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Studio *in vitro* e *ex vivo* dell'attività antiossidante di *Casimiroa* spp, *Croton lechleri*, *Ribes nigrum* e *Boswellia serrata* nella prevenzione dell'aterosclerosi

In vitro and *ex vivo* antioxidant activity of *Casimiroa* spp, *Croton lechleri*, *Ribes nigrum*, and *Boswellia serrata* in atherosclerosis prevention

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Oxidative and glycoxidative stress are postulated to be the primary events in the pathogenesis of type 2 diabetes mellitus and its vascular implications. Further, LDL oxidation in the vessel wall plays a key role in atherogenesis, also related to damage from oxygen species (ROS). Moreover, the risk for development of atherosclerosis is by approximately three-fold increased in patients with diabetes. The medicinal plants are widely used in folk medicine for the treatment of cardiovascular diseases and diabetes mellitus. The genus *Casimiroa* (Rutaceae) includes few species which have their habitat in Central America and Mexico; among these, the most common are C*asimiroa edulis* Llave et lex. and *Casimiroa pubescens* Ramirez. The decoction of leaves and seeds are traditionally used for treating hypertension. The sap of *Croton lechleri* (Euphorbiaceae), a South American tree, is used topically in the treatment of wound healing and orally, in a dilute form, mainly for gastric ulcers and intestinal diseases. The gum resin of *Boswellia serrata* (Burseraceae), which grows in dry mountainous regions of India, Northern Africa and Middle East, has been traditionally used to treat various chronic inflammatory diseases. *Ribes nigrum* (Grossulariaceae), a species native to central and northern Europe and northern Asia, is a traditional medicine for the treatment of inflammatory disorders such as rheumatic diseases.
The aim of this research was to investigate the antioxidant activity of these medicinal plants by means of several experimental methods *in vitro* and *ex vivo* to outline their role in the prevention and/or treatment of cardiovascular diseases related to oxidative stress.
The antioxidant activity was evaluated by DPPH· method, and ORAC (Oxygen Radical Absorbance Capacity) assay. Also, the total phenolic content (TPC) was determined by the use of Folin-Ciocalteu reagent, and the total flavonoid content (TFC) by complexation with chloride aluminium. The activity of the plant extracts on LDL oxidation was studied by monitoring the formation of conjugated dienes, and the quantification of thiobarbituric acid reactive substances (TBARS). Finally, their inhibitory effect on advanced glycation end products (AGEs) formation were evaluated by means of BSA-glucose/ribose fluorescence assay.
In DPPH· assay, *Croton lechleri* sap and blackcurrant (*Ribes nigrum*) bud extract showed higher scavenging activity in comparison with *Casimiroa* extracts, whereas in the ORAC assay the *Casimiroa* leaf extracts showed a high ORAC value and *Croton lechleri* an activity even higher. In TPC test, *Croton lechleri* showed the highest value (713.76 ± 32.23 mg GAE/g). In the LDL oxidation assay, the plant extracts exhibited considerable protective effects by prolonging the oxidation lag phase; for example, at the concentration of 0.8 μg/ml *Croton lechleri* increased the lagtime by 58.6%, and in the antiglycation study all extracts inhibited the AGEs formation significantly in the BSA-glucose model. The results from this research suggest that the medicinal plants *Croton lechleri*, *Casimiroa* spp. and *Ribes nigrum*, even if in different manner, may have implications in the prevention of atherosclerotic vascular diseases, whereas *Boswellia serrata* showed a minor role.

Abstract

Studio *in vitro* ed *ex vivo* dell'attività antiossidante di *Casimiroa* spp, *Croton lechleri*, *Ribes nigrum* e *Boswellia serrata* nella prevenzione dell'aterosclerosi

Si ritiene che lo stress ossidativo e glicossidativo sia uno degli eventi primari nella patogenesi del diabete mellito di tipo 2 e delle sue implicazioni vascolari. Inoltre, l'ossidazione delle lipoproteine a bassa densità (LDL) all'interno delle pareti vascolari svolge un ruolo chiave nel processo di aterogenesi, oltre ad essere correlata ai danni prodotti dalle specie reattive dell'ossigeno (ROS). Peraltro, il rischio di sviluppo di aterosclerosi è di circa tre volte maggiore nei pazienti affetti da diabete. Le piante medicinali sono ampiamente utilizzate nella medicina popolare per il trattamento delle malattie cardiovascolari e del diabete mellito. Il genere *Casimiroa* (Rutaceae) comprende alcune specie il cui habitat è America Centrale e Messico; tra queste, le specie più comuni sono *Casimiroa edulis* Llave et lex. e *Casimiroa pubescens* Ramirez. Il decotto di foglie e semi è tradizionalmente utilizzato per il trattamento dell'ipertensione. Il latice di *Croton lechleri* (Euphorbiaceae), un albero sudamericano, viene utilizzato come cicatrizzante per uso topico, e, per via orale in forma diluita, per il trattamento di ulcere gastriche e malattie intestinali. La resina gommosa di *Boswellia serrata* (Burseraceae), pianta che cresce nelle regioni aride montuose di India, Nord Africa e Medio Oriente, è tradizionalmente usata per il trattamento di varie malattie infiammatorie croniche. *Ribes nigrum* (Grossulariaceae), una specie originaria dell'Europa centrale e settentrionale e Asia settentrionale , è utilizzato in medicina tradizionale per il trattamento di malattie infiammatorie come le malattie reumatiche.
Lo scopo di questa ricerca prevede lo studio dell'attività antiossidante di queste piante medicinali mediante diversi metodi sperimentali *in vitro* ed *ex vivo*, per delineare il loro ruolo nella prevenzione e/o trattamento di malattie cardiovascolari legate allo stress ossidativo.
L'attività antiossidante è stata valutata tramite il saggio DPPH• e il saggio ORAC (*Oxygen Radical Assorbanza Capacity*). Inoltre, sono stati valutati il contenuto fenolico totale (TPC), mediante l'uso del reattivo di Folin-Ciocalteu, e il contenuto di flavonoidi totale (TFC), mediante complessazione con alluminio cloruro. L'attività degli estratti vegetali sull'ossidazione delle LDL è stata studiata monitorando la formazione di dieni coniugati, e la quantificazione di sostanze reattive dell'acido tiobarbiturico (TBARS). Infine , il loro effetto inibitorio sulla formazione di prodotti finali della glicazione avanzata (AGE) è stato valutato mediante test fluorimetrico con BSA-glucosio/ribosio.
Dai risultati ottenuti tramite il saggio DPPH•, si osserva che il latice di *Croton lechleri* e il gemmoderivato di ribes nero (*Ribes nigrum*) hanno mostrato un'attività *scavenging* più elevata rispetto agli estratti di *Casimiroa*, mentre nel saggio ORAC gli estratti di foglie di *Casimiroa* hanno mostrato un elevato valore di capacità antiossidante, e il latice di *Croton lechleri* un'attività ancora più elevata. Nel saggio TPC, *Croton lechleri* ha mostrato il valore più alto (713,76 ± 32,23 mg GAE/g). Nel saggio di ossidazione delle LDL, gli estratti vegetali hanno mostrato effetti protettivi notevoli prolungando la fase di latenza di ossidazione; ad esempio, alla concentrazione di 0,8 mg/ml *Croton lechleri* ha determinato un prolungamento del tempo di latenza del 58,6%. Nello studio antiglicativo tutti gli estratti hanno significativamente inibito la formazione di AGEs nel modello BSA-glucosio. I risultati di questa ricerca suggeriscono che le piante medicinali *Casimiroa* spp., *Croton lechleri* e *Ribes nigrum*, anche se in modo diverso, possono avere implicazioni nella prevenzione delle malattie vascolari aterosclerotiche, mentre *Boswellia serrata* risulta possedere un ruolo minore.

ABBREVIATIONS

$O_2^{\bullet -}$: superoxide

$^{\cdot}OH$: hydroxyl radical

$^1O_2$: singlet oxygen

$A^{\cdot}$: radical

$A^{+\cdot}$: radical cation

AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride

AGEs: advanced glycosylation end products

AH: antioxidant

AOC: antioxidant capacity

ApoB: apolipoprotein B

ATP: adenosine triphosphate

ASC: ascorbic acid

AUC: area under the curve

bFGF: basic fibroblast growth factor

BHA: butylated hydroxyanisole

BHT: butylated hydroxytoluene

Bs: oleo-gum extract of *Boswellia serrata*

BSA: Albumine Bovine fraction V lyophilized powder

CAT: catalase

CD59: CD59 glycoprotein

Ce1: methanolic seed extract of *Casimiroa edulis*

Ce2: aqueose leaf extract of *Casimiroa edulis*

CML: carboxymethyl-lysine

Cp1: methanolic seed extract of *Casimiroa pubescens*

Cp2: methanolic leaf extract of *Casimiroa pubescens*

$CuSO_4$: copper sulphate

CVD: cardiovascular disease

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EC: endothelial cell

EC_{50}: half maximal effective concentration

EDTA: ethylenediaminetetraacetic acid

FDA: Food and Drug Administration

FL: fructose-lysine

FoxLDL: fully oxidized LDL

GAE: gallic acid equivalents

GPx: glutathione peroxidase

GSH: glutathione

H_2O_2: hydrogen peroxide

HAT: hydrogen atom transferring

HDL: high-density lipoprotein

HIV/AIDS: human immunodeficiency virus infection / acquired immunodeficiency syndrome

HUVEC: human umbilical vein endothelial cells

IGF-1: insulin-like growth factor 1

IL-1: interleukin 1 family

LDL: low-density lipoprotein

LOO•: lipid peroxy radical

LOOH: lipid hydroperoxide

LOX-1: lectin-like Ox-LDL receptor

Mφ: macrophages

MCP-1: monocytes chemoattractant protein-1

MCSF: macrophage colony stimulating factor

MDA: malondialdehyde

MM-LDL: minimally modified LDL

MMP: matrix metalloproteinases

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB: epidermal growth factor

NO⋮ nitric oxide

ORAC: oxygen radical absorbance capacity

Ox-LDL: oxidized low-density lipoprotein

PBS: phosphate buffered saline

pD_2: -log EC50

PDGF: platelet-derived growth factor

QE: quercetin equivalents

RCS: reactive carbonyl species

Rn: 2% hydroalcoholic solution of the extract of the buds of *Ribes nigrum*

RNS: reactive nitrogen species

ROS: reactive oxygen species

SdD: dry residue obtained by lyophilisation of *Croton lechleri* sap

SET: single electron transfering

SMC: smooth muscle cell

SOD: superoxide dismutase

SRs: scavenger receptors

TBA: thiobarbituric acid

TBARS: thiobarbituric acid reactive substances

TCA: trichloroacetic acid

TEAC: trolox equivalent antioxidant capacity

TFC: total flavonoid content

TNF-α: tumor necrosis factor

TPC: total phenolic content

VSMC: vascular smooth muscle cells

INTRODUCTION

- **Atherosclerosis**

Cardiovascular diseases (CVDs) are the leading cause of death globally: more people die annually from CVDs than any other cause. About 17.3 million people died from CVDs in 2008, representing 30% of all global deaths (Alwan, 2011); of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke (*Global atlas on cardiovascular disease prevention and control*2011) .

Atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. This pathological condition, commonly referred to as a hardening or furring of the arteries (Maton, 1993), is a specific form of arteriosclerosis in which an artery wall thickens as a result of the accumulation of calcium and fatty materials such as cholesterol and triglyceride. It is a chronic inflammatory response in the arterial walls affecting blood vessels caused largely by the accumulation of macrophages and white blood cells and promoted by low-density lipoproteins (LDL), without adequate removal of fats and cholesterol from the macrophages by functional high-density lipoproteins (HDL). It is caused by the formation of multiple plaques within the arteries. The atheromatous plaque usually has three distinct components:

1. the atheroma ("lump of gruel", from greek ἀθήρα *(athera)*, meaning "gruel"), which is the nodular accumulation of a soft, flaky, yellowish material at the center of large plaques, composed of macrophages nearest the lumen of the artery;
2. underlying areas of cholesterol crystals;
3. calcification at the outer base of older/more advanced lesions.

Atherosclerosis is a chronic disease that remains asymptomatic for decades (Ross, 1993). Atherosclerotic lesions or atherosclerotic plaques are separated into two broad categories: stable and unstable, also called vulnerable (Ross, 1999). The pathobiology of atherosclerotic lesions is very complicated but generally, stable atherosclerotic plaques, which tend to be asymptomatic, are rich in extracellular matrix and smooth muscle cells, while, unstable plaques are rich in macrophages and foam cells and the extracellular matrix separating the lesion from the arterial lumen (also known as the fibrous cap) is usually weak and prone to rupture (Finn, Nakano, Narula, Kolodgie, & Virmani, 2010). Ruptures of the fibrous cap expose thrombogenic material, such as collagen to the

circulation and eventually induce thrombus formation in the lumen (Didangelos, Simper, Monaco, & Mayr, 2009). Upon formation, intraluminal thrombi can occlude arteries outright (e.g. coronary occlusion), but more often they detach, move into the circulation and eventually occluding smaller downstream branches causing thromboembolism. Apart from thromboembolism, chronically expanding atherosclerotic lesions can cause complete closure of the lumen. Interestingly, chronically expanding lesions are often asymptomatic until lumen stenosis is so severe (usually over 80%) that blood supply to downstream tissue(s) is insufficient, resulting in ischemia.

These complications of advanced atherosclerosis are chronic, slowly progressive and cumulative. Most commonly, soft plaque suddenly ruptures, causing the formation of a thrombus that will rapidly slow or stop blood flow, leading to death of the tissues fed by the artery in approximately 5 minutes, resulting in an infarction. A coronary thrombosis of a coronary artery cause myocardial infarction (a heart attack), and the same process in an artery to the brain cause a stroke. A combination of both stenosis and aneurysmal segments narrowed with clots in very advanced disease can cause a claudication from insufficient blood supply to the legs.

Atherosclerosis affects the entire artery tree, in particular larger, high-pressure vessels such as the coronary, renal, femoral, cerebral, and carotid arteries. These are termed "clinically silent" when infarctions involve only very small amounts of tissue and the person having the infarction does not notice the problem and does not seek medical help, or when they do, physicians do not recognize what has happened.

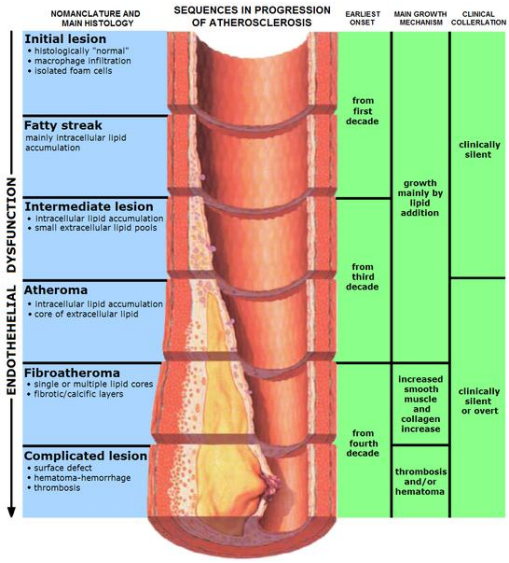

Figure 1. Representation of the progression of atherosclerosis.
From: http://en.wikipedia.org/wiki/Atherosclerosis

The lipid peroxidation, the hallmark of fatty streak formation, is the earliest manifestation of atherosclerosis. Macrophages (Mφ) take up unmodified (native) LDL at low regulated rates, whereas structurally modified LDL (Ox-LDL) is avidly taken up by Mφ to form foam cells (Brown & Goldstein, 1983). This occurs at uncontrolled rates, not subject to negative feedback, via specific scavenger receptors (SRs) (Nedeljkovic, Gokce, & Loscalzo, 2003; Steinberg & Witztum, 2002). These findings provided the theory that Ox-LDL plays a pivotal role in atherosclerotic initiation and development; providing a mechanistic link between hypercholesterolaemia and CVD.

Native LDL accumulates in the extracellular subendothelial space of arteries and can be oxidatively modified by all major arterial wall cell types, including endothelial cells, Vascular Smooth Muscle Cells (VSMC) and Mφ (Ting et al., 1997). Both the lipid and protein moieties of lipid particles can be oxidized, yielding a broad spectrum of Ox-LDL species, differing structurally and functionally, dependent on the degree of oxidative modification (Steinberg & Witztum, 2002).

Three lines of evidence support that LDL *in vivo* oxidation contributes to the formation and progression of atherosclerotic plaques. First, oxidatively modified LDL accumulates and is extracted from atherosclerotic lesions, correlating with atherosclerotic risk (Chisolm, Hazen, Fox, & Cathcart, 1999). Second, immunohistochemistry reveals that epitopes in atherosclerotic lesions react with antibodies raised against Ox-LDL. Third, human and animal studies demonstrate the presence of autoantibodies, which react with Ox-LDL, suggesting the presence of Ox-LDL *in vivo*, or a similar epitope (Steinberg, 1997).

Human *ex vivo* data on lipid peroxidation showed a significant positive correlation between patients with acute coronary syndromes and plasma/arterial wall Ox-LDL levels in coronary atherectomy specimens (Ehara et al., 2001). This evidence supports previous investigations (Toshima et al., 2000) suggesting that plasma Ox-LDL may be a useful marker of CVD. The power of plasma Ox-LDL to predict the burden of atherogenesis, and the type of epitope most representative of Ox-LDL *in vivo* remains to be determined (Tsimikas & Witztum, 2001).

Ox-LDL can be indirectly atherogenic by inducing extensive humoral and cellular responses; extending beyond foam cell formation. Both minimally modified LDL (MM-LDL) and fully oxidized LDL (FoxLDL) stimulate monocyte-endothelial cell interactions

and the expression of adhesion molecules in different ways; promoting atherogenesis and plaque instability (Frei, 1999). MM-LDL can stimulate pro-inflammatory signals causing increased adherence and penetration of monocytes to endothelial cells via inducing expression of monocytes chemoattractant protein-1 (MCP-1) and macrophage colony stimulating factor (MCSF), stimulating Mφ differentiation and up-regulation of SRs (Steinberg, 1997).

By contrast, LOX1's major ligand FoxLDL which is directly chemotactic for monocytes, VSMC, and T cells; stimulates Mφ and VSMC mitosis; induces endothelial cell cytotoxicity and inhibits Mφ motility (Frei, 1999). Additionally, in cell culture studies, FoxLDL stimulates juxtaglomerular cell renin release in the kidney, associated with enhanced O_2^- production (Galle & Heermeier, 1999). Moreover, experimental evidence suggests that the binding of Ox-LDL with LOX-1 induces ROS production, VSMC apoptosis and modulation of MMP activity, potentially causing plaque instability (Szmitko et al., 2003; Thomson, Puntmann, & Kaski, 2007).

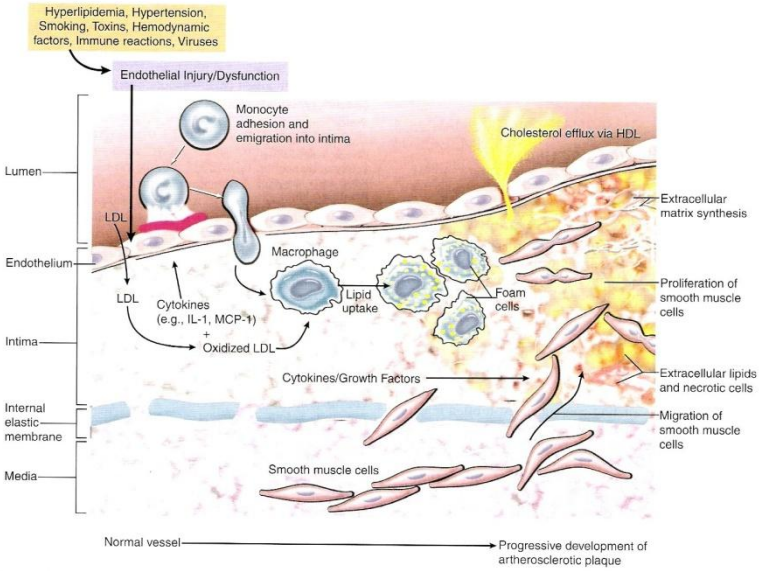

Figure 2. Representation of the sequences of cellular interactions in developing atherosclerosis. From: (Kumar, Vinay,, Abbas, Abul K., Fausto, Nelson., Robbins, Stanley L., Cotran,Ramzi S.,, 2005)

- **ROS and Antioxidants**

Reactive oxygen species (ROS) are highly reactive molecules or molecular fragments that are continuously produced in all aerobic organisms, mostly as a consequence of aerobic respiration. With the help of the mitochondrial respiratory chain, aerobic organisms are able to attain a far greater energy production efficiency compared to anaerobic organisms.

However, one disadvantage of aerobic respiration is continuous electron leakage to O_2 during mitochondrial ATP synthesis. 1–5% of total oxygen consumed in aerobic metabolism therefore produces the superoxide anion, ($O_2^{\cdot -}$), the first reduction product of O_2 (Dreher & Junod, 1996). Besides oxidative phosphorylation, low levels of ROS are continuously formed in peroxisomes, the cytochrome P450 system and inflammatory cells, including neutrophils, eosinophils and macrophages. Some exogenous sources of radicals also exist, including ionizing radiation, ozone, and many chemotherapeutic drugs. The term ROS covers several types of reactive oxygen metabolites, including free radicals, which are defined as a molecule containing one or more unpaired electrons on its outermost orbital, for example, superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen $^1O_2$, (Tab. 1) (Wiseman & Halliwell, 1996). The term ROS also encompasses some non-radicals such as hydrogen peroxide (H_2O_2). The life-span of different ROS varies considerably, from less than 1 ns of $^{\cdot}OH$ to even hours of H_2O_2, depending on numerous cellular environment factors (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). Besides the high reactivity, another important feature of ROS is that their reactions with non-radicals tend to result in the formation of new radicals.

The term ROS can also be taken to cover nitric oxide-derived reactive molecules, such as peroxynitrite. These molecules play important roles in many physiological processes; however, if the amount of ROS exceeds the capacity of the ROS-suppressing machinery, oxidative stress is said to occur. This imbalanced redox status is sufficiently potent to induce damage in all cellular macromolecules, including DNA (Wiseman & Halliwell, 1996). ROS are nowadays considered as a significant class of carcinogens participating in cancer initiation, promotion and progression (Klaunig et al., 1998). However, they also have important roles in intracellular and intercellular signaling. Nowadays H_2O_2 is recognized as a key intracellular messenger at subtoxic levels in certain important signal pathways, such as epidermal growth factor and NF-κB activation (Bae et al., 1997; Rhee, 1999). In addition, H_2O_2 plays a crucial role as a mediator of the effects of platelet-derived growth factor (PDGF), epidermal growth factor and angiotensin II. This is underlined by the observation that all of these signaling pathways are completely blocked after the specific inhibition of H_2O_2 (Bae et al., 1997; Sundaresan, Yu, Ferrans, Irani, &

Finkel, 1995; Ushio-Fukai et al., 1999). H_2O_2 and $NO^{\cdot}$ are also involved essentially in apoptotic pathways (Finkel, 1998; Karihtala & Soini, 2007).

Table 1. Formation of the biologically most important reactive oxygen species.

Reaction	Note
$O_2+e^- \rightarrow O_2^{\cdot -}$	Superoxide formation (various sources)
$2\ O_2^{\cdot -}+2\ H^+ \rightarrow H_2O_2+O_2$	Hydrogen peroxide formation, catalyzed by SODs
$Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+ OH+OH^-$	Fenton reaction
$O_2^{\cdot -}+H_2O_2 \rightarrow \cdot OH+OH^-+O_2$	Haber-Weiss reaction (iron-catalyzed)

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Valko et al., 2007).

Based on their mechanism of action, the antioxidants can be divided in two types.

- Type I : "Chain breaker" .

They are able to inactivate free radicals by donating hydrogen atom or transferring a single electron to the free radical species. They are compounds that, thanks to their negative redox potential, are able to provide electrons to the free radicals, thus restoring the chemical balance of the system. Their effectiveness depends on the stability of the radicals in which they are transformed; therefore, the more efficient the delocalization of unpaired electrons produced in the reaction with free radicals, the greater its antioxidant power. Antioxidants of this type can dis-activate the radical species through two basic mechanisms: by transfer of a hydrogen atom (Hydrogen Atom Transfer, HAT) or by transfer of a single electron (Single Electron Transfer, SET). The final result is the same, but the kinetics and the potential of the reactions are different (Prior, Wu, & Schaich, 2005). In reality, these mechanisms may also take place at the same time, but it will be

chemical structure of antioxidant, together with its solubility properties, partition coefficient and solvent, to determine the prevalent mechanism of action. The bond-dissociation energy and the ionization potential are the two main factors that affect the mechanism and efficiency of antioxidant (Wright, Johnson, & DiLabio, 2001). Antioxidants "donors of a hydrogen atom" act according to the following scheme:

$$X\cdot + AH \rightarrow XH + A\cdot$$

Generally, one substance can act as an antioxidant if once oxidized, its radical form is not reactive or little reactive towards other molecules. HAT reactions are solvent and pH independent and, generally occur quite quickly, ending in a few seconds or a few minutes. Conversely, reactions SET shall run slowly and are pH-dependent. SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals:

$$X\cdot + AH \rightarrow X^- + AH^{\ddagger\cdot}$$
$$AH^{\ddagger\cdot} + H_2O \leftrightarrow A\cdot + H_3O^+$$
$$X^- + H_3O^+ \rightarrow XH + H_2O$$
$$M(III) + AH \rightarrow AH^+ + M(II)$$

Belong to this group of antioxidants *tert*-butyl-hydroxyanisole (BHA), the tert-butyl hydroxytoluene (BHT), tert-butyl-hydroxyquinone (TBHQ), propyl-gallate (PG), tocopherols and phenolic compounds.

- Type II: "Metal scavenger". Prevent the formation of free radicals by acting as metal chelating agents. Metal ions such as iron or copper are potent pro-oxidants that accelerate lipid oxidation lowering the activation energy of the reactions of initiation, generating alkyl radicals from fatty acids (1) or inducing the formation of singlet oxygen (much more reactive than normal triplet oxygen present in the air that we breathe) mediated by superoxide anion (2):

$$Fe^{3+} + RH \rightarrow Fe^{3+} + R\cdot \qquad (1)$$
$$Fe^{2+} + {}^3O_2 \rightarrow Fe^{3+} + O_2^-\cdot \rightarrow {}^1O_2{}^* + e- \qquad (2)$$

The metals also perpetuate lipid oxidation, producing free radicals via the Fenton reaction (3). This reaction is the major source of formation of alkoxy radicals, which are the most reactive and damaging ROS in biological systems:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH \cdot + OH^- \qquad (3)$$

Other free radicals formed by the metals are produced from the decomposition of lipid hydroperoxides (reactions (4) and (5)), in which the metal reacts either in the reduced form (Fe^{2+}) or in the oxidized form (Fe^{3+}); the latter, however, was found to produce radicals at a rate 10 times lower.

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO \cdot + OH^- \qquad (4)$$

$$Fe^{3+} + ROOH \rightarrow Fe^{2+} + ROO \cdot + H^+ \qquad (5)$$

Examples of metal-acid scavenger are ethylenediaminetetraacetic acid (EDTA) (Fig. 3), citric acid, ascorbic acid and some amino acids.

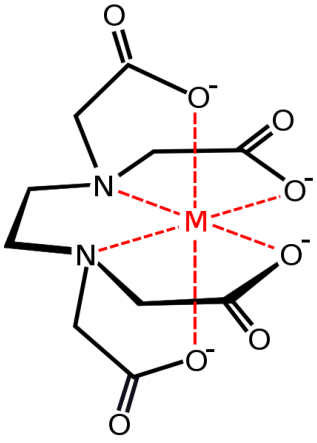

Figure 3. Chemical structure of Metal-EDTA chelate.

In nature, between the two classes of antioxidants there are not so precise limits; substances such as phenolic compounds can behave simultaneously both as chain breaker and as metal scavenger.

The plaque formation is proposed to be initiated at sites of endothelial damage inducing adhesion molecule and chemotactic factor expression. This leads to the tethering, activation and attachment of monocytes and T lymphocytes to endothelial cells, with consequent migration into the subendothelial space. Transformation of monocytes into macrophages (Mφ) generates further ROS which, alongside potential ROS production from other cell types, generates oxidized low density lipoprotein (Ox-LDL) promoting foam cell formation. Foam cells on combining with leucocytes formulate fatty streaks which can, with continued down-stream effects of ROS alongside inflammatory

pathways, contribute to advanced plaque formation encouraging plaque instability and thrombotic events (Madamanchi, Vendrov, & Runge, 2005; McCormick, Gavrila, & Weintraub, 2007). Fig. 4 demonstrates the proposed mechanisms of ROS production and plaque formation.

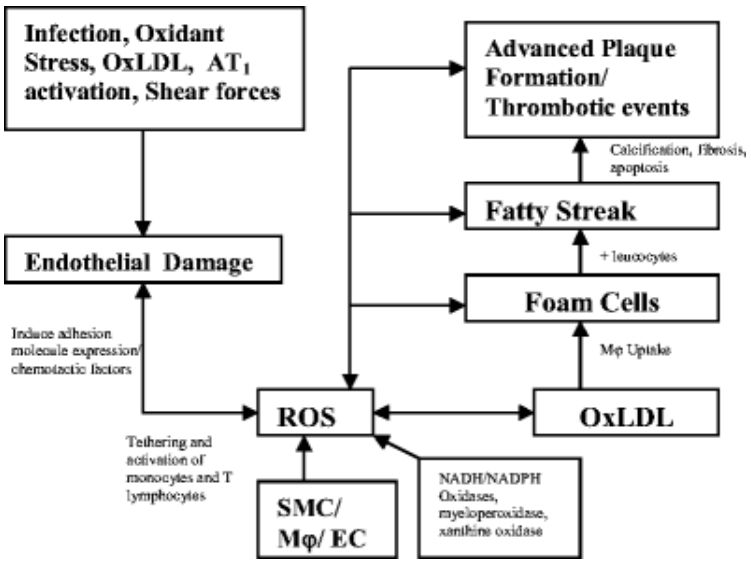

Figure 4. Summary of the role of ROS in plaque formation. From: (Thomson et al., 2007)

ROS generation causes apoptosis via caspase induction and collagen matrix degradation by activating matrix metalloproteinases (MMP), factors implicated in plaque instability (Irani, 2000; Nedeljkovic et al., 2003). Collagen, an important component of the matrix of atheromatous plaques is generated by a vitamin C-dependent process (Libby & Aikawa, 2002). Thus, at least theoretically, antioxidant vitamins may be significant in stabilizing plaques and inhibiting or slowing down advanced atheroma formation and disruption.

- **Hyperglycaemia promotes atherosclerosis**

Atherosclerosis is a leading cause of morbidity and mortality in patients suffering from diabetes mellitus. The risk for development of atherosclerosis is increased by approximately three fold in patients with diabetes as a result of a number of processes which are still poorly understood. One hypothesis is that increase modification of low density lipoprotein (LDL) by oxidation and/or glycation may enhance the atherogenic process in individual with diabetes. There is increasing evidence that both LDL and plasma from individuals with diabetes may be more susceptible to oxidation (RW.ERROR - Unable to find reference:93).

One of the important possible mechanisms responsible for the accelerated atherosclerosis in diabetes is the non-enzymatic reaction between glucose and proteins or lipoproteins in arterial walls, collectively known as Maillard, or browning reaction. Glucose forms chemically reversible early glycosylation products with reactive amino groups of circulating or vessel wall proteins (Schiff bases), which subsequently rearrange to form the more stable Amadori-type early glycosylation products. Equilibrium levels of Schiff-base and Amadori products (the best known of which is hemoglobin A_{1C}) are reached in hours and weeks, respectively (Fig. 5). Some of the early glycosylation products on long-lived proteins (e.g. vessel wall collagen) continue to undergo complex series of chemical rearrangement to form advanced glycosylation end products (AGEs). Once formed, AGE-protein adducts are stable and virtually irreversible. Although AGEs comprise a large number of chemical structures, carboxymethyl-lysine-protein adducts are the *in vivo* predominant AGEs (Aronson & Rayfield, 2002).

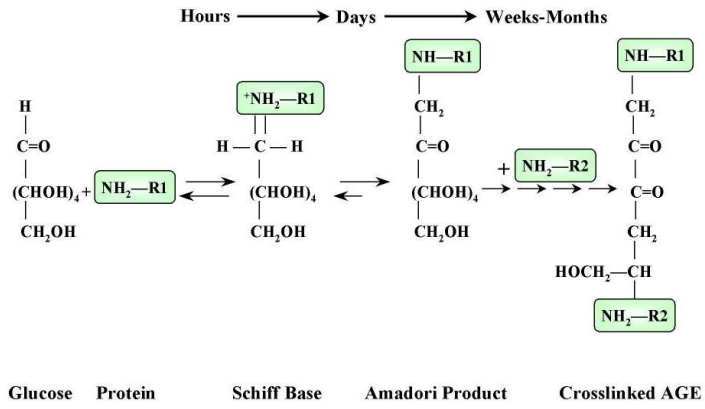

Figure 5. Formation of advanced glycosylation end products (AGEs). From: (Aronson & Rayfield, 2002)

AGEs can accelerate the atherosclerotic process by diverse mechanisms, which can be classified as non-receptor dependent and receptor-mediated.

- Non-receptor dependent mechanisms includes the cross-linking collagen and enhanced synthesis of extracellular matrix compounds, trapping of LDL, and quenching of nitric oxides; functional alterations of regulatory proteins such as bFGF and complement regulatory protein CD59; lipoprotein modifications, such as LDL glycosylation, reduced LDL receptor recognition, and increased susceptibility of LDL to oxidative modification (Fig. 6).

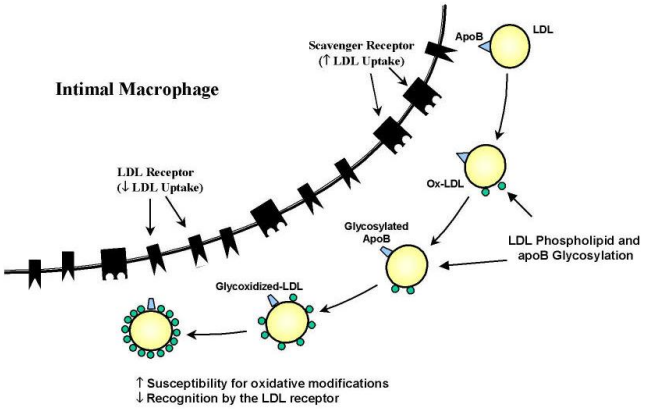

Figure 6. Potential mechanisms by which LDL glycosylation increases the atherogenicity. Advanced glycosylation of the phospholipid component of LDL is accompanied by the progressive oxidative modification of unsaturated fatty acid residues. Glycosylation of LDL apoB reduces its recognition by the LDL receptor and increases uptake through the scavenger receptor. From: (Aronson & Rayfield, 2002)

Receptor mediated mechanism involves promoting inflammation, secretion of cytokines such as TNF-α, IL-1, etc.; induction of cellular proliferation, such as stimulation of PDGF and IGF-1 secretion from monocytes and possibly SMC; and endothelial dysfunction, such as increased permeability of EC monolayers, procoagulant activity, expression of adhesion molecules and intracellular oxidative stress (Fig. 7), (Hsieh et al., 2007).

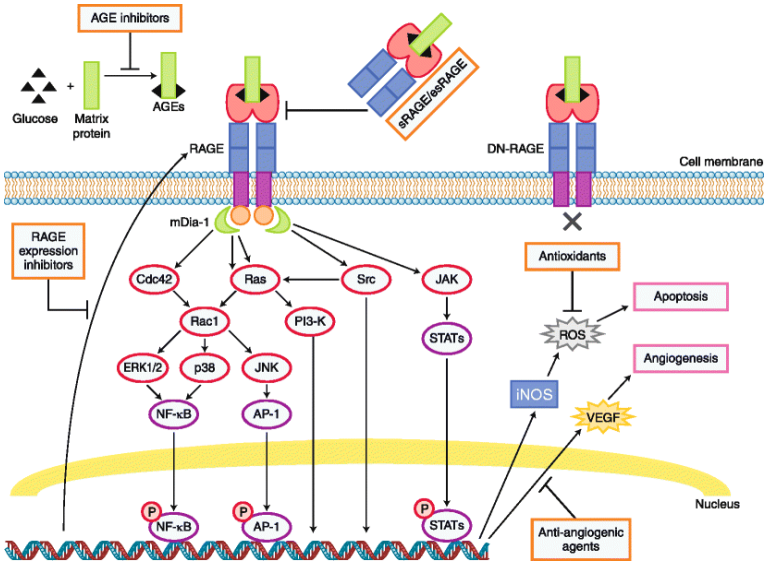

Figure 7. AGE/RAGE-mediated proinflammatory signaling and interventions. From: (Zong, Ward, & Stitt, 2011)

In short, both hyperglycemia and glycation clinically are associated with diabetic complications, while LDL glycation is thought to play an important role in the pathogenesis of vascular and neurodegenerative diseases (Aronson & Rayfield, 2002; Basta, Schmidt, & De Caterina, 2004).

- **Medicinal Plants**

Since very old times, herbal medications have been used for relief of symptoms of disease (Maqsood, Singh, Samoon, & Balange, 2010). The need for bioactive compounds with medicinal properties presents a tremendous challenge and has encouraged scientists to explore, in detail, plants that are potential sources of promising compounds (Holetz et al., 2002; Novais et al., 2003). Despite the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. Much interest in medicinal plants emanates from their use in folk medicines as well for their prophylactic properties, especially in developing countries. A large number of medicinal plants have been investigated for their antioxidant properties, either in the form of raw extracts or of their chemical constituents, which may be effective to prevent the destructive processes caused by oxidative stress (Zengin, Aktumsek, Guler, Cakmak, & Yildiztugay, 2011). Although the toxicity profile of most medicinal plants has not been thoroughly evaluated, it is generally accepted that medicines derived from plant products are safer than their synthetic counterparts (Vongtau et al., 2005; y Tejidos, Sobre el Ciclo Estral, 2007).

Furthermore, natural plant extracts and purified constituents have been evaluated for their role in preventing the formation of AGEs. So far, phenolic antioxidants have been found to be the most promising agents, and their activities against AGE formation *in vitro* have been shown, with a few exceptions, to correlate highly with their free radical scavenging activities. However, several clinical trials have failed to provide conclusive evidence for the efficacy of natural antioxidant therapy in diabetic patients. Therefore, it would be of great interest discovering natural AGE inhibitors that can suppress the formation of AGEs, both through preventing glycoxidation (scavenging free radical and/or chelating metal ions) and by sequestering reactive carbonyl species (especially 1,2-dicarbonyls, the key intermediates in the glycation of proteins). So far, very few natural products have been found to have scavenging activities against the reactive carbonyl species (RCS) (Peng et al., 2008).

Therefore, the aim of this research was to investigate the antioxidant and antiglycation activities of selected medicinal plants by means of several experimental methods *in vitro* and *ex vivo* to outline their role in the prevention and/or treatment of cardiovascular diseases related to oxidative stress.

- ***Croton lechleri*** Muell.-Arg

Croton lechleri (Euphorbiaceae) is a tree which grows in the low mountainous areas of the Peruvian Andean region, as well as in Colombia, Ecuador and Bolivia and it is known for its medicinal properties. The bark, when slashed, releases a red latex called "sangre de drago" or "sangre de grado" or "dragon's blood" (Fig. 8). The blood-red latex (sap) is a common household remedy in Peru and in other Latin American countries, where indigenous tribes use the sap internally and externally to stop bleeding, help heal wounds, and treat intestinal ailments.

In vitro and *in vivo* studies support the traditional use the viscous latex, which exhibits antioxidant, antiviral and anti-inflammatory activities, in addition to being efficacious in the treatment of different types of diarrhea, including cholera. The oral administration of a preparation, termed SP-303, isolated from the bark latex by Ubillas *et al*. (Ubillas et al., 1994), leads to positive results in the treatment of traveler's diarrheas and diarrheal episodes in AIDS patients and was approved as Crofelemer (Fulyzaq®) by the FDA in December 2012 to treat diarrhea in HIV/AIDS patients on antiretroviral therapy (Yeo, Crutchley, Cottreau, Tucker, & Garey, 2013). Crofelemer is an oligomeric proanthocyanidin mixture primarily composed of (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin monomer units linked in random sequence (Tradtrantip, Namkung, & Verkman, 2010). When applied to the skin for treating abrasions and blisters, the red sap forms a seal, protecting the lesion. Thus, it is applied topically to reduce the symptoms of insect bites with a reduction of swelling and redness. The sap has been used in the treatment of several types of tumors. Since free radicals may participate in the early stages of carcinogenesis, recently antioxidant activity was evaluated against the oxidative damages induced by apomorfine in *Saccaromices cerevisiae* (De Marino et al., 2008).

The sap derived by *C. lechleri* and related *Croton* species has been thoroughly investigated, both in terms of phytochemical profile and bioactivity, disclosing a unique phytocomplex characterized by peculiar lignans, proanthocyanidins, flavonols, steroids, and alkaloids. The characteristic secondary metabolites are proanthocyanidins, which account for up to 90% of dry weight and many polyphenolic components such as catechin, epicatechin, gallocatechin, epigallocatechin and dimeric procyanidins B-1 and B-4. Several minor constituents were also identified: clerodane diterpenoids such as korberin A and B, bincatriol, crolechinol, crolechinic acid and the dihydrobenzofuran

lignan 3',4-*O*-dimethylcedrusin. Work on *Croton lechleri* led to the isolation of a benzylisoquinoline-like alkaloid taspine in the sap and thaliporphine and glaucine in the leaves. Taspine and the lignan 3',4-*O*-dimethylcedrusin are thought to be responsible for the wound healing actions of *sangre de drago*, because of their stimulatory actions on wound repair (De Marino et al., 2008; D. Gupta, Bleakley, & Gupta, 2008).

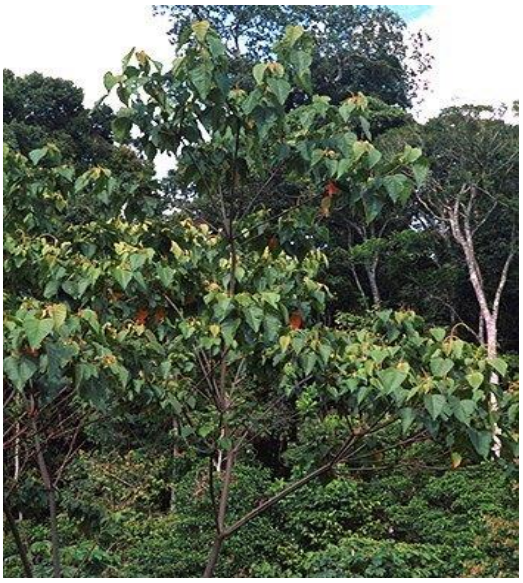

Figure 8. The *Croton lechleri* tree and the latex derived from the incision of its bark. From: http://ccbolgroup.com/sangreE.html and http://www.inriodulce.com/links/medicinalplants.ht

- ***Casimiroa* spp.**

Casimiroa edulis Llave et Lex (Rutaceae) (Fig. 9) popularly called "Zapote blanco", is a tree distributed in the temperate zones of Mexico and central America. The use of the tree in folk medicine is known from prehistoric times, where concoctions of the leaves or seeds are taken for their interesting sedative-like and sleep inducing effects. Furthermore, the seeds are also known to be used in the treatment of dermatological conditions (Romero, Escobar, Lozoya, & Enriquez, 1983).

Most of the studies have been performed on the seeds, bark and fruits of *Casimiroa edulis*, and afforded a number of alkaloids, coumarins, flavonoids, zapotin, 3,5-trimethoxyflavone and limonoids (Awaad et al., 2012).

In pharmacological studies on *Casimiroa edulis*, alcohol extracts of seeds and aqueous extracts of leaves were found to have hypnotic, anticonvulsant and antihypertensive

effects (Magos & Vidrio, 1991). There have been many other pharmacological activities reported for samples of the plant taken from Mexico and America.

Regarding, *Casimiroa pubescens* Ramirez, popularly known as rat sapote or bighorn sapote, is also used as a sedative, but unlike *Casimiroa edulis*, few chemical and pharmacological investigations were done to support its use against depression or anxiety (Suárez, 2012).

Figure 9. The tree, fruits and seed of *Casimiroa edulis*. From:
http://www.phoenixtropicals.com/whiteSapote.html
http://www.fairchildgarden.org/Articles/id/566/read/White-Sapote-Unique-and-Delicious/

- ***Ribes nigrum* L.**

The genus *Ribes* belongs to the family of *Grossulariaceae* and has about 150 different species. *Ribes nigrum* (Fig. 10), known as Black Currant, is a perennial small shrub, which is widely distributed in Europe and North Asia, and is cultivated in many countries for its usage of the fruits in the food industry (Sasaki et al., 2013).

The fruit of the black currant, black currant berries, are favored for their organoleptic properties such as distinctive color and intense flavor, which is due to phenolic compounds such as anthocyanins, and the presence of sugars, acids, and volatile compounds. Black currants are primarily cultivated for juice and beverage production and also processed for jams, jellies, purées, teas, as functional food products, and to some

extent, it is consumed fresh. The berries have significant antioxidant activity in part attributed to their relatively high content of ascorbic acid (vitamin C).The content of ascorbic acid in commercial cultivars ranges from 130–200 mg/100 mL fresh juice, but even higher levels (over 350 mg/100 mL) have been detected in some breeding materials. However, the antioxidant activity is also attributed to the high levels of phenolic compounds. The most important compounds are the anthocyanins, with an average content of approximately 250 mg /100 g in fresh fruits. In addition to anthocyanins, black currants also contain significant amounts of hydroxycinnamic acids, flavan-3-ols and flavonols, with potential health-promoting properties. There is convincing evidence about the positive contribution of black currant on human health, including effects on vascular function. Due to its health-promoting properties, black currants could be an important fruit in the daily diet. (Vagiri et al., 2013).

The leaves of *R. nigrum* have been used as a traditional medicine for treatment of rheumatic disease in Europe, and have been shown to exhibit antioxidant and anti-inflammatory effects. (Garbacki, Tits, Angenot, & Damas, 2004)

The most important industrial product of black currant is berries; however, leaves and buds due to their characteristic color and excellent flavor have also found some applications as a raw material for the food and cosmetic industries. The information on antioxidant properties of black currant buds is rather scarce. Recently it was reported that buds (opened at the end of March) and leaves (in June) had a higher content in phenolics and antioxidants than fully ripened berries (in July) (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006).

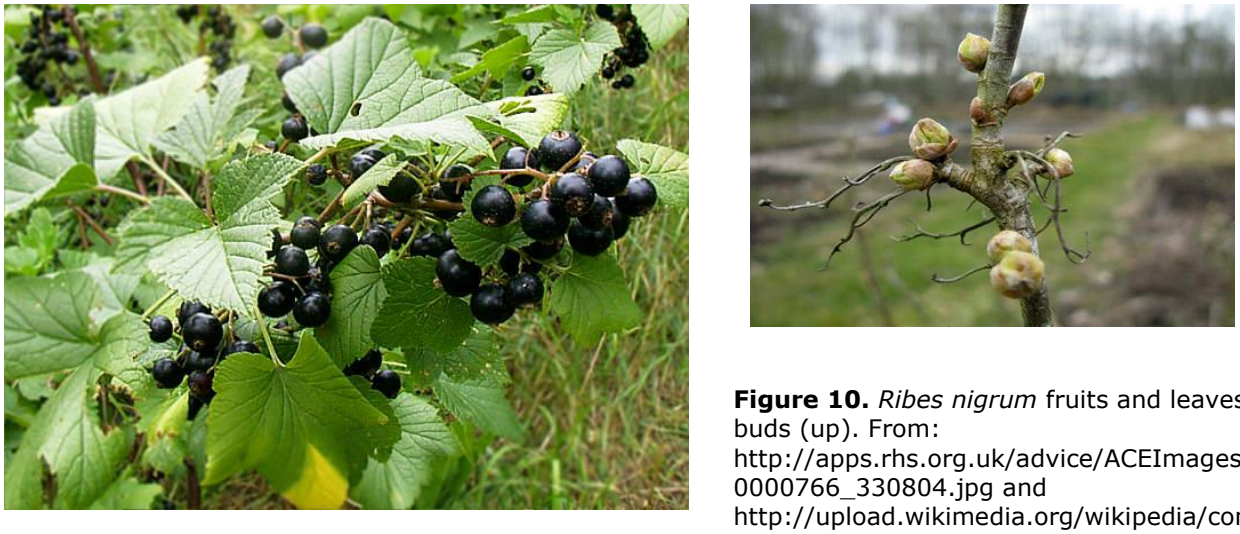

Figure 10. *Ribes nigrum* fruits and leaves (left) buds (up). From: http://apps.rhs.org.uk/advice/ACEImages/RHS_SCN0000766_330804.jpg and http://upload.wikimedia.org/wikipedia/commons/a/a8/Schwarzejohannisbeere.jpg

- ***Boswellia serrata* Roxb ex Colebr**

The gum resin of *Boswellia serrata* (Burseraceae), a kind of deciduous tree grown in the dry part of China and India, has been considered throughout the ages to have a wealth of healing properties and which has long been used, as reported in ancient Ayurvedic medical texts, as a powerful anti-inflammatory agent (I. Gupta et al., 1997). In fact, *Boswellia serrata* resins (Fig. 11) have been used for the treatment of rheumatoid arthritis and other inflammatory diseases such as Crohn's disease. In pharmacological studies, the anti-inflammatory activity has been attributed to its ability in regulating immune cytokine production and leukocyte infiltration. Extracts from *Boswellia serrata* have been shown to possess anti-bacterial, anti-fungal, anti-carcinogenic, and anti-neoplastic properties. Clinically, this resin has been shown to reduce the peritumoral edema in glioblastoma patients and reverse multiple brain metastases in breast cancer patients. Also, efficacy, safety and tolerability profile of essential oil formulation containing *Boswellia serrata* oil has been confirmed for the treatment of acute soft tissue injuries (AHMED, ABDEL-RAHMAN, SALEM, SHALBY, & LOKMAN, 2013). The pharmacological effects of *Boswellia serrata* gum resin extract are mainly attributed to boswellic acids (BAs), which were proposed to act as inhibitors of 5-lipoxygenase, nuclear factor kappa-B (NFκB)-pathway, human leukocyte elastase, cathepsin G, and microsomal prostaglandin E_2 synthase (mPGES)-1. Several pilot clinical trials investigating the efficacy of BSE in the treatment of inflammatory disorders like Crohn's disease, ulcerative colitis, inflammatory bowel disease, rheumatoid arthritis, osteoarthritis, and asthma as well as phase I toxicity studies suggest promising beneficial therapeutic effects with no serious, long-term or irreversible adverse effects. Moreover, BSE was assigned the orphan drug status for the reduction of peritumoral edema by the EMA agency, in 2002 (Gerbeth et al., 2013).

Figure 11. *Boswellia serrata* gum resin. From:http://www.greenclays.com/organic-boswellia-serrata.php

AIM OF THE STUDY

Many natural products have been reported to contain large amounts of antioxidants other than the well-known vitamin C, E and carotenoids. These antioxidants play a benefic role in delaying, intercepting, and preventing oxidative reactions which if not controlled are mostly undesirable. Interesting is the discovery of natural antioxidants of plant origin.
The aim of this study was to assess the antioxidant activity of some selected traditionally used medicinal plants.
The plant extracts assessed in this study are: **Ce1**, methanolic seed extract of *Casimiroa edulis*; **Ce2,** aqueous leaf extract of *Casimiroa edulis*, **Cp1,** methanolic seed extract of *Casimiroa pubescens*, **Cp2,** methanolic leaf extract of *Casimiroa pubescens;* **SdD,** dry residue obtained by lyophilisation of *Croton lechleri* sap; **Rn**, hydroalcoholic bud extract of *Ribes nigrum*; and **Bs**, oleo-gum extract of *Boswellia serrata.*
In previous studies of our laboratory, we focused on phytochemical characterization and vasorelaxation of coumarin compounds from *Casimiroa* genus and their extracts (Bertin, Chen et al. 2013, Bertin, Garcia-Argaéz et al. 2011, Froldi, Bertin et al. 2011), on phytochemical characterization and studies on the *in vitro* vascular modulation and antiproliferative activities of *Croton lechleri* sap (Montopoli, Bertin et al. 2012, Bolcato Jenny 2010), on chemical characterization and *in vitro* study of anti-inflammatory activity of *Boswellia serrata* (Rancan Serena 2013). Further, it was observed that in literature the studies on the antioxidant activity on these plant extracts are mostly incomplete. Thus, the present study was designed to evaluate these plant extracts for the antioxidant capacity with different *in vitro* and *ex vivo* assays, based on different scavenging mechanisms to obtain a complete antioxidant profile.

The following steps were applied:

- Determination *in vitro* antioxidant activity using DPPH · assay (based on SET mechanism) and ORAC assay (based on HAT mechanism);
- Assessment of several statistical programs for EC_{50} estimation in DPPH ·assay;
- Determination non-enzymatic antioxidant content by measuring the total polyphenolic content (TPC) and total flavonoid content (TFC);
- Determination *ex vivo* antioxidant activity to evaluate inhibitory effect on LDL peroxidation using TBARS test and determination of conjugated dienes;
- *In vitro* study of anti-glycation activity based on BSA-glucose/ribose model.

MATERIALS AND METHODS

- **Chemicals and solutions**

AAPH 2,2'-Azobis(2-amidinopropane) dihydrochloride 98% (*Acros Ognanics*) $C_8H_{18}N_6 \cdot 2HCl$ (PM = 271.19 g/mol)

$AlCl_3$ (*Sigma-Aldrich*) (PM = 133.34 g/mol)

Aminoguanidine-bicarbonate 97% (*Sigma-Aldrich*) $NH_2NHC(=NH)NH_2 \cdot H_2CO_3$ (PM = 136.11 g/mol)

BHT Butylated hydroxytoluene (*Sigma-Aldrich*); $C_{15}H_{24}O$ (PM = 220.35 g/mol)

BSA Albumine Bovine fraction V lyophilized powder (*Sigma-Aldrich*) (PM ~ 68.000 g/mol)

$CuSO_4$ $5H_2O$ Copper(II) sulfate pentahydrate (*Sigma-Aldrich*) (PM = 249.69 g/mol)

D(+)-Glucose Anhydrous (*J.T.Baker*) $C_6H_{12}O_6$ (PM = 180.16 g/mol)

DMSO Dimethyl sulfoxide 99.9% (*Carlo Erba*)

DPPH ·2,2-difenil-1-picrilidrazile (*Sigma-Aldrich*) $C_{18}H_{12}N_5O_6$ (PM = 394.32 g/mol)

D(-)-Ribose (*Sigma-Aldrich*) $C_5H_{10}O_5$ (PM = 150.13 g/mol)

Folin-Ciocalteu's phenol reagent (*Merck*)

Fuorescein free acid (*Sigma-Aldrich*) $C_{20}H_{12}O_5$ (PM = 332.31 g/mol)

Gallic acid monohydrate (*Sigma-Aldrich*) $(HO)_3C_6H_2CO_2H \cdot H_2O$ (PM = 188.13 g/mol)

Methanol (HPLC grade) (*Sigma-Aldrich*)

Na_2-EDTA Ethylenediaminetetraacetic acid disodium salt dihydrate (*Sigma-Aldrich*); $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$ (PM = 372.24 g/mol)

PBS Phosphate buffered saline (*ex vivo* experiments) NaCl 137 mM; KCl 2.7 mM; $Na_2HPO_4 \cdot 2H_2O$ 10 mM; KH_2PO_4 2 mM, pH 7.4

PBS Phosphate buffered saline (*in vitro* experiments) $NaH_2PO_4 \cdot 2H_2O$ 41.25 mM; $Na_2HPO_4 \cdot 2H_2O$ 54.23 mM, pH 7.4

Quercetin dihydrate (*Sigma-Aldrich*) $C_{15}H_{10}O_7 \cdot 2H_2O$ (PM=338.27 g/mol)

TBA 2-thiobarbituric acid (*Sigma-Aldrich*); $C_4H_4N_2O_2S$ (PM = 144.15 g/mol)

TCA trichloroacetic acid (*Merck*); CCl_3COOH (PM = 163.39 g/mol)

Trolox® (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% (*Sigma-Aldrich*) $C_{14}H_{18}O_4$ (PM = 250.29 g/mol)

- **Plant materials**

All the extracts of *Casimiroa* spp. have been given from colleagues of Instituto de Química, Univesidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, Coyoacán 04510, México D.F.

Ce1, methanolic seed extract of *Casimiroa edulis*

Ce2, aqueose leaf extract of *Casimiroa edulis*

Cp1, methanolic seed extract of *Casimiroa pubescens*

Cp2, methanolic leaf extract of *Casimiroa pubescens*

SdD dry residue obtained by lyophilisation of *Croton lechleri* sap, collected through incision of the bark from trees growing in the province of Napo, Ecuador. The voucher code number (SdD 007) for the crude drug was deposited in the Department of Pharmaceutical and Pharmacological Sciences of Padua University.

Rn 2% hydroalcoholic solution of the extract of the buds of *Ribes nigrum* (*Cento Fiori srl Forlì*).

Bs oleo-gum extract (EPO, Milano Italy).

***In vitro* methods**

- **DPPH ·assay**

DPPH ·(2,2-diphenyl-1-picrylhydrazyl) is a stable free-radical compound that appears as a dark-colored crystalline powder (Fig. 12).

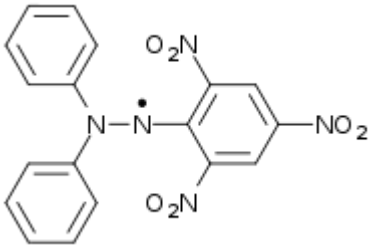

Figure 12. Chemical structure of DPPH·.

The delocalisation of the spare electron over the molecule DPPH ·gives rise to the deep violet colour, characterised by an absorption band in methanol solution centred at about 520 nm, Figure 13. When a solution of DPPH• is mixed with a substance that can donate a hydrogen atom or an electron gives rise to the reduced form with the loss of the violet colour, with a residual pale yellow colour due to the picryl group still present (Molyneux, 2004).

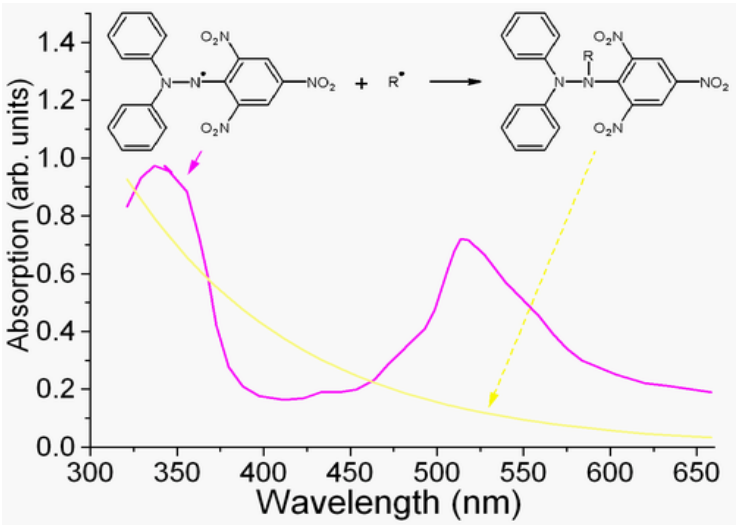

Figure 13. DPPH· radical has a deep violet color in methanolic solution, and it becomes colorless or pale yellow when reduced. From: http://en.wikipedia.org/wiki/DPPH

Thus, when a solution of DPPH ·is placed in contact with a substrate electron donor or hydrogen it passes to a stable no-radical form, with a change of the color of the solution to pale yellow, and the extent of discoloration is proportional to the scavenging activity against the DPPH ·radical. This, it can be monitored by spectrophotometric analysis at a wavelength of 517 nm.

The analysis is simple, sensitive and fairly rapid and needs only a UV–Vis spectrophotometer, this explains its widespread use in antioxidant screening. The results are normally expressed using the EC_{50} value, defined as the concentration of antioxidant that causes a 50% decrease in the DPPH absorbance.

- Experimental Protocol:

The DPPH ·radical scavenging assay was performed according to the method reported by (Brand-Williams, Cuvelier, & Berset, 1995) with some modifications.

At the beginning, it was prepared a methanol solution of 70 µM DPPH• kept in an amber glass bottle with screw cap. At the same time, the solutions of the extracts were prepared from stock solutions. The DPPH• methanolic solution was subdivided in amber vials, and then the samples were added to obtain the final concentrations, within a range from 0.1 to 1000 µg/ml.

The vials were shaken vigorously, incubated for 60 minutes in the dark, at room temperature. After incubation, the samples were read by a spectrophotometer (λ = 517 nm). The control solution was done with DPPH• supplemented with methanol instead of sample solution. In this determination were used quartz cuvettes with a cross section of 10 mm and a spectrophotometer Beckman Coulter model DU 800.

The DPPH ·scavenging effect was calculated using the following equation:

$$DPPH\ \ s.e.\ (\%) = \left[\frac{A_0 - (A - A_b)}{A_0}\right] \cdot 100$$

A_0 is the absorbance of control solution, A is the absorbance of the solution of DPPH• treated with the plant sample, and A_b is the absorbance of the methanolic solution of the sample, this procedure allows to eliminate any interference of solvent absorbance on spectrophotometric determination.

All solutions were prepared daily and stored at room temperature, protected from light. After the spectrophotometric reading, the antioxidant efficacy was determined using the

EC_{50} value, using appropriate software (Chen, Bertin, & Froldi, 2013). Several experimental evidences have indicated a non-linear relationship between the antioxidant concentration and the DPPH · radical scavenging activity (Eklund et al., 2005; Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005); as a consequence, the determination of EC_{50} becomes quite problematic, revealing a variable goodness of fit for the plotted regression models. So it was performed a comparison study to identify the more suitable program for the EC_{50} estimation from experimental data obtained by DPPH · assay by comparing various statistical programs. For this, six computational programs and four different regression models were employed to estimate the EC_{50} value, using various standard natural antioxidants (Tab. 2).

Table 2: The statistical programs used in the comparative study

Statistical program	Equation for EC_{50} calculation	Note
GraphPad Prism 5.01(4P)	$Y = \frac{1}{1 + 10^{\{[\log EC_{50} - \log (x)] \cdot \text{Hillslope}\}}}$	*Y*: response; *x*: concentration of the agonist; bottom: baseline; top: maximum response; Hillslope: steepness of the antiradical curve. x_b: concentration of the sample at the inflection point; *s*: asymmetry of the curve.
GraphPad Prism 5.01(5P)	$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{(1 + 10^{[(\log x_b - \log x) \cdot \text{Hillslope}]})^s}$	
BLeSq 0.9.1	$y = \ln (p/1 - p)$	*p*: probability
OriginPro 8.5	$Y = A_1 + \frac{A_2 - A_1}{1 + 10^{[(log\ x_0 - log\ x)p]}}$	A_1: baseline; A_2: maximum response; *p*: slope of the curve; x_0 concentration at the inflection point, EC_{50} value.
SigmaPlot 12	$Y = Y_0 + \frac{a}{(1 + e^{[-(x - x_0)/b]})^c}$	x_0: concentration at the inflection point; *c*: asymmetry factor; *b*: slope; Y_0 and *a*: min and max of *Y* values.
BioDataFit 1.02	$Y = A_1 + \frac{A_2 - A_1}{1 + 10^{[(log\ x_0 - log\ x)p]}}$	A_1: baseline; A_2: maximum response; *p*: slope of the curve; x_0 concentration at the inflection point, EC_{50} value.
IBM SPSS Statistics Desktop19.0	Relative function for EC_{50} calculation, equation not available	

To this purpose six standard compounds were used: quercetin, (+)-catechin, l-ascorbic acid, caffeic acid, chlorogenic acid, and *N*-acetyl-cysteine. Each compound was assayed at eight different concentrations, within the range of 0.1–300 μmol/l, and then the experimental data were processed by six different statistical programs to obtain estimated EC_{50} values. However, these ones may be considered as theoretical values, because they are derived from a range of antioxidant concentrations, where experimental points are rather far from the actual EC_{50} value. For this reason, in order to determine the most reliable EC_{50} value, successively the DPPH• scavenging assay was still performed for each antioxidant using several concentrations closer to the estimated EC_{50}. The evaluation of the antiradical curve done in a smaller range of antioxidant concentrations, as near as possible to the estimated EC_{50} value, enables a more accurate specification of the EC_{50} for the mathematical interpretation. For this, to perform a more accurate analysis, we enclosed the EC_{50} value within a narrow range. Once the EC_{50} fell in a narrow range, it may be calculated by using a simple mathematical method based on the principle of right-angled triangle (Alexander, Reading, & Benjamin, 1999). This method was applied to all the antioxidant compounds to obtain the actual EC_{50} values (Fig. 14).

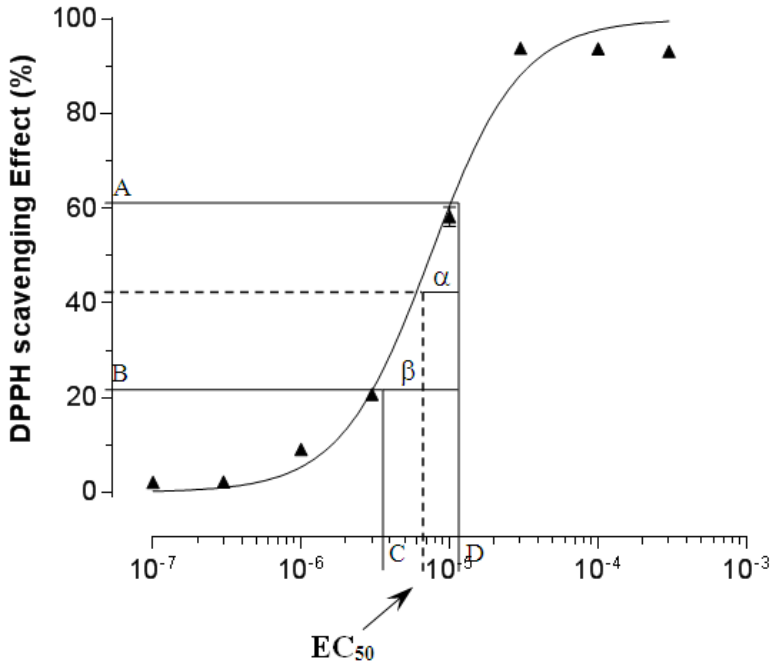

Figure 14. The EC_{50} derivation from the concentration–response curve of catechin, obtained by GraphPad Prism five-parameter regression, with highlighted the nearest actually recorded responses (A and B) of each experimental concentration (C and D); on either side of the EC_{50} forming a right-angled triangle, according the method of (Alexander et al., 1999).

To apply this method, two assumptions have to be accepted: (1) that the maximum response is reached, and (2) that the responses to the experimental concentrations of the

two recorded points on either side of the 50% response should be as close as possible to the point of the EC_{50}, in order to consider the sigmoid curve as a straight line. As shown in FigureY, $\Delta\alpha$ and $\Delta\beta$ are two similar triangles, where the corresponding sides have lengths in the same ratio. *A*, *B*, *C*, and *D* are known values from the experimental data; since we have already normalised the data in percentage, the EC_{50} response is the 50% of the maximal response. Therefore, we applied the follow equation (Alexander et al., 1999):

$$EC_{50} = D - \frac{(A - 50\%\ \text{max response}) \cdot (D - C)}{(A - B)}$$

We estimated the goodness of the regression for the programs, adopting the following equation:

$$\sigma^2 = \frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2$$

where μ is the actual EC_{50}, N is the number of reference compounds (i.e. 6) and x_i is the estimated EC_{50} value for each antioxidant. The program that has showed the lowest variance was considered as the best statistical program tested.

- **ORAC assay**

The oxygen radical absorbance capacity (ORAC) assay is a widely used method to characterize the antioxidant capacity of different materials such as biological fluids, essential oils, spices, foods, dietary supplements, or cosmetic products.

In this assay, a peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product; therefore, the reaction can be easily quantified by fluorescence. The peroxyl radical used is 2,2′-azobis(2-amidinepro-pane) dihydrochloride (AAPH), which reacts with fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one) that is the fluorescent probe. The ORAC reaction is performed at 37 ℃, and since it is temperature sensitive, this is strictly-controlled throughout all the experiment.

The ORAC assay depends on the free radical damage to a fluorescent probe that correlates with a decrease of the fluorescence intensity; this is an index of the degree of free radical damage. In the presence of an antioxidant, there will be an inhibition of free radical damage, with a protection of the probe fluorescence, Fig. 15. The uniqueness of the ORAC assay is that the reaction is driven to completion and the quantitation is

achieved using “area under the curve” (AUC). In particular, the AUC method allows ORAC to determine both inhibition time and inhibition percentage of the free radical damage into a single value (Fig. 16).

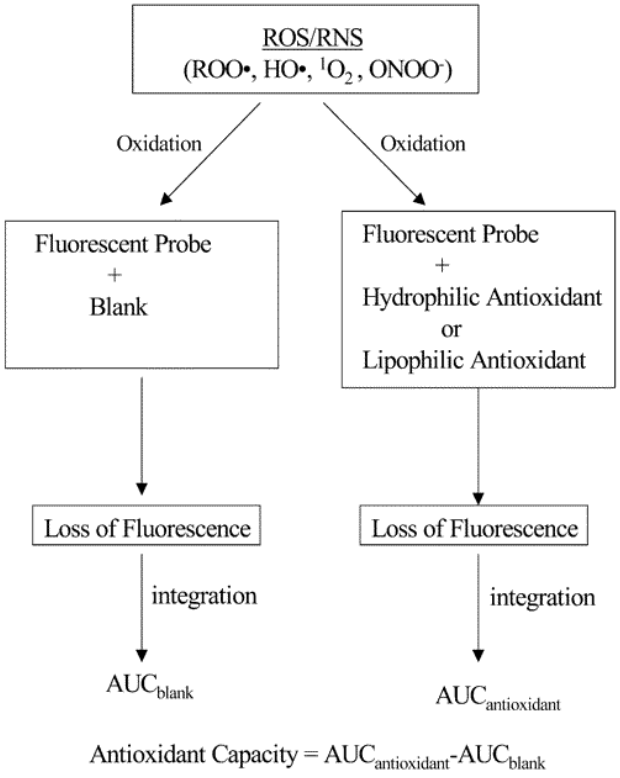

Figure 15. Schematic illustration of the principle of the ORAC assay. From: (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002)

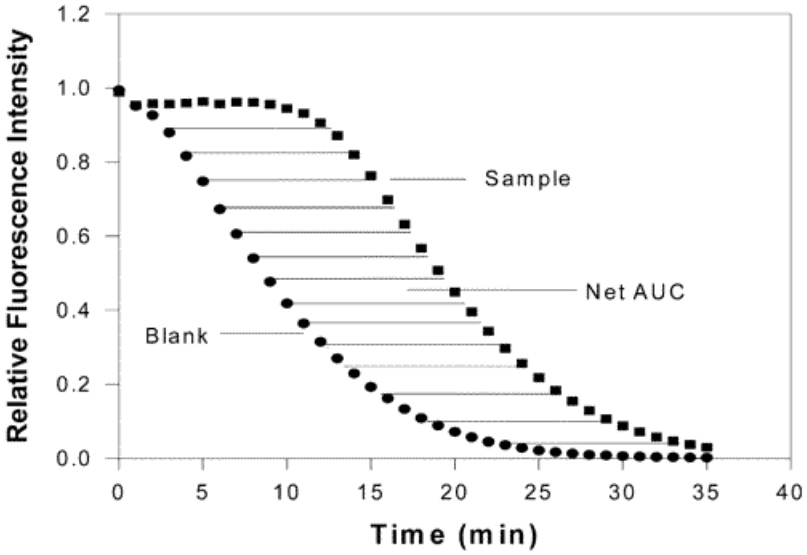

Figure 16. Illustration of calculation of the ORAC value expressed as the net area under the curve (AUC). From: (Huang et al., 2002)

The net area under the curve (AUC) of the standards and samples was calculated. The standard curve is obtained by plotting Trolox® concentrations against the net AUC of the measurements for each concentration. Final ORAC values are calculated using the regression equation between Trolox® concentration and the net AUC and are expressed as micromole Trolox® equivalents per liter for liquid samples or per gram for solid samples. The AUC is calculated as:

$$AUC = 0.5 + f_1/f_0 + ... f_i/f_0 + f_{59}/f_0 + 0.5(f_{60}/f_0)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*.

The net AUC is obtained by subtracting the AUC of the blank from that of a sample. ORAC values are usually reported as Trolox equivalents.

- Experimental protocol

ORAC assays were performed as described by (Gillespie, Chae, & Ainsworth, 2007) with some modifications. Briefly, prepare the sample solution and the solution of fluorescein in PBS to a final concentration of 0.08 μM, kept on ice and protected from light.

At the same time prepare the solutions of standard antioxidant (Trolox®) and the generator of peroxy radicals (AAPH). Trolox is solubilized in PBS so as to obtain a 10^{-4} M stock solution, from which it is prepared by progressive dilution solutions of 50 µM , 25 µM, 12.5 µM and 6.25 µM. The AAPH solution was prepared in PBS at a concentration 0.15 M, all solutions must be prepared freshly and kept on ice and protected from light. Then we set the microplate reader (VictorTM X3, PerkinElmer) for a kinetic reading of fluorescence at 37 °C for one hour, with the wavelength of excitation at 485 nm and emission at 530 nm, preheating the instrument to 37 °C for 10 min. In a 24-well plate were added 1500 µL of fluorescein solution 0.08 µM , 250 µL of buffer solution or standard solution of Trolox® (6.25 - 50 µM) or sample solution, then add 250 µL of AAPH solution in each well and proceed directly to the fluorescence reading through regular scans at intervals of one minute to 60 minutes total.

Once obtained the decay curves of the fluorescence signal, calculate the AUC relative to each well by subtracting the average value of the AUC of the blank from the AUC of Trolox® and the test sample. It was obtained in this way, the net AUC and, through it, the calibration line and the corresponding equation to obtain the final value expressed in TEAC (Trolox equivalent antioxidant capacity).

- **Determination of the Total Phenolic Content (TPC)**

The TPC assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at 760 nm with Beckman Coulter model DU 800. Although the exact chemical nature of the reaction is unknown, it is believed that sequences of reversible one- or two-electron reduction reactions lead to blue species (possibly, $PMoW_{11}O_{40}$). The total phenolic content of the extracts was determined using the Folin-Ciocalteu reagent (V. Singleton & Rossi, 1965). The reaction mixture contained 50 µl of diluted vegetable extract, 4.2 ml of freshly prepared diluted Folin-Ciocalteu reagent, and 750 µl of 22% sodium carbonate. The mixtures were kept in dark, at ambient conditions, for 2 h to complete the reaction. Then, the absorbance at 760 nm was measured. A standard curve with five concentrations of gallic acid standard solution (0 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 250 µg/ml and 500 µg/ml) was generated. The TPC of each extract is expressed as milligrams of gallic acid equivalents (GAE) per g of extract.

- **Determination of Total Flavonoid Content (TFC)**

This method for the quantification of flavonoids is based on the spectrophotometric determination of a complex flavonoid-$AlCl_3$, which provides a bathochromic displacement and the hyperchromic effect.

The principle of aluminium chloride colorimetric method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids. Thus, the total flavonoid content of the plant extracts was estimated by aluminium chloride ($AlCl_3$) colorimetric method (Fernandes, Ferreira, Randau, de Souza, & Soares, 2012).

The extracts were diluted with methanol to 5 mg/ml. Briefly, 200 µl $AlCl_3$ 2.5% (w/v) was added to 400 µl of each diluted solution and the solution were made up to 2.5 ml by adding 1.9 ml of distillate water. After 15 min of incubation at room temperature, the absorbance was measured by spectrophotometer Beckman Coulter model DU 800 at 410 nm. The same procedure was repeated without the addition of $AlCl_3$ for preparation of the contrast solution. The standard curve of known concentrations of quercetin was generated by preparing and testing five concentrations of quercetin standard solution, which were 0.0 µg/ml, 12.5 µg/ml, 25 µg/ml, 500 µg/ml, 1000 µg/ml. Total flavonoid content (TFC) was expressed as milligrams of quercetin equivalents per g of extract.

***Ex vivo* methods**

- **Thiobarbituric Acid Reactive Substances (TBARS) test**

The TBARS test is the most common method for measuring malondialdehyde (MDA) in food products and biological samples. MDA is a major degradation product of lipid hydroperoxides, Fig. 17. TBARS test is based on spectrophotometric quantitation of the pink complex formed after reaction of MDA with two molecules of thiobarbituric acid (TBA)(Fig. 18). This method was used to determine the human LDL oxidation *ex vivo*.

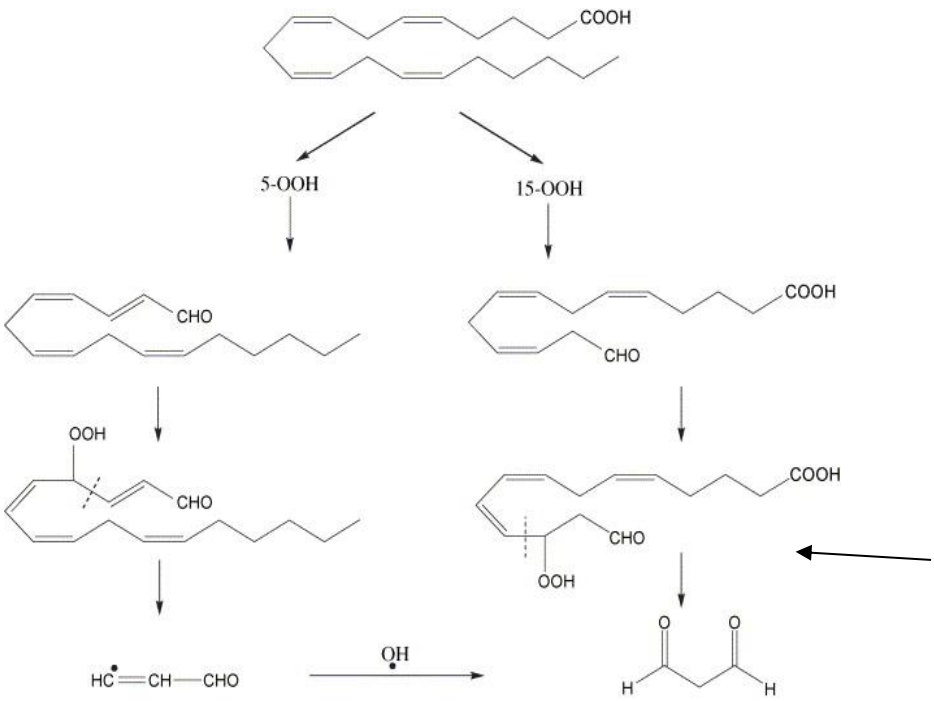

Figure 17. The two mechanisms proposed by (Esterbauer, Schaur, & Zollner, 1991) and colleagues (1991) based on the successive hydroperoxide formation and β-cleavage of the fatty acid chain to give a hydroperoxyaldehyde; MDA is then generated by β-scission or by reaction of the final acrolein radical with a hydroxyl radical.

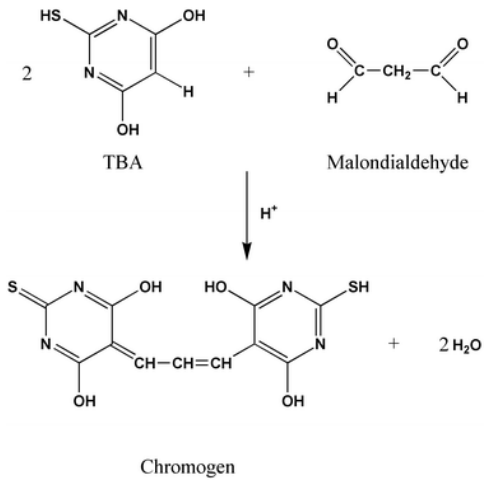

Figure 18. Chromophore formed by condensation of MDA with TBA. From: (Botsoglou et al., 1994)

- Experimental protocol

After carrying out an exhaustive dialysis of human LDL in EDTA–free PBS, transfer 480 µL of the LDL suspension (25 µg/mL) in each of the six microtubes with safety lock (Eppendorf Safe-Lock Tubes 2.0 mL), and then add 24 µL of diluted methanolic solution of the sample and incubate for about 15 minutes. Then add 12 µl of aqueous solution of

0.4 mM $CuSO_4$, obtaining a final concentration of 10 µM in the reaction mix. Close the microtubes and keep them in a water bath for 1 hour at 37 ℃, to facilitate the process of oxidation on LDL. After incubation, the microtubes were transferred on ice, 50 µL of an aqueous solution of Na_2-EDTA was added to chelate the $CuSO_4$ and stop the oxidation process. Then proceed with the addition of butylated hydroxytoluene (BHT, 25 µl, 2 g/L), 250 µL of trichloroacetic acid (TCA, 100 g/L and 500 µL of TBA 6.7 g/L; BHT is an alkylated phenol antioxidant action, while the TCA is used to acidify the reaction environment and promote the binding of TBA with malondialdehyde produced during peroxidative degradation of LDL. The microtubes were filled with N_2 gas and stirred gently, then moved into boiling water for 20 minutes. After this period, in which is formed the adduct MDA-TBA_2, the microtubes were transferred on ice and centrifuged at 3000 g for 5 minutes. The supernatant was transferred in quartz cuvettes for spectrophotometric reading, at a wavelength of 532 nm with Beckman Coulter model DU 800.

The absorbance is converted into equivalent of MDA using its molar extinction coefficient ε

$$\varepsilon_{MDA} = 1.56 \text{ X } 10^5 \text{ M}^{-1} \text{ cm}^{-1}$$

Thus for the Beer -Lambert law $A = \varepsilon \cdot c \cdot l$, it can be obtain the concentration c of MDA :

$$c = \frac{A}{\varepsilon \cdot l} = \frac{A}{1{,}56 \cdot 10^5}$$

- **Determination of Conjugated Dienes**

The primary products of lipid peroxidation are hydroperoxides of the general structure: -CH=CH-CH=CH-CHOOH-, with an absorption maximum around 234 nm. Since ox-LDL is, like native LDL, fully soluble in buffer, the generation of such conjugated lipid hydroperoxides can directly be measured by recording the UV spectrum of the aqueous LDL solution. An example for such an experiment is shown in Figure 18. The kinetic of the diene formation, i.e. the change of the absorbance vs. time, can be clearly divided into three phases (Fig. 19). A first phase, during which the dienes very slowly increase, a

second phase during which they very rapidly increase to a maximum value, and at the end, a third phase during which the dienes decrease. The first two phases can be termed as lag-phase and propagation phase. During the lag-phase, the endogenous lipophilic antioxidants of LDL protect the polyunsaturated fatty acids against oxidation, and thus prevent that the lipid peroxidation process may come into the rapid propagating chain phase. The protective action of the antioxidants progressively decreases since they are inactivated and consumed in free radical scavenging. When the LDL particle is depleted of its antioxidants, the lipid peroxidation process enters in the propagation phase in which the polyunsaturated fatty acids are rapidly converted to conjugated lipid hydroperoxides, as indicated by the increase of the 234 nm absorbance. The transition from the lag-phase to the propagation phase is not abrupt, but a continuous process. We define the end of the lag-phase as the interval (minutes) between the intercept of the linear least-square slope of the curve with the initial-absorbance axis as shown in Figure 18. The last phase of the LDL oxidation is characterized by decomposition of the lipid hydroperoxides formed during the propagation phase. These decomposition reactions are extremely complex and can lead to many compounds showing UV absorbance in the 210-240 nm range; for example, 2-alkenals or 4-hydroxyalkenals, typical products of lipid peroxidation, absorb at 220-225 nm region (Esterbauer, Striegl, Puhl, & Rotheneder, 1989).

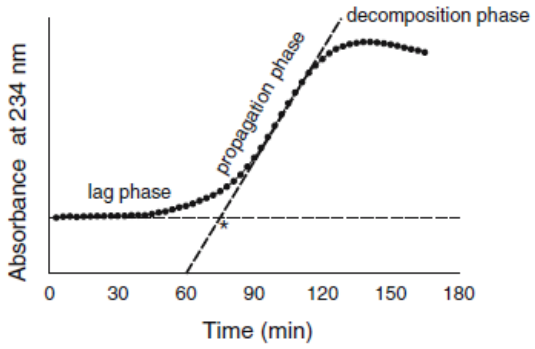

Figure 19. The three phases of LDL oxidation: lag phase, propagation phase, and decomposition phase. From: (Scheffer, Teerlink, & Heine, 2005)

- Experimental protocol

After carrying out an exhaustive dialysis of human LDL in EDTA-free PBS, transfer 480 µL of suspension of LDL (25 µg/mL) in each of the six microtubes, then add 24 µL of diluted methanolic solution of the sample and incubate the mixture for about 15 minutes. Then add 12 µL of aqueous solution of 0.4 mM $CuSO_4$, to obtain a final concentration of 10 µM in the reaction mixture. Transfer the contents of each microtube in a quartz cuvette and proceed to the kinetic reading with a spectrophotometer Beckman Coulter model DU 800 at 234 nm and at 37 °C, performing scans at regular intervals of 5 minutes.

- **Determination of the Advanced Glycation Endproducts**

All proteins are subject to glycation reactions, and so far no exception has been reported. The glycation reaction between amine residues of protein with glucose is very rapid and initially reversible, producing a labile Schiff-base. The product may then react further, through an Amadori-rearrangement, to give a relatively stable fructosamine. This Amadori product is the characteristic product of glycated proteins. Finally, in long-living proteins, a cascade of slow cross-link reactions may result in advanced glycation end products (AGEs) (Fig. 20) (Sobal, Menzel, & Sinzinger, 2000).

Therefore, glycation reactions are consist of two stages. In the first step, glucose and the amino groups of lysine residues react with each other to form fructose-lysine (FL). The subsequent processes are dehydration, rearrangements and cyclization. Later, further reactions result in the formation of advanced glycation end products AGEs (browning- or Maillard products), Figure 19. From these reactions, the main characterized products are carboxymethyl-lysine (CML) and pentosidine. CML can be formed by free radical cleavage of FL and pentosidine is a glucose-derived cross-link involving arginine and lysine residues. Most AGEs can easily be measured by fluorescence (excitation at 370 nm and emission at 440 nm) or by an ELISA technique using anti-AGE antibodies (Sobal et al., 2000).

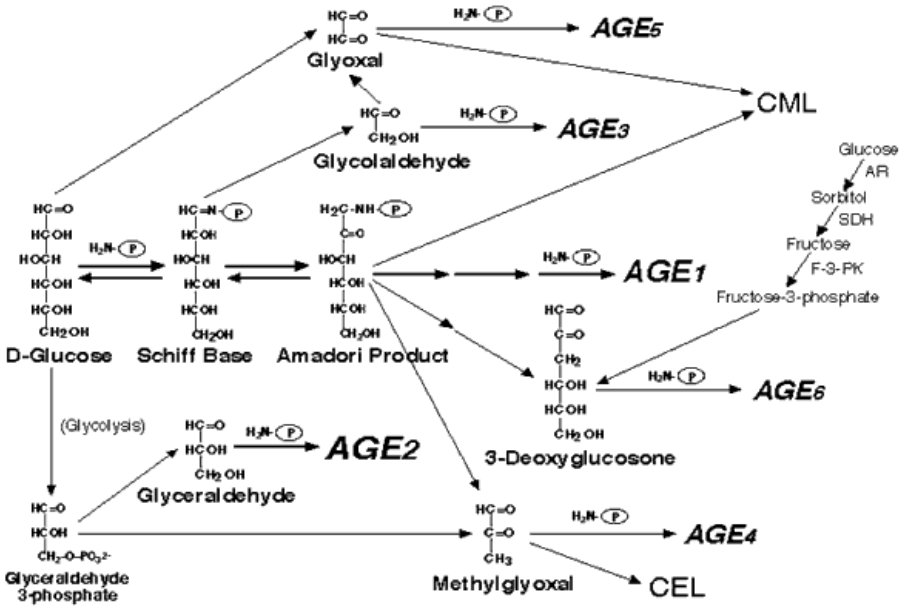

Figure 20. The formation of advanced glycation end products.
From: http://www.liquida.it/louis-camille/

- Experimental protocol

The methodology was based on that of (Perez,R M Perez Gutierrez, Rosa, 2012). BSA was incubated with glucose or ribose in phosphate buffered-saline (PBS) (pH 7.4) in the presence of extract at 37℃ for 5 or 7 days. In each test solution there are: BSA (50 mg/mL), glucose (0.8 M) or ribose (0.1 M), sample (5 to 100 μg/mL) and 0.02% sodium azide.

All the reagents and samples were sterilized by filtration through 0.2 μm membrane filters. The protein, the sugar and the prospective inhibitor were included in the mixture simultaneously. Aminoguanidine (50 mM) was used as an inhibitor positive control. Reactions without any inhibitor were also setup. Each solution was kept in the dark in a capped tube. After 5 or 7 days of incubation, fluorescence intensity (excitation wavelength of 355 nm and emission wave-length of 460 nm) was measured for the test solutions. Percent inhibition was calculated as follows:

$$Inhibition\ \% = \left[1 - \frac{(As - Ab)}{(Ac - Ab)}\right]$$

where As = fluorescence of the incubated mixture with sample, Ac is the fluorescence of the incubated mixture without sample as a positive control, and Ab is the fluorescence of incubated mixture without sugar (blank control).

STATISTICAL ANALYSIS

Results were expressed as means ± standard error of the mean (SEM) of at least three measurements. Statistical analysis was performed using Student's *t*-test and $P < 0.05$ was considered to be significant. And in DPPH ·assay, EC50 estimation was obtained by use of GraphPad Prism® 5P.

RESULTS

In vitro methods

DPPH ·assays are usually classified as SET (Single Electron Transfer) reactions. These radical indicators may be neutralized by direct reduction via electron transfer or by radical quenching via hydrogen atom transfer (Prior et al., 2005). In general, SET-based assays measure antioxidant reductive capacity.

The addition of the extracts to the DPPH solution induced a rapid decrease in its absorbance, determined at 517 nm. Fig. 21 shows the effect of different plant extracts in comparison with ascorbic acid on the inhibition of DPPH · radical. Our investigation shows that free radical scavenging ability of *Croton lechleri* was similar to ascorbic acid under the test conditions.

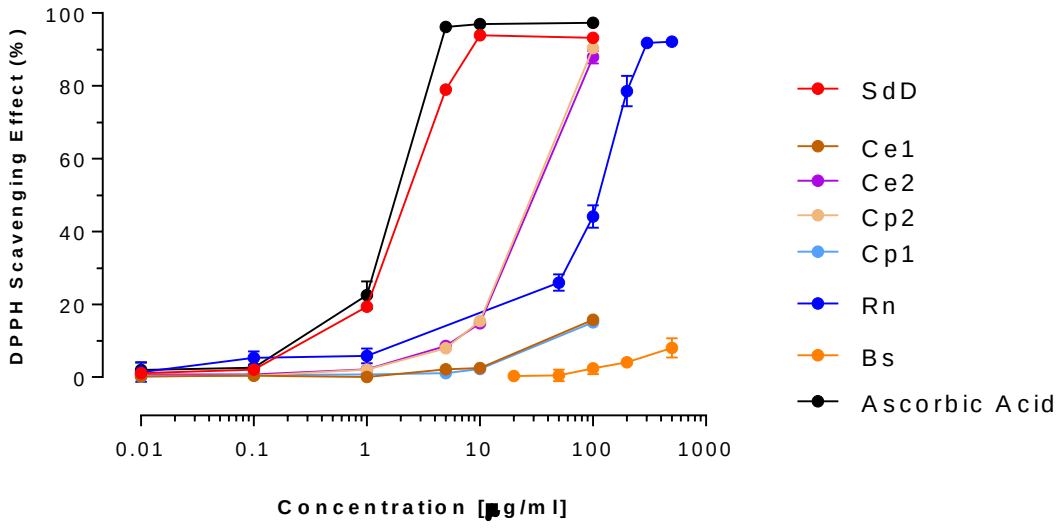

Figure 21 DPPH· assay: scavenging effects of the plant extracts determined using spectrophotometry. Each value is the mean ± SEM (n=5).

The EC_{50}, defined as the concentration of antioxidant that causes a 50% decrease in the DPPH ·absorbance, is generally used as an indicator of antioxidant capacity for plant extracts and pure compounds. Therefore, for each substance the EC_{50} value was determined using GraphPad Prism® 5P (Tab. 3), this to compare the antioxidant potency of all of the extracts (Chen et al., 2013).

Table 3 The antioxidant potency of the plant extracts determined by use of DPPH· assay.

Extract	EC_{50}* (µg/ml)
SdD	2.74
Ce2	33.17
Cp2	41.72
Rn	109.90
Ascorbic acid	1.77

* For the extracts which did not reach the 50% inhibition it was not possible to obtain the EC_{50} value.

Though the antioxidant potential of *Croton lechleri* was found to be slighter lower than that of ascorbic acid, anyway, the study revealed the prominent antioxidant activity of the sap (Table 3). Further, our investigation shows that the two *Casimiroa* leaf extracts have a good free radical scavenging activity.

Before using GraphPad Prism® 5P as the statistical program of choice for EC_{50} estimation, it was performed a comparative study to find the best statistical program to estimate EC_{50} values in DPPH · assay, using five statistical programs and six standard compounds. Estimated EC_{50} values by statistical programs are considered as theoretical values, while the actual EC_{50} values were determined by performing the same assay on a smaller range and using a simple mathematical method based on the principle of right-angled triangle, see Materials and Methods (Tab. 4).

Table 4. The EC_{50} values for standard antioxidants expressed as pD_2 (-log EC_{50}) ± SD, obtained after statistical elaboration with six softwares; in the last row, the actual EC_{50} values were obtained by applying the right-angled triangle method (see methods).

	Quercetin	(+)-Catechin	L-Asc. acid	Caffeic acid	Chlor. acid	*N*-acetyl-cyst.
GraphPad [a]	5.392 ±0.046	5.130 ±0.047	4.869 ±0.032	5.007 ±0.038	5.309 ±0.035	4.466 ±0.039
GraphPad [b]	5.316 ±0.046	5.109 ±0.021	4.830 ±0.058	4.993 ±0.011	5.293 ±0.023	4.578 ±0.035
Blesq [c]	5.272 ±0.041	5.097 ±0.054	4.851 ±0.049	5.018 ±0.051	5.246 ±0.042	4.388 ±0.050
Blesq [d]	5.304 ±0.076	5.000 ±0.076	4.893 ±0.039	5.018 ±0.051	5.305 ±0.035	4.496 ±0.010
BioDataFit	5.411 ±0.030	5.166 ±0.035	4.881 ±0.023	4.992 ±0.024	5.314 ±0.016	4.576 ±0.023
OriginPro	5.411 ±0.030	5.166 ±0.035	4.881 ±0.023	4.992 ±0.024	5.314 ±0.016	4.576 ±0.023
SigmaPlot	5.316 ±0.046	5.109 ±0.021	4.830 ±0.058	4.993 ±0.011	5.293 ±0.023	4.578 ±0.035
SPSS	5.354 ±0.212	5.159 ±0.131	4.870 ±0.136	5.023 ±0.138	5.287 ±0.125	4.440 ±0.130
Actual EC_{50}	5.261 ±0.021	5.095 ±0.004	4.793 ±0.006	4.930 ±0.016	5.203 ±0.008	4.521 ±0.028

[a] GraphPad log (inhibitor) vs. normalized response model (variable slope).
[b] GraphPad five-parameter regression model.
[c] Blesq logit regression model.

[d] Blesq logit regression model with outliers elimination.

The relative variance of the estimated EC_{50} of each antioxidant was calculated for the different statistical programs; SigmaPlot and GraphPad Prism 5P implemented with a five-parameter equation showed the minor variance and seem to work with a better approximation in relation to actual EC_{50} values. Given that GraphPad Prism was almost exclusively developed for biological and pharmacological use, and GraphPad Prism 5P is the only that could easily load all datasets together and could plot them on the same graphic, with an automatic update after every data changing in the spreadsheet cells, we suggest the five-parameter regression model as an efficient statistical strategy for curve-fitting, EC_{50} determination and data processing (Chen et al., 2013).

ORAC (oxygen radical absorbance capacity) assay is based on HAT (Hydrogen Atom Transfer) mechanism, it measures the antioxidant inhibition of peroxyl radical induced oxidations, and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (Ou, Hampsch-Woodill, & Prior, 2001). The results obtained in the ORAC assay are shown in Fig. 22, and summarized in Tab. 5.

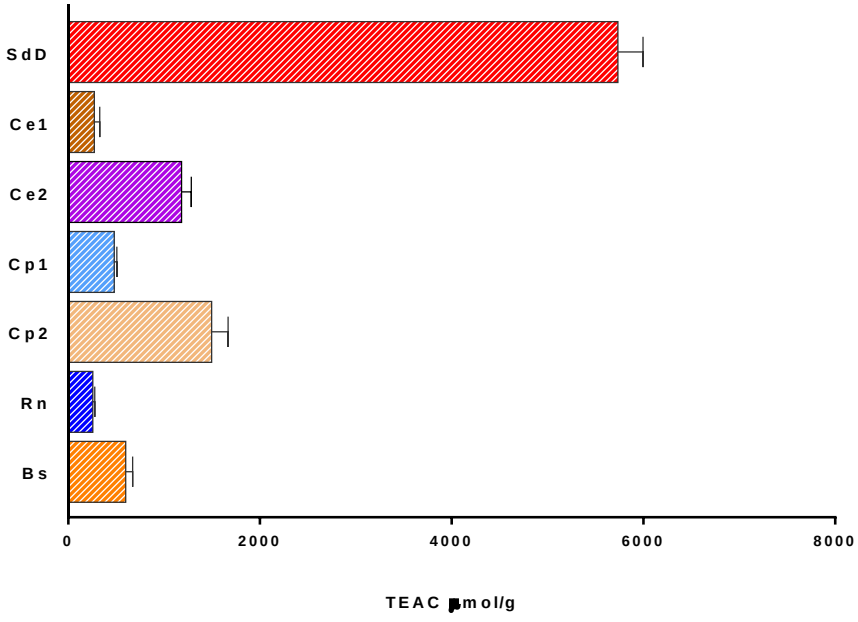

Figure 22 The ORAC results for the extracts are expressed as micromole of Trolox equivalents per gram.

Table 5. The TEAC values of the extracts obtained by ORAC assay.

Extract	TEAC (µmol/g)
SdD	5735 ± 261
Cp2	1496 ± 171
Ce2	1181 ± 102
Cp1	479 ± 28
Ce1	273 ± 54
Bs	599 ± 73
Rn	256 ± 21

Each value in the table is represented as mean ± SEM (n = 5).

Using this antioxidant assay, the two *Casimiroa* leaf extracts (Ce2 Cp2) showed high ORAC values, and the *Croton lechleri* sap had higher activity.

In order to obtain a deeper knowledge on the antioxidant capacity, the determination of total phenolic content and total flavonoid content were carried out in all the extracts considered in this research (Fig. 23).

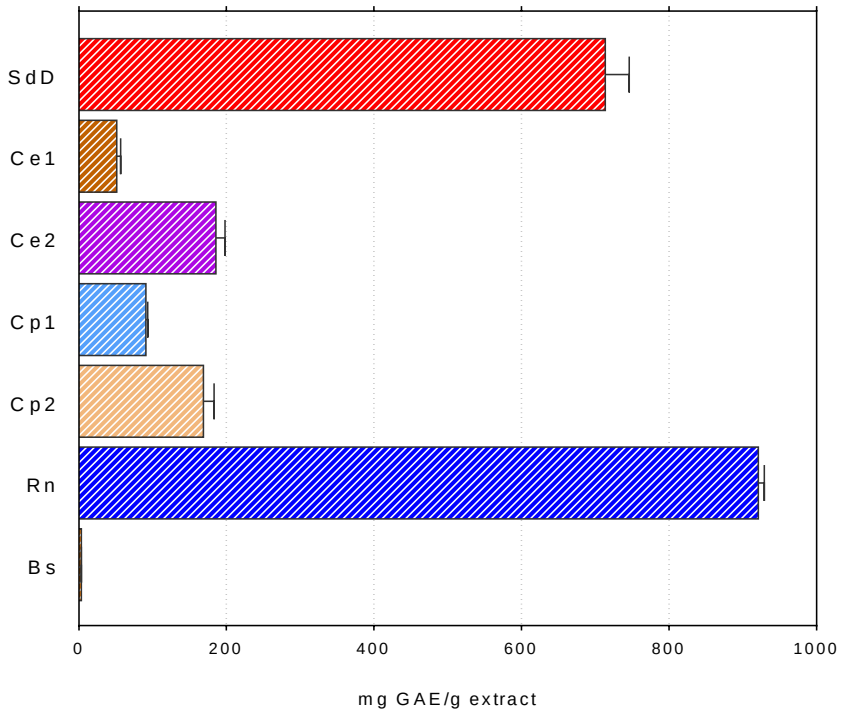

Figure 23 Total phenolic content (TPC) determined in the analyzed extracts, expressed as mg GAE (gallic acid equivalents)/g extract. Results are the means ± SEM of at least three experiments.

The TPC values were expressed as milligram gallic acid equivalents (GAE) per gram of dry extract. *Ribes nigrum* showed the highest amount of phenols equal to 921.04 ± 8.27 mg GAE/ g of extract, followed by *Croton lechleri* which showed an amount of 713.76 ± 32.22 mg GAE. The extracts of *Casimiroa* spp. showed a minor quantity of phenolic compounds including in a range from 50 to 200 mg GAE, while *Boswellia serrata* showed a negligible content.

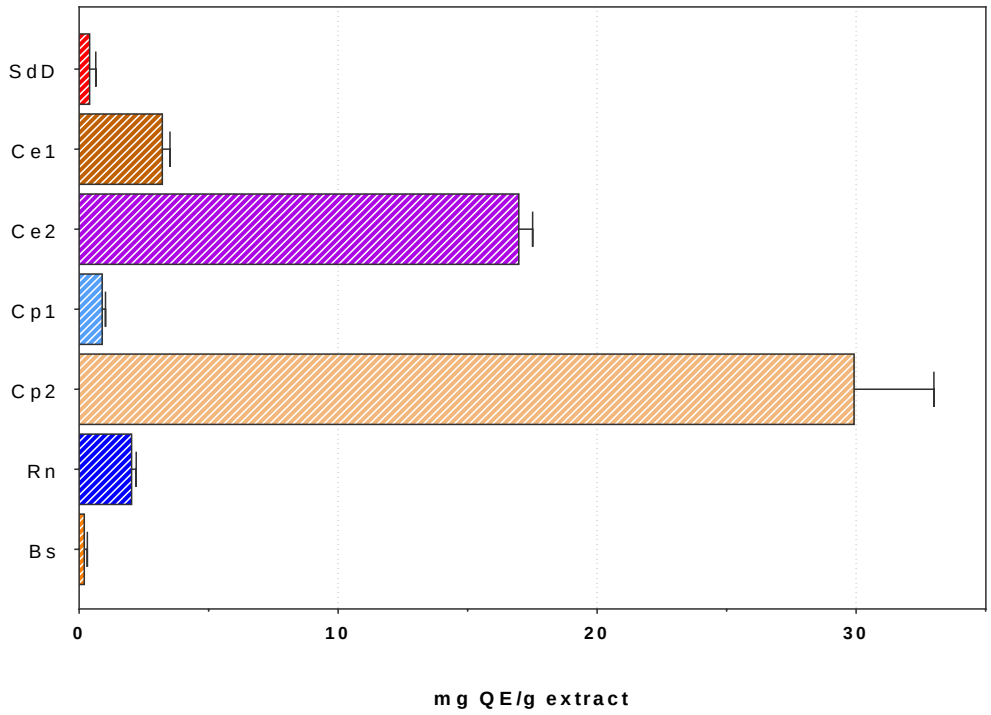

Figure 24 Total Flavonoid Content (TFC) of the extracts are expressed as mg QE (quercetin equivalents)/g extract. Each value is reported as mean ± SEM of at least 3 experiments. Ce…..

It is well known that various phenolic compounds cause different responses in this assay. The molar response of this method is roughly proportional to the number of phenolic hydroxyl groups in a given substrate, but the reducing capacity is enhanced when two phenolic hydroxyl groups are oriented ortho or para (Frankel, Waterhouse, & Teissedre, 1995). Since these structural features of phenolic compounds are reportedly also responsible for antioxidant activity, measurements of phenols in these extracts may be related to their antioxidant properties.

Further, it was determined the total flavonoid content of each extract which was expressed as milligrams quercetin equivalents (QE) per gram of dry extract (Fig.24). The two *Casimiroa* leaf extracts showed highest flavonoid content equal to 29.92 ± 3.07 mg QE/g extract (Cp2) and 16.98 ± 0.53 mg QE/g extract (Ce2), respectively. A lower level

was found in the other extracts. A low correlation (R^2= 0.05) was shown between total phenolic and total flavonoid content (data not shown).

- ***Ex vivo* antioxidant methods**

In order to have a deeper knowledge of the antioxidant property of these extracts, we also studied them using two experimental protocols based on the oxidation of human low density lipoprotein (LDL).

TBARS assay measures the MDA formed as the split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of a lipid substrate. It is postulated that the formation of MDA from fatty acids with less than three double bonds (e.g., linoleic acid) occurs via the secondary oxidation of primary carbonyl compounds (e.g., non-2-enal) (Fernández, Pérez-Álvarez, & Fernández-López, 1997). The TBARS procedure is widely used for its simplicity even though the reaction is not very specific and conditions-dependent.

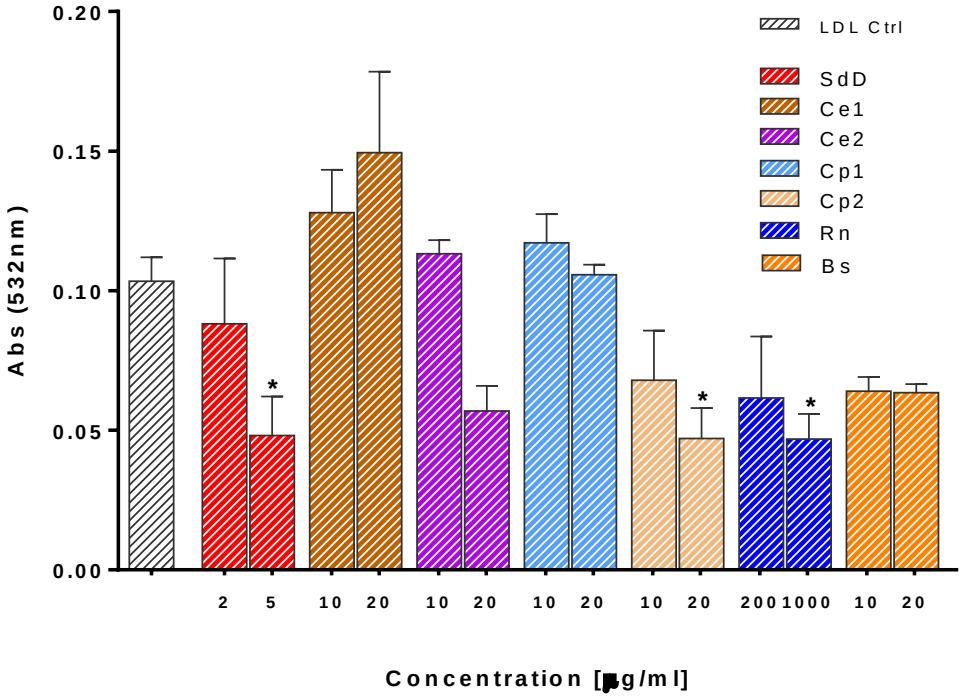

Figure 25 The effect of the plant extracts on copper-induced LDL oxidation. LDL (25µg/ml) was incubated for 1 h at 37°C with 10 µM of Cu^{2+} in the absence (LDL ctrl) or presence of the different extracts. Oxidation was determined by TBARS. Results represent mean ± SEM of at least three experiments. (p < 0.05)

The results of the Fig. 25 show that at 10 µg/ml the *Casimiroa* extracts have no inhibitory effects on the TBARS formation, but at a higher concentration (20 µg/ml) the two leaf extracts showed a moderate inhibition, 54.47% and 45.02% for Ce2 and Cp2, respectively. At the same concentrations the *Boswellia serrata* extracts showed almost the same inhibition of about 38%. Otherwise, the *Croton lechleri* sap, at a very low concentration of 5 µg/ml, caused a significant decrease on TBARS formation (80.57%), while *Ribes nigrum* only at the high concentration of 1000 µg/ml inhibited the TBARS formation of a similar amount.

To confirm the data obtained by TBARS assay, it was carried out also a continuous monitoring of oxidation of human low density lipoproteins based on the quantification of conjugated dienes performing a kinetic reading at 234 nm, at which conjugated dienes, a primary product of LDL oxidation, have an intense absorption. This method is more specific and sensitive; Fig. 26 shows an example of one of the kinetics performed with the extracts. The *Croton lechleri* clearly caused an inhibition dose-dependent of the human LDL oxidation in comparison with the control (without inhibitor).

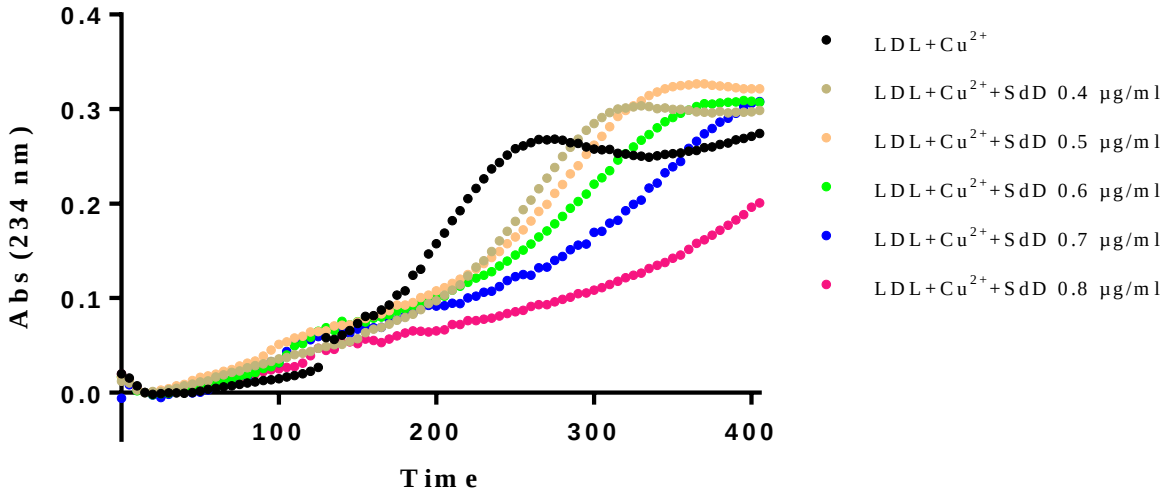

Figure 26. Example of determination of conjugated dienes with increasing concentrations of extract of *Croton lechleri*

The Tab. 6 reports the results of this assay expressed as lag-time, parameter which correlates with the period before the rapid increase of the conjugated dienes (see methods). Again, at low concentrations minor than 1 µg/ml *Croton lechleri* prolonged in

a concentration-dependent manner the lag-phase. The leaf extracts of *Casimiroa* (Ce2 and Cp2) mildly increased the lag-phase, at the relatively low concentration from 2 to 7 µg/ml; the seed extracts (Ce1 and Cp1) appear to be much less actives than the leaf extracts, showing a similar inhibition at an almost ten-fold higher concentration. *Ribes nigrum* and *Boswellia serrata* at the concentrations chosen in this test did not show any activity (Tab. 6).

Table 6 Effect of the plant extracts on the lag phase of LDL oxidation.

Extracts	Concentration (µg/ml)	Ctrl Lagtime (min)	Lagtime (min)	Rate of inhibition (%)
SdD	0.4	195 ±50	220 ±52	14.5
	0.6		260 ±109	32.5
	0.8		305 ±121	58.6
Ce1	20	198 ±29	222 ±10	13.6
	30		336 ±16	71.5
Cp2	3	193 ±24	240 ±30	24.4
	5		290 ±41	50.1
	7		350 ±75	82.0
Cp1	20	239 ±27	264 ±49	9.9
	30		319 ±73	32.5
Ce2	2	179 ±43	206 ±39	14.9
	5		261 ±48	47.1
Rn	200	234 ±7	253 ±41	8.5
	1000		243 ±30	3.8
Bs	10	234 ±7	246 ±16	5.2
	15		257 ±16	9.9

Each value in the table is represented as mean ± SEM of at least three experiments.

In order to investigate the inhibitory effect of these extracts on advanced glycation end-products (AGEs), the final products of the non-enzymatic reaction between reducing sugars and amino groups in proteins, lipoproteins, and nucleic acids, a further research

step was carried out using an assay based on co-incubation of BSA with D(+)-glucose or D(-)-ribose and each extract. At the end of the incubation, the AGEs formation was measured by determining the fluorescence by excitation at 355 nm and emission at 460 nm.

The Fig. 27A shows the inhibitory effect of plant extracts on the glycation of (Bovine Serum Albumin) BSA, induced by 0.1 M ribose (5 days incubation), a potent glycation inducer. In this condition, it was observed a dose-dependent inhibition; at 50 µg/ml, *Croton lechleri*, Ce2 and Cp1 showed an inhibition of 18.07 ± 6.91%, 10.85 ± 7.04% and 10.81 ± 4.25%, respectively, while the positive control aminoguanidine (50 mM) showed an inhibition of 56.77 ± 5.88%.

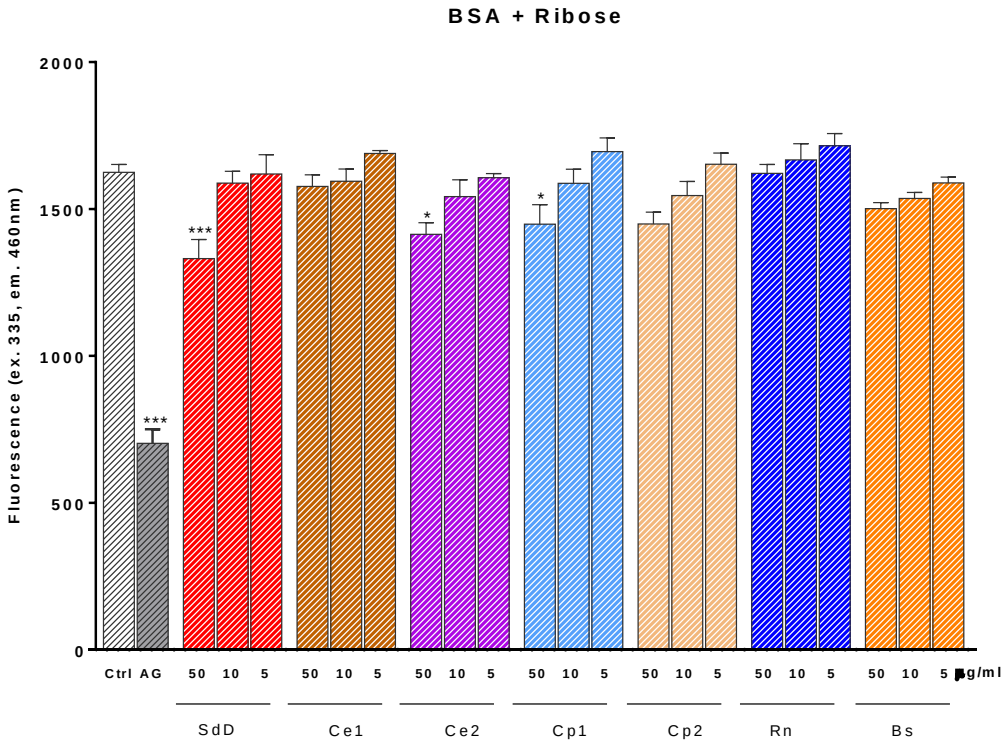

Figure 27A Effects of the extracts on the formation of AGEs resulting from BSA (50 µg/ml) glycation induced by 0.1M ribose. Each value represents mean ± SEM of at least three experiments.

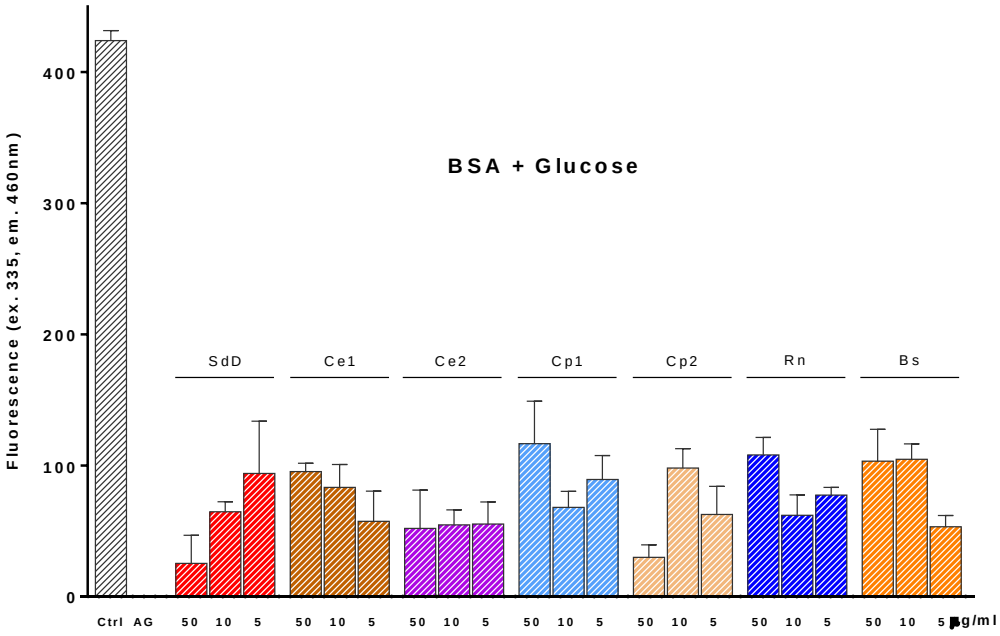

Figure 27B Effects of the extracts on the formation of AGEs resulting from BSA (50 µg/ml) glycation induced by 0.8 M glucose. Each value represents mean ± SEM of at least three experiments.

The Fig. 27B represents the glycation of BSA induced by glucose, a more physiological but in the same time weaker *in vitro* glycation inducer. The incubation time was prolonged to 7 days and the concentration used in the treatment was increased to 0.8 M, in comparison to ribose. Aminoguanidine inhibited completely the glycation while all extracts showed a remarkable inhibitory effect on BSA glycation, even at low concentrations but no dose-response relation was observed probably due to the incompletion of the reaction.

DISCUSSION

Cardiovascular diseases are the leading cause of deaths worldwide, though since the 1970s, cardiovascular mortality rates have declined in many high-income countries (Fuster & Kelly, 2010). At the same time, cardiovascular deaths and disease have increased at a fast rate in low- and middle-income countries (Finegold, Asaria, & Francis, 2013). The causes of cardiovascular disease are diverse but atherosclerosis and/or hypertension are the most common (Dantas, Jiménez-Altayó, & Vila, 2012).

Oxidative stress, an imbalance between formation of reactive oxygen species (ROS) and antioxidants *in vivo*, appears to be important in both the early and later stages of the atherosclerotic process. ROS, which include free radicals such as superoxide anion radicals, hydroxyl radicals and non-free radical species such as H_2O_2 and singlet oxygen, are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury, inflammation, cardiovascular diseases, diabetes and aging process. It is generally assumed that frequent consumption of plant derived phytochemicals from vegetables, fruit, tea and medicinal herbs may contribute to the shift of balance toward an adequate antioxidant status (Mahomoodally, Subratty, Gurib-Fakim, & Choudhary, 2012).

Several reports tend to show that numerous plant derived natural products are effective antioxidants, and many medicinal plants with a long history of use in folk medicine in different countries against a variety of diseases have turned out to be rich sources of antioxidants (Lee et al., 2005; Mathisen, Diallo, Andersen, & Malterud, 2002). The advantage of natural antioxidants is their safety and that large oral doses are well tolerated (Green, Brand, & Murphy, 2004). Many antioxidant compounds, naturally occurring in plant sources, have been identified as free radical or active oxygen scavengers. Recently, interest has considerably increased in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenesis (Ito, Fukushima, & Tsuda, 1985). Natural antioxidants may protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods. Hence, studies on natural antioxidants have great importance. In the present investigation, we studied the antioxidant property of four extracts obtained from medicinal plants which have been used in folk medicine for centuries in the treatment of several diseases, so their possible application in the prevention of atherosclerosis could be an interesting reinforcement of

their use in ethnomedicine. “Dragon’s blood” is a bright red resin that is obtained from different species of four distinct plant genera; *Croton*, *Dracaena*, *Daemonorops*, and *Pterocarpus*.

Croton lechleri Mull. Arg., own of Mexico, Venezuela, Ecuador, Peru and Brazil, is possibly the best-known source of this sap. When the trunk of the tree is cut or wounded, a dark red resin oozes out (RW.ERROR - Unable to find reference:172). It is used in folk medicine as cicatrizing, anti-inflammatory (Pieters et al., 1993; Ubillas et al., 1994), anti-microbial (Ubillas et al., 1994) and anticancer (Hartwell, 1969), as well as for the treatment of disorders of the digestive system (Ubillas et al., 1994).

The antitumor, antimutagenic, antidiarrhoeal and anti-inflammatory activities of *Croton lechleri* have been intensively studied, but few preliminary or partial studies on its antioxidant activity were documented in the literature in spite of the 90% of the dry weight of the sap is composed of phenolic compounds, including proanthocyanidins, catechin, epicatechin, gallocatechin and epigallocatechin (Cai et al., 1991).

Casimiroa edulis Llave et Lex (Rutaceae) popularly called ‘Zapote blanco’, is a tree distributed in the temperate zones of Mexico and central America. The use of this plant in folk medicine is known from prehistoric times; its leaf or seed concoctions are taken for the sedative-like and sleep inducing effects (Romero et al., 1983).

In early pharmacological researches, alcohol extracts of seeds (Magos & Vidrio, 1991) and aqueous extracts of leaves (Magos, Vidrio, & Enr íquez, 1995) of *Casimiroa edulis* were found to have hypnotic, anticonvulsant and antihypertensive effect. In our previous work, the extracts of *Casimiroa edulis* and *Casimiroa pubescens* were revealed to possess a vasodilation activity on arterial vessel (Bertin, Garcia-Arga éz, Mart ìnez-V àzquez, & Froldi, 2011). Therefore, we chose to further study the *Casimiroa genus* to determine its antioxidant activity for the possible use of these plant extracts in the treatment of CVD taking advantage of the dual action of vasodilation and antioxidant.

The black currant *(Ribes nigrum*), a woody shrub in the family Grossulariaceae, is grown for its berries which are a rich sources of phenolic compounds such as flavonoids and other polyphenols. In folk medicine it was used in the treatment of arthritis, rheumatic complaints, diarrhea, spasmodic cough and has demonstrated a good anti-inflammatory property (EMA, 2010).

Boswellia serrata (Burseraceae), an oleo-gum-resin obtained from a medium size tree of India, has been used for a variety of therapeutic purposes such as cancer, inflammation, arthritis, asthma, psoriasis, colitis and as hypolipidemic remedy. Its anti-inflammatory

property is widely documented while its antioxidant studies are few and often limited to some preliminary *in vitro* assays. So a more detailed study of the antioxidant activity is required as a complementary mechanism to the anti-inflammatory activity in the prevention of atherosclerosis.

The DPPH• method is one of the most commonly used to determine antioxidant activity; it is based on the determination of the radical-scavenging activity. It measures the reducing ability of antioxidants toward DPPH• and is considered to be mainly based on an SET (single electron transfer) reaction, and hydrogen-atom abstraction is a marginal reaction pathway (Huang, Ou, & Prior, 2005). By this method, in this research, it was shown that the antioxidant activity varied widely between the extracts; the highest activity was presented by the *Croton lechleri* sap (EC_{50}=2.74 µg/ml) with an activity comparable to that of ascorbic acid, a well-known antioxidant vitamin.

Further, it was carried out also the ORAC assay of all the extracts and again the *Croton lechleri* showed the highest activity (TEAC = 5,74 x 10^3 ± 0,64 x 10^3 µmol/g). Differently, from DPPH · assay, the ORAC assay is based on HAT (hydrogen atom transfer) reaction and measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (Ou et al., 2001).

Moreover, the four *Casimiroa* extracts (Ce1, Cp1, Ce2, Cp2) showed similar activity in both DPPH• and ORAC assays, and to be noted that in both assays the leave extracts (Ce2 and Cp2) showed higher activity than the seed extracts (Ce1 and Cp1).

We also observed that *Ribes nigrum* demonstrated a higher activity in the DPPH ·assay than in the ORAC assay, this may indicate that it acts as an antioxidant mainly by SET mechanism, and this was confirmed by its high value in the total phenolic content assay, which is also based on SET reaction (Huang et al., 2005). Since in the DPPH• assay, the two seed extracts of *Casimiroa* (Ce1 and Cp1), together with the extract of *Boswellia serrata*, showed a poor activity that did not reached the 50% of inhibition and for this it was not possible to calculate EC_{50} values. It was observed that all extracts showed similar trend in both DPPH ·and total phenolic content assays This phenomenon may be because that first two assays share the same mechanism (SET), and second, phenols are responsible for the majority of the antioxidant activity in most plant- derived products (V. L. Singleton, Orthofer, & Lamuela-Raventos, 1999).

The phenolic compounds are found as a group of approximately 8000 natural compounds which have a phenol as a common structural feature (Shahidi, Janitha, & Wanasundara, 1992). These compounds are divided into three major class, according to the number of phenol subunits in the molecule: 1) simple phenols – phenolics containing one phenol unit; 2) flavonoids – phenolics containing two phenol subunits; and 3) tannins – phenolics consisting of at least three phenol subunits. They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening. Many studies have suggested that flavonoids exhibit biological activities in mammalian, including antiallergenic, antiviral, and anti-inflammatory. Also most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. The capacity of flavonoids to act as antioxidants has been the subject of several studies in the past years, and structure-activity relationships have been established (Pietta, 2000).

Several epidemiological studies provide support for a protective effect of the consumption of fresh fruits and vegetables against cancer (Ingram, Sanders, Kolybaba, & Lopez, 1997), heart disease (Gey, 1995), and stroke (Peterson & Dwyer, 1998). Thus, it is possible that also flavonoids contribute to the protective effect of fruits and vegetables. This possibility has been evidenced by several *in vitro*, *ex vivo*, and animal studies (Gorinstein, Bartnikowska, Kulasek, Zemser, & Trakhtenberg, 1998).

Unfortunately, the humans trials are still limited and somewhat controversial (Wang & Goodman, 1999). Data on biological markers, such as blood levels of flavonoids and their metabolites, are not widely available, thus making it difficult to determine individual or combined role of the flavonoids in relation to other antioxidants (Pietta, 2000).

In our study, we carried out a measurement of total flavonoid content by aluminium chloride method; surprisingly, the extracts of *Croton lechleri* and *Ribes nigrum*, which in the total phenolic content assay showed the highest values, were found to have a very low level of flavonoids, 0.41 ± 0.24 mg QE/g extract and 2.03 ± 0.18 mg QE/g extract respectively. This may be explained because the major components of these two extracts are proanthocyanidins (*Croton lechleri*) and anthocyanins (*Ribes nigrum*) that are oligomers of flavan-3-ol units linked mainly through C_4 to C_8 bonds, and glucosides of anthocyanidins (derived from flavonols, which lack the ketone oxygen at the 4-position) which do not directly react with aluminium chloride.

DPPH• and ORAC assay are considered indirect assays of *in vivo* antioxidant capacity. They are very useful in screening studies because they provide often reproducible data.

The biological relevance of these methods has been argued, since the results of these *in vitro* assays could indicate the potential *in vivo* activity. Obviously, it should be underlined that the mentioned assays do not measure bioavailability, *in vivo* stability, and preservation of antioxidants in the tissues, and their reactivity in situ (Huang et al., 2005). It has been suggested that these assays may underestimate the real physiological antioxidant capacity of extracts (Serrano, Goñi, & Saura-Calixto, 2007). Anyway, since these measurements are rapid and easy to perform, they are widely used for *in vitro* screening of antioxidant activity. It is argued that the use of various analytical methods for evaluation of antioxidant activity can lead to get more knowledge about antioxidant potentials of the studied compounds (Laguerre, Lecomte, & Villeneuve, 2007); therefore, we studied also the antioxidant activity of our plant extracts against the human Low-Density Lipoprotein (LDL) oxidation, by conjugated dienes measurement and TBARS test.

The oxidation of LDL is started by the copper-induced lipid peroxidation through the oxidative deterioration of polyunsaturated lipids. The initiation of a peroxidation sequence in a biological membrane as in whatever polyunsaturated fatty acid depends from the abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which then easily reacts with an oxygen molecule to give a peroxy radical (LOO•). Peroxy radicals can abstract a hydrogen atom from another molecule or a hydrogen atom to give a lipid hydroperoxide, LOOH. A probable alternative fate of peroxy radicals is to form cyclic peroxides; these cyclic peroxides, lipid peroxides, and cyclic endoperoxides fragment to aldehydes including MDA and polymerization products. MDA is the major product of lipid peroxidation (Singh, Chidambara Murthy, & Jayaprakasha, 2002).

The intermediate product of this propagation reaction is the conjugated dienes that has an absorption at 234 nm; thus, with a simple spectrophotometric reading, it can be followed the entire process of the LDL oxidation. While, with the TBARS test the entity of the oxidation was done by the determination of the MDA, final product of LDL oxidation.

In conjugated dienes measurement, at the high concentration of ≥ 100µg/ml all extracts inhibited totally the oxidation process except *Ribes nigrum*, which even at very high concentration did not show a significant inhibition. Since LDL oxidation is based on HAT mechanism (Huang et al., 2005), this data could confirm that *Ribes nigrum* reacts as antioxidant mostly basing on SET reaction. Moreover, for a suitable comparison of the extracts, in the present experiments the concentrations of each extracts were chosen in

order to obtain a complete three phase-curve for everyone in less than 10 hours; this assay is more sensitive but much more time consuming, the results obtained are in line with those obtained by the ORAC assay. And this confirm that these two assays share the same scavenging mechanism, the HAT reaction. While the TBARS assay is less specific, reason for which it is in some way criticized in literature; the no specificity probably results from the acid-heating step of the TBA assay that causes the formation of TBA/MDA-like derivatives (Liu, Yeo, Doniger, & Ames, 1997). Anyway, in TBARS at the concentrations of 10 and 20 μg/ml all extracts of *Casimiroa* genus and *Boswellia serrata* showed the same trend observed in diene conjugated determination, except for *Croton lechleri* which at 5 μg/ml inhibited significantly oxidation of LDL, while *Ribes nigrum* even at very high concentration (200 μg/ml) did not exert a significant inhibition.

TBARS test and conjugated dienes determination are direct methods that are established on studying the effects of antioxidants on the oxidative degradation of a system for the biological relevance (individual lipids, lipid mixtures – oils, lipid membranes, low density lipoprotein, DNA, blood, plasma, etc.) (Roginsky & Lissi, 2005). Direct approach of evaluation that applies various lipid model systems rather than the indirect approach was preferred, where the antioxidant activity is assessed artificially by means of so called one-dimensional antioxidant capacity (AOC) assays (Laguerre et al., 2007). The kind of oxidative substrate in the model systems and the conditions of system play an important role for choosing the methods. Direct methods are often time consuming and they do not achieve the demand for quick and easy assessments.

In the present study, it was also used a simple screening method to measure the inhibitory effects of the plant extracts on formation of fluorescent AGEs *in vitro*. AGE accumulation *in vivo* has been implicated as a major pathogenic process in diabetic complications, including neuropathy, nephropathy, retinopathy, and cataract and other health disorders such as atherosclerosis, Alzheimer's disease, and normal aging. Thus, the discovery and investigation of AGE inhibitors might offer a potential therapeutic approach for the prevention of diabetic or other pathogenic complications (Peng et al., 2008).

In our experimental system were used high concentrations of glucose to speed up the glycation reaction, thus allowing to undertake the glycation process evaluation in an appropriate time-scale, which it occurs very slowly under physiological conditions (Matsuura et al., 2002). Also, to simulate glycation, we repeated the same test using as glycation inducer the ribose, which is 100 times more potent than glucose (Baynes & Monnier, 1989).

Glycation of serum albumin has been widely studied in recent years, and bovine serum albumin (BSA) is commonly used as experimental substrate (Wei, Chen, Chen, Ge, & He, 2009). Given that our main focus was the study of the role of glycation on LDL in the pathogenesis of atherosclerosis, BSA was used as the protein in the glycation model, this for two reasons: first, the protocol requires use of high amounts of protein (up to 50 mg/ml) which was not possible to get with LDL from blood sampling; second, glycation of BSA and LDL are based on the same mechanism - glucose reacts with lysine residues of target proteins (Ghaffari & Mojab, 2010; Jahouh, Hou, Kovâ?, & Banoub, 2012).
In the BSA-glucose protocol, all the plant extracts inhibited significantly the glycation, while in the BSA-ribose protocol only the *Croton lechleri* sap and two extracts of *Casimiroa* Cp1 and Ce2 inhibited significantly the formation of AGEs.

The results obtained in this research work reinforced the claims made in ethnomedicine, giving good prospective of the use of these plant extracts; especially, the *Croton lechleri* sap and leaf extract of *Casimiroa*, in the prevention of the cardiovascular diseases and diabetic complications. In the future, more investigations of *in vivo* antioxidant effect and studies of cytotoxicity are needed. Further, these medicinal plants may also be used to find new compounds endowed with protective action against LDL oxidation and glycation, typical processes of metabolic syndrome.

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