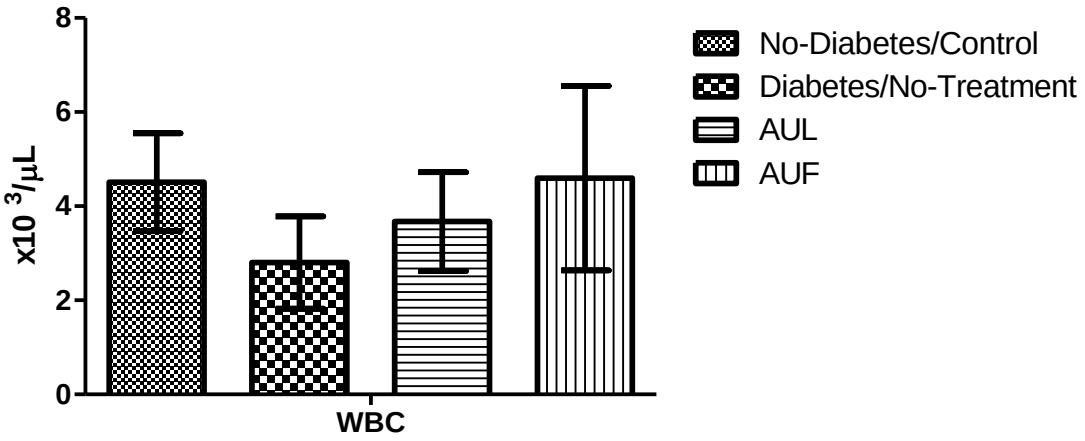

Figure 49: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **white blood cell levels**.

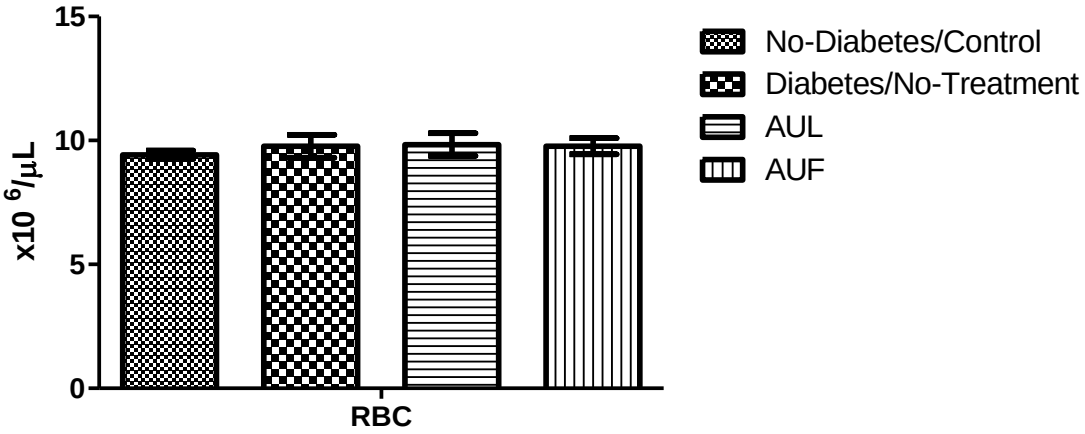

Figure 50: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **red blood cell levels**.

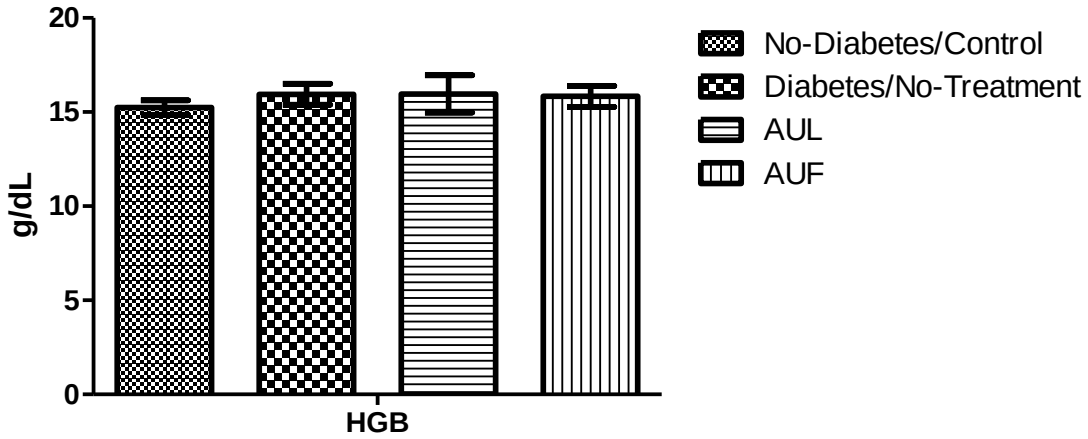

Figure 51: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in **haemoglobin levels**.

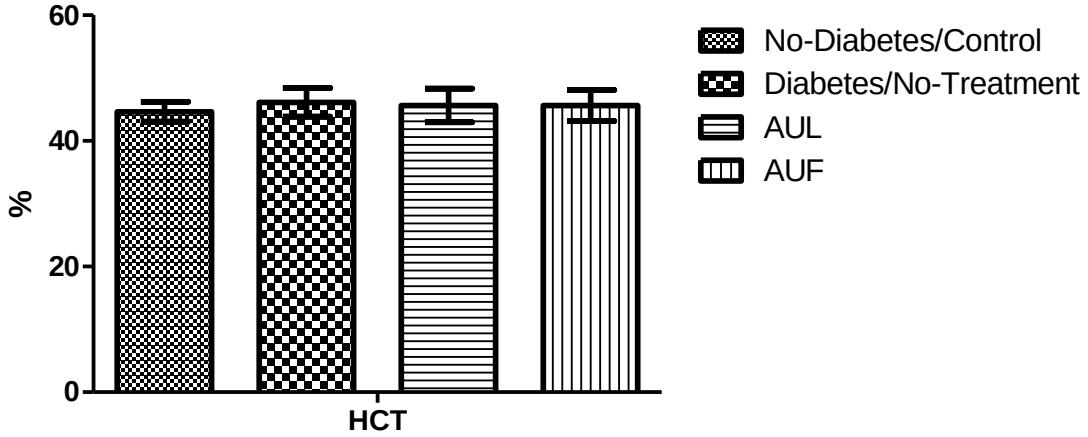

Figure 52: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **haematocrit**.

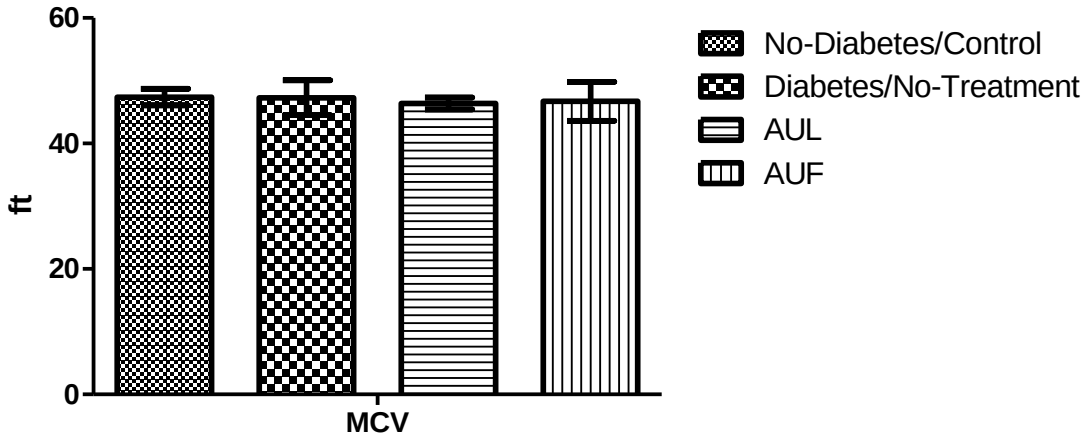

Figure 53: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **mean corpuscular volume**.

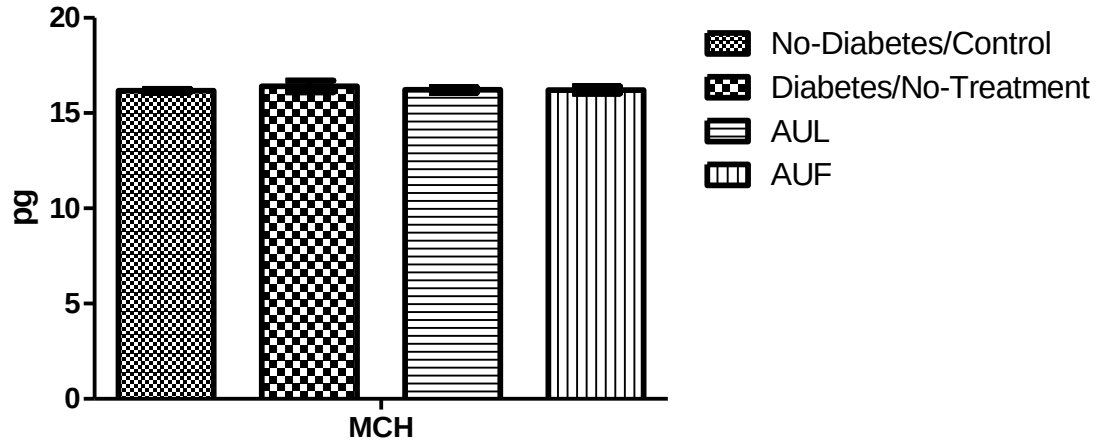

Figure 54: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **mean corpuscular hemoglobin**.

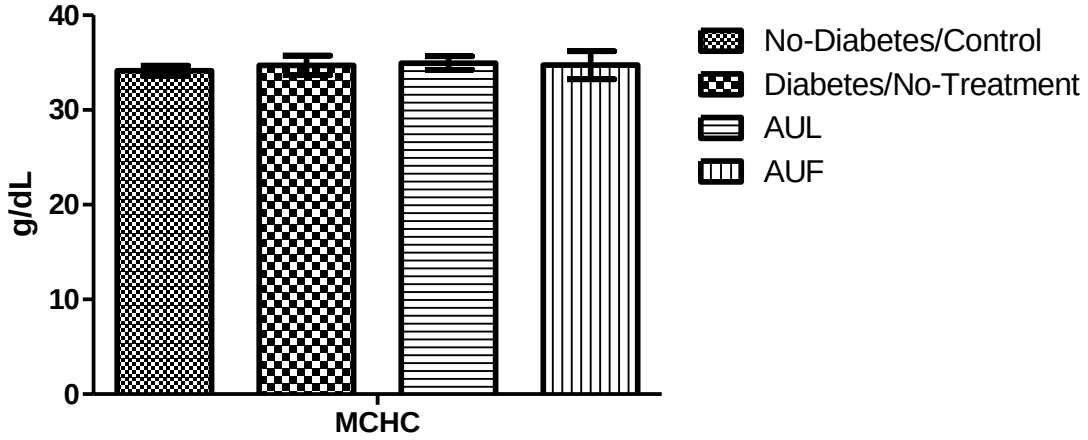

Figure 55: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **mean corpuscular haemoglobin concentration**.

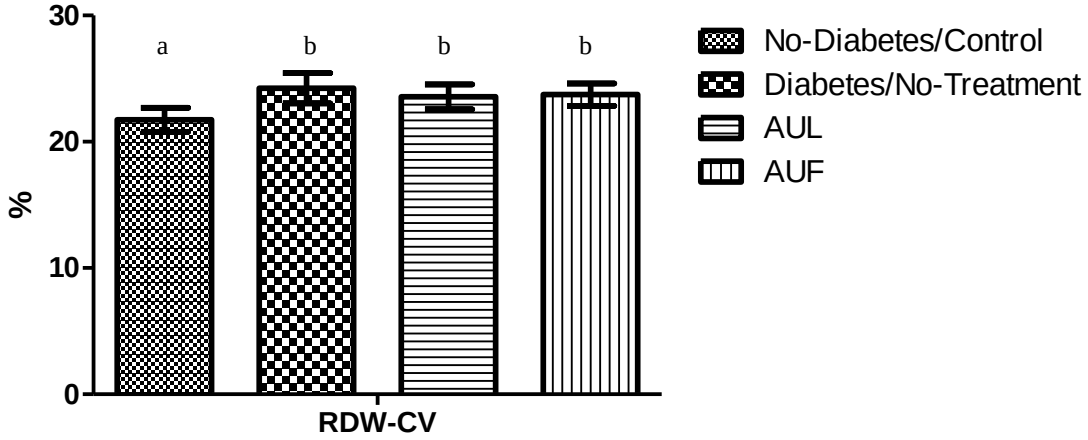

Figure 56: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **red blood cell distribution width**. Bars with SD bars without common superscript differ significantly ($p < 0.05$).

Figure 57: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **platelet distribution width**.

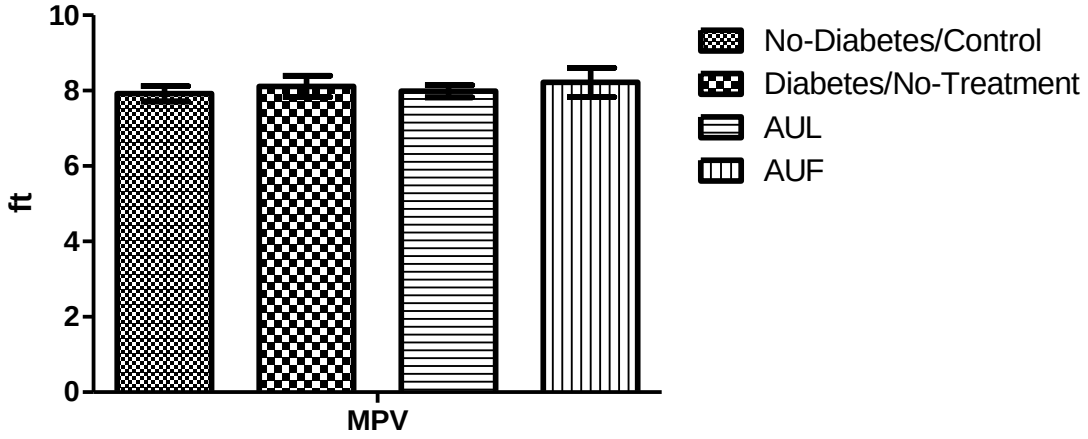

Figure 58: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **mean platelet volume**.

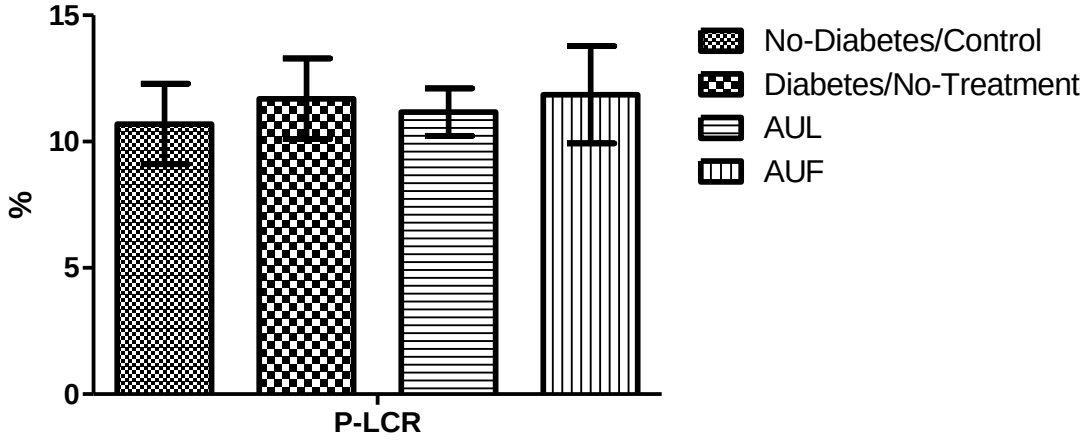

Figure 59: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **platelet larger cell ratio**.

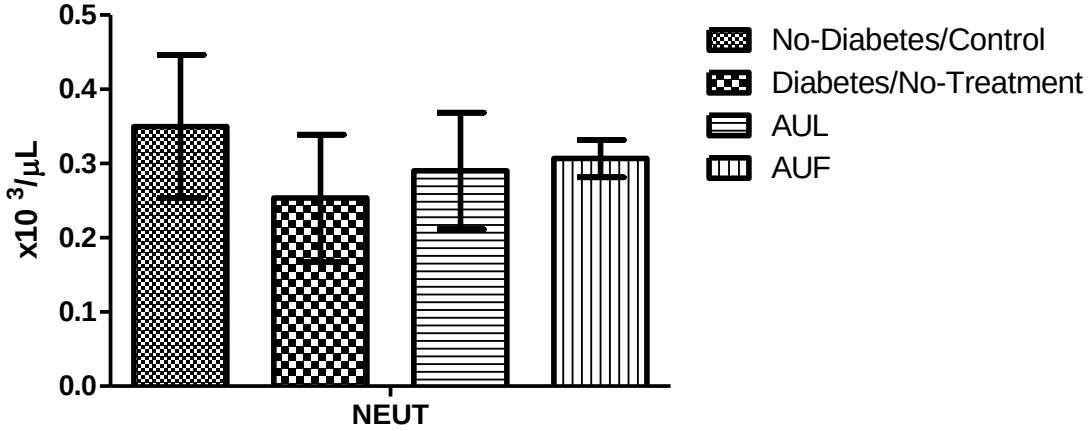

Figure 60: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **neutrophils levels**.

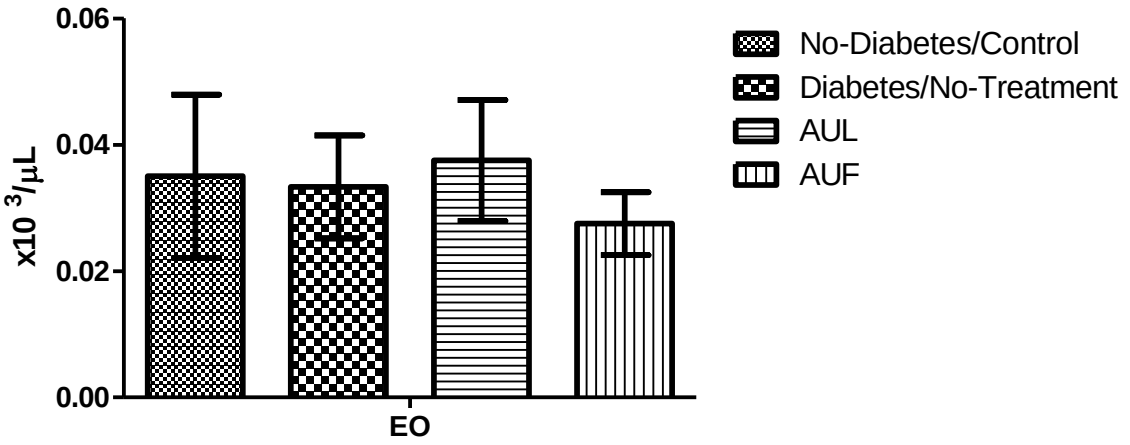

Figure 61: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **eosinophils levels**.

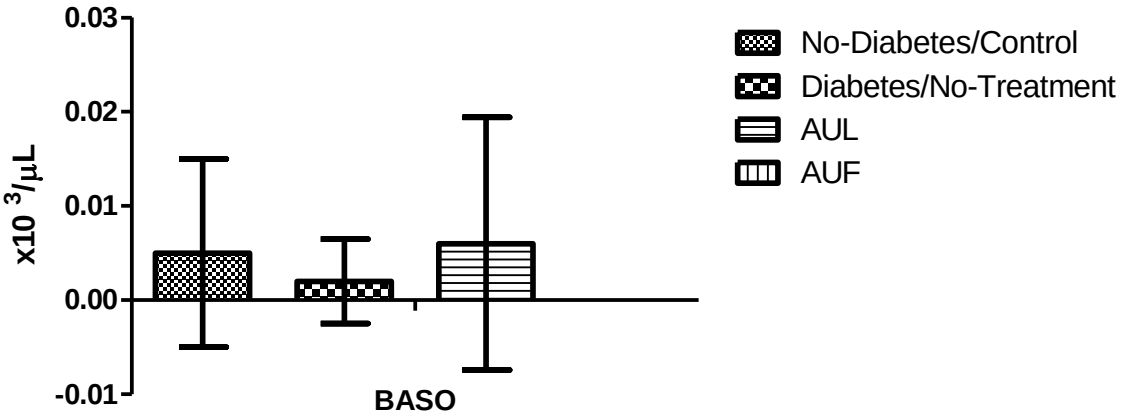

Figure 62: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **basophils levels**.

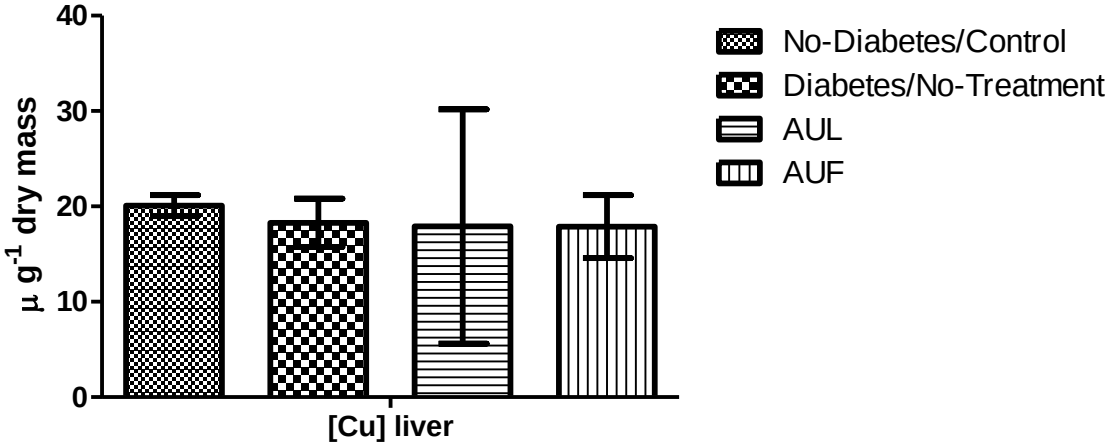

Figure 63: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **copper concentration in liver tissue (µg $^{-1}$ dry mass)**.

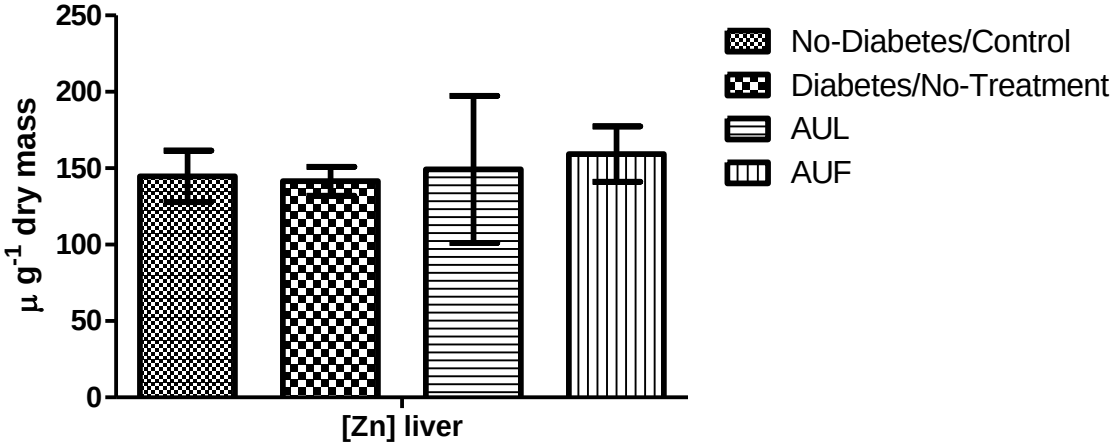

Figure 64: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **zinc concentration in liver tissue (µg $^{-1}$ dry mass)**.

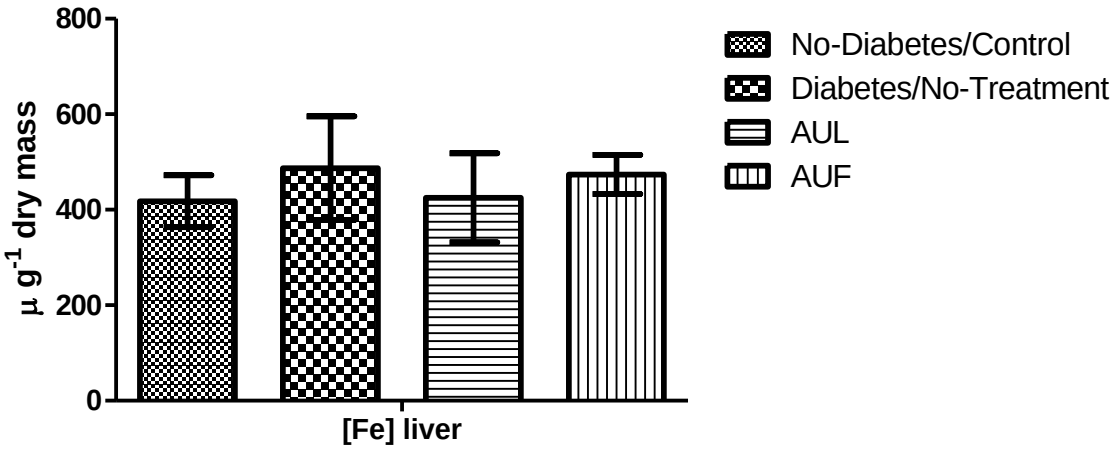

Figure 65: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **iron concentration in liver tissue (µg $^{-1}$ dry mass)**.

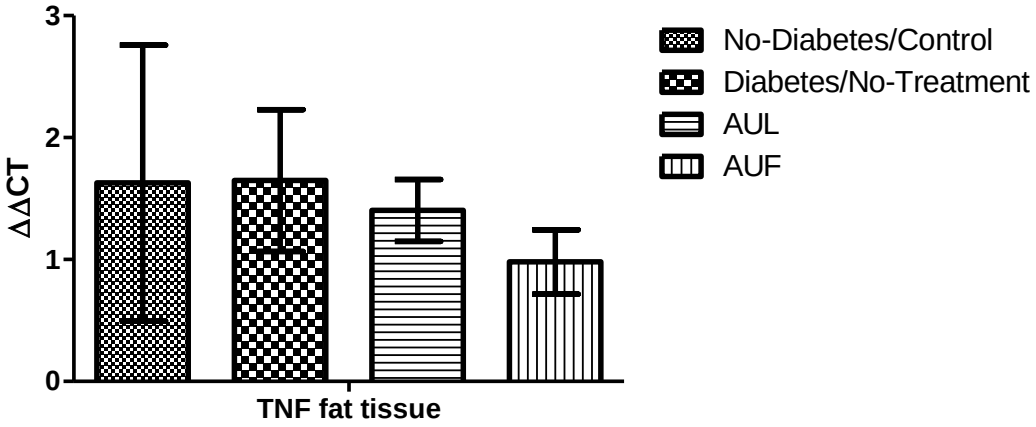

Figure 66: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of TNF alpha gene in fat tissue**.

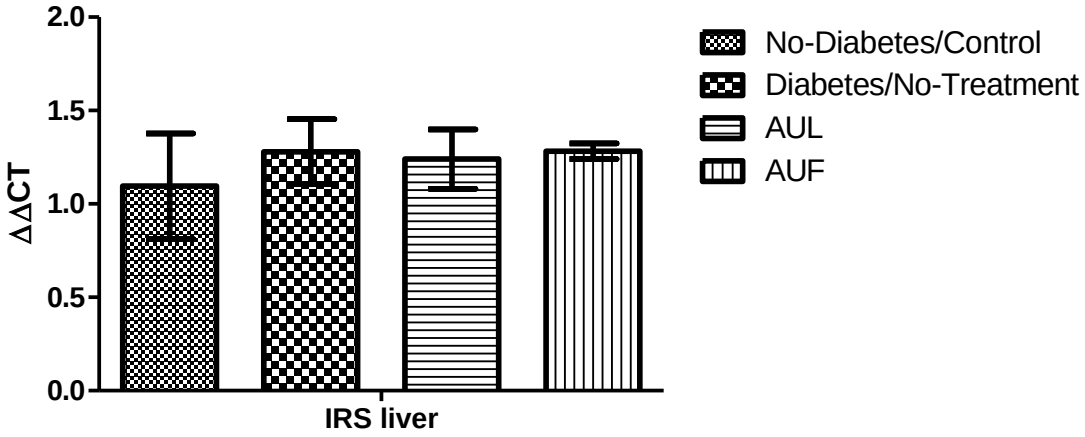

Figure 67: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of IRS gene in hepatic tissue**.

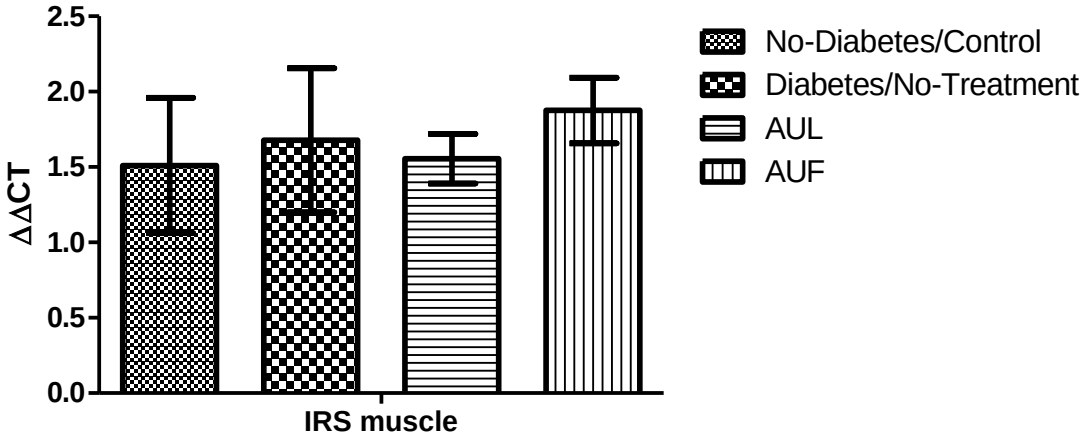

Figure 68: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of IRS gene in muscular tissue**.

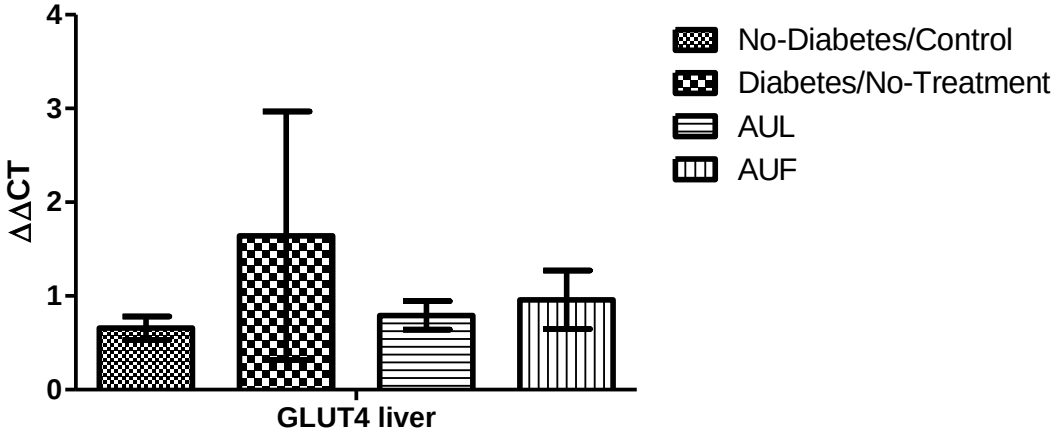

Figure 69: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and Arbutus unedo L. leaf (AUL) and fruit (AUF) aqueous extract in the **expression of GLUT-4 gene in hepatic tissue.**

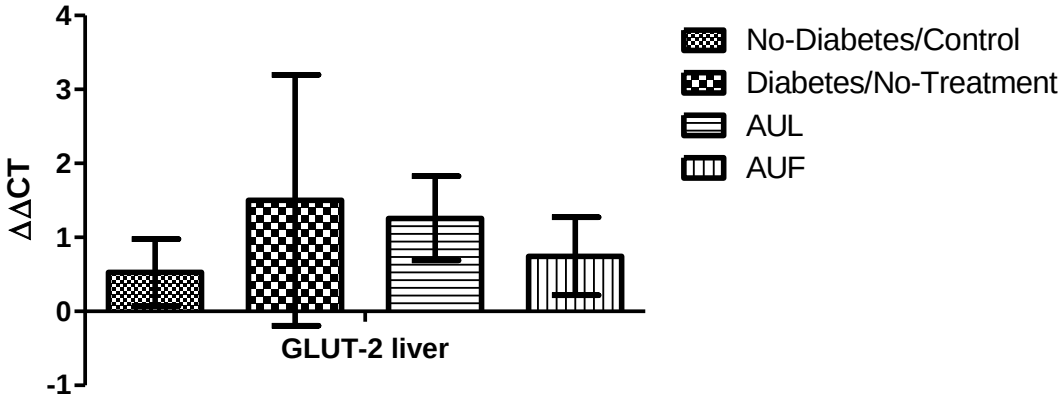

Figure 70: Effect of high-fat diets (No-Diabetes/Control), STZ injection (Diabetes/No-treatment) and *Arbutus unedo* L. leaf (AU_L) and fruit (AU_F) aqueous extract in the **expression of GLUT-2 gene in hepatic tissue**.