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# Anti-inflammatory activity of plant extracts: an effective in vitro model of study based on primary culture of porcine endothelial cells

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# 1 Abstract

Inflammation is a protective response exerted by the organism to eliminate an injurious stimulus. Endothelial cells play a fundamental role in different physiological processes since, changing their phenotype, modulate the complement and coagulation cascades, inflammation and innate and adaptive immunity. Medicinal plants have been the main remedy to treat different ailments and their study has permitted the development of many drugs.

The aim of the present research was to study the phytochemical composition and the anti-inflammatory activity of four medicinal plants (*Cucumis sativus* L, *Boswellia serrata* Roxb. Colebr, *Salvia sagittata* Ruiz & Pav and *Clinopodium tomentosum* Kunth), traditionally used to treat different diseases, on primary culture of porcine aortic endothelial cells (pAECs) stimulated with Lipopolysaccharide (LPS).

This approach is relevant in terms of screening possible positive activities of medicinal plants in relation to the Replacement and Reduction principles in agreement with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and its implementation in Italy through "Decreto Legislativo" n. 26/2014.

Different extraction processes and techniques have been employed to verify their phytochemical and biological effects (HPLC, RT-PCR, Western blot). The phytochemical analysis revealed the presence of different bioactive compounds (11-keto-β-boswellic acid and β-boswellic acid in *Boswellia serrata* extracts, different phenolic acids and flavonoids with rosmarinic acid as the most abundant component in *Salvia sagittata* and *Clinopodium tomentosum* extracts). In the biological analysis, *Cucumis sativus*, *Boswellia serrata*, *Salvia sagittata* and *Clinopodium tomentosum* reduced the LPS-induced cytotoxicity with the decrement of different inflammatory cytokines and increment of protective molecules and anti-inflammatory cytokines.

In conclusion, our results have clearly demonstrated that the medicinal plants studied have attenuated lipopolysaccharide induced inflammatory response in endothelial cells. Moreover the studies confirm the relevance of primary culture of pAECs as an interesting screening model to explore the huge variety of Ecuadorian medicinal plants.

# 2 Introduction

## 2.1 Endothelial cells and their role in health and disease

Endothelial cells, for their strategic position, play a fundamental role in different physiological processes such as in inflammatory diseases. Normally, they are in dynamic equilibrium with their environment preventing thrombus formation by expression and secretion of anti-coagulant, anti-adhesive, and anti-inflammatory molecules.

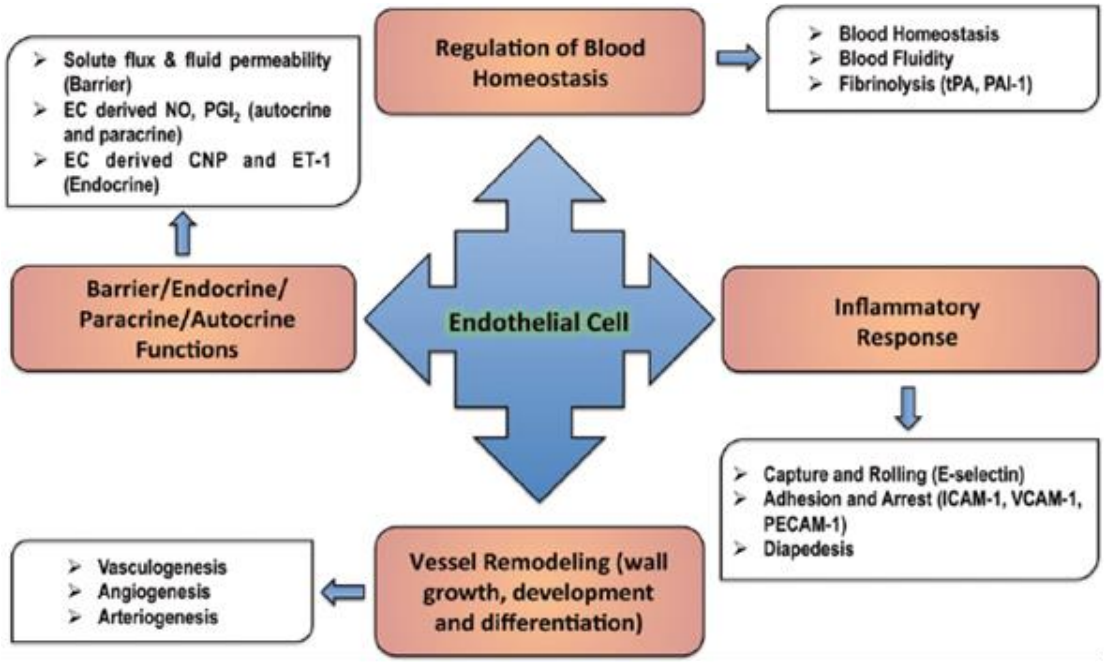


**Figure 1**. Functions of endothelial cells (Triggle et al. 2012)

However, under the stimulation of many different stressors, the endothelium shows changes, which allow it to participate in the inflammatory response; this phenomenon is known as: "endothelial cell activation" (Hunt & Jurd 1998). This term was coined in the 1960s by Willms-Kretschmer to underline a change in the endothelial morphology as well as in the endothelial function. Then, in the 1980s it was demonstrated that interleukin 1 and tumour necrosis factor, induced expression of pro-adesive, pro coagulant and inflammatory genes switching the endothelial resting phenotype into an activated one. These changes do not necessarily evolve into injury or dysfunction, therefore it was reintroduced the term "endothelial cell activation" (Pober 1988).

It is now well demonstrated that the phenotype of activated endothelial cells involves the up-regulation of pro-inflammatory genes, including secretion of inflammatory cytokines and chemokines. This state can still be counterbalanced by the synthesis of protective molecules (IkB-a, A20, Bcl-2) that can still revert the activated phenotype to normal phenotype (Aird 2008).

Otherwise the activation of transcription factor NF-kB if not contrast can evolve into apoptosis or necrosis of endothelial cells

Moreover, endothelial cell activation is divided in two distinct stages, an early event named ‘‘EC stimulation’’ and a later event named ‘‘EC activation’’. These two phases are referred to as ‘‘Type I EC activation’’ and ‘‘Type II EC activation,’’ respectively (Bach 1994; Pober & Sessa 2007; Roumenina et al. 2016)

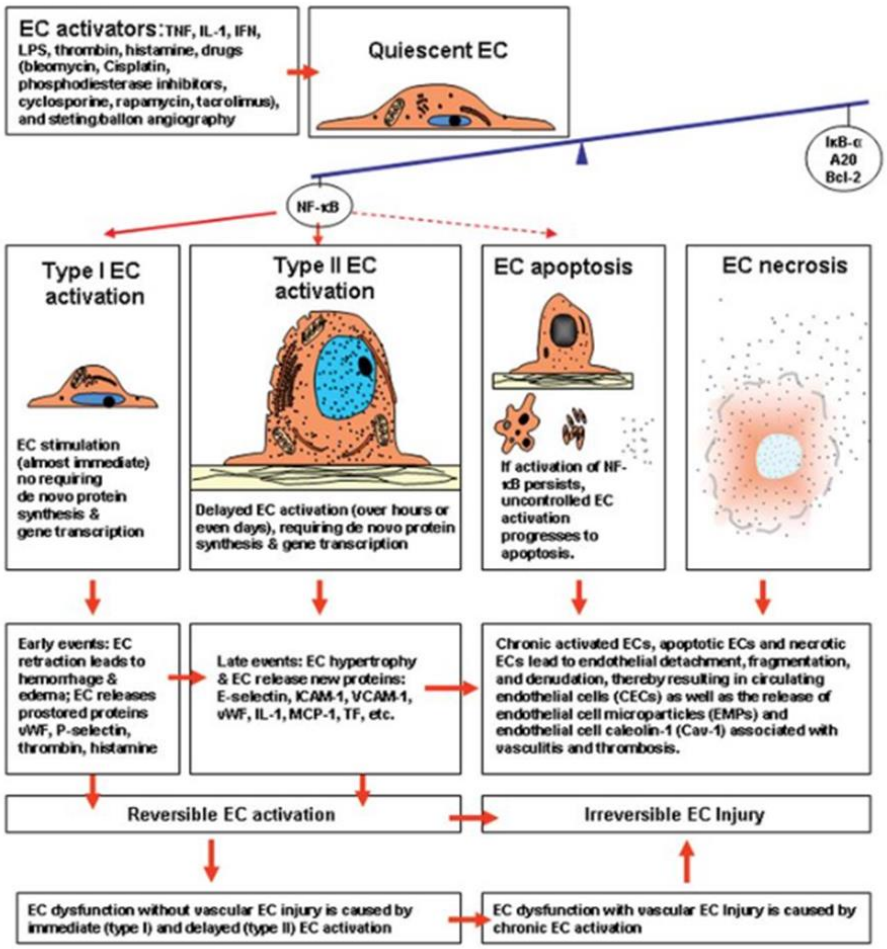


**Figure 2.** Endothelial cell activation (Zhang et al. 2010)

***Type I activation:*** occurs immediately following stimulation and does not require de novo protein synthesis or gene transcription. The surface of the type I-activated ECs is capable of shedding endothelial adhesion and antithrombotic molecules, such as P-selectin,thrombin, heparin, antithrombin III, and thrombomodulin (Bach 1994). This activation typically occurs after interaction of different ligands that bind to the extracellular domains of heterotrimeric G-protein-coupled receptor (GPCRs), as example histamine H1 receptors signal through the intracellular G-protein $\alpha_q$ subunit (Pober & Cotran 1990) catalysing exchange of GDP for GTP. The GDP to GTP

exchange triggers the dissociation of the G-protein $\alpha_q$ subunit and activates β isoforms of phospholipase C (PLCβ), catalysing the release of inositol-1,4,5-trisphosphate (InsP3) from the membrane lipid phosphatidylinositol-4,5- bisphosphate (PtdIns (4,5) P2). InsP3 induces transient intracellular elevations in cytosolic free Ca2+ by releasing Ca2+ from endoplasmic reticulum stores. In endothelial cells, these changes may take the form of oscillatory, transient Ca2+ levels, or may, at greater signal strength, produce an elevated plateau of cytosolic Ca2+ levels (Birch et al. 1994). The entry of extracellular Ca2+ may be sustain elevated cytosolic Ca2+ levels (Lückhoff & Clapham 1992). The exchange of GDP for GTP, on the small G protein RHO (RAS homologue), is facilitated by the activation of GPCRs. Thus, the type I activation response is mediated by combine RHO activation and elevation in cytosolic free Ca2+. Specifically, Ca2+-mediated activation of cellular phospholipase A2 (cPLA2), an enzyme that cleaves membrane phosphatidylcholine into arachidonic acid and lysophosphatidylcholine, results in an increased of blood flow.

By cyclooxygenase-1 (COX1; known as prostaglandin H synthase 1 (PGH1)) the free arachidonic acid is rapidly and sequentially converted to PGH2 and then by prostacyclin synthase to PGI2 (Niu et al. 2003). The adaptor protein calmodulin form a complex with the cytosolic Ca2+ ions that activates nitric-oxide synthase 3 (NOS3) to produce NO, which synergizes with PGI2. The formation of Ca2+–calmodulin complex and the temporary rise in cytosolic Ca2+ leads to the activation of myosin-light-chain kinase (MLCK) (Egan & FitzGerald 2006), which phosphorylates myosin light chain (MLC). The combination of MLC phosphatase inhibition (mediated by RHO-dependent kinase) and MLCK activation increases MLC phosphorylation. The phosphorylation of MLC initiates the contraction of actin filaments that are attached to tight junction and adherens junction proteins resulting gaps opening between adjacent endothelial cells. In post-capillary venules, these responses are particularly evident where tight junction elements are least expressed and relevant GPCRs are maximally expressed (Heltianu et al. 1982). The leakiness of venules may be enhanced by the production of NO and PGI2 in venular endothelial cells (Teixeira et al. 1993). A provisional matrix formed by plasma proteins, leaking from the blood into the tissues, supports the migration, attachment and survival of invading neutrophils (Frangogiannis 2006).

The exocytosis of WPBs is also causing by the activation of MLC in venular endothelial cells, which bring P-selectin to the luminal cell surface (Birch et al. 1992 and 1994). At the same time, an endothelial-cell derived acyl form of platelet-activating factor (PAF) is formed by the rapidly acetylation of lysophosphatidylcholine (Prescott et al. 1984). The collective display of P-selectin and PAF on the endothelial luminal plasma membrane procure a dual signal that causes the

tethering of circulating neutrophils (due to P-selectin) followed by cell regulation and integrin activation (due to PAF), initiating neutrophil extravasation (Lorant et al. 1991).

In intracellular Ca2+ rising in endothelial cells also has a major role in leukocyte recruitment. Most part of leukocytes seem to cross through the wall vessel by passing between adjacent endothelial cells (Marchesi 1961).

The endothelial cell membranes not only contain adherens and tight junction proteins, but also express a platelet-endothelial cell adhesion molecule 1 (PECAM1 or CD31) and, two proteins that participate in homophilic interactions with neutrophils and monocytes in a manner that is essential for transmigration (Schenkel et al. 2002).

Occasionally leukocytes also may cross through the endothelial cell body (Feng et al. 1998).

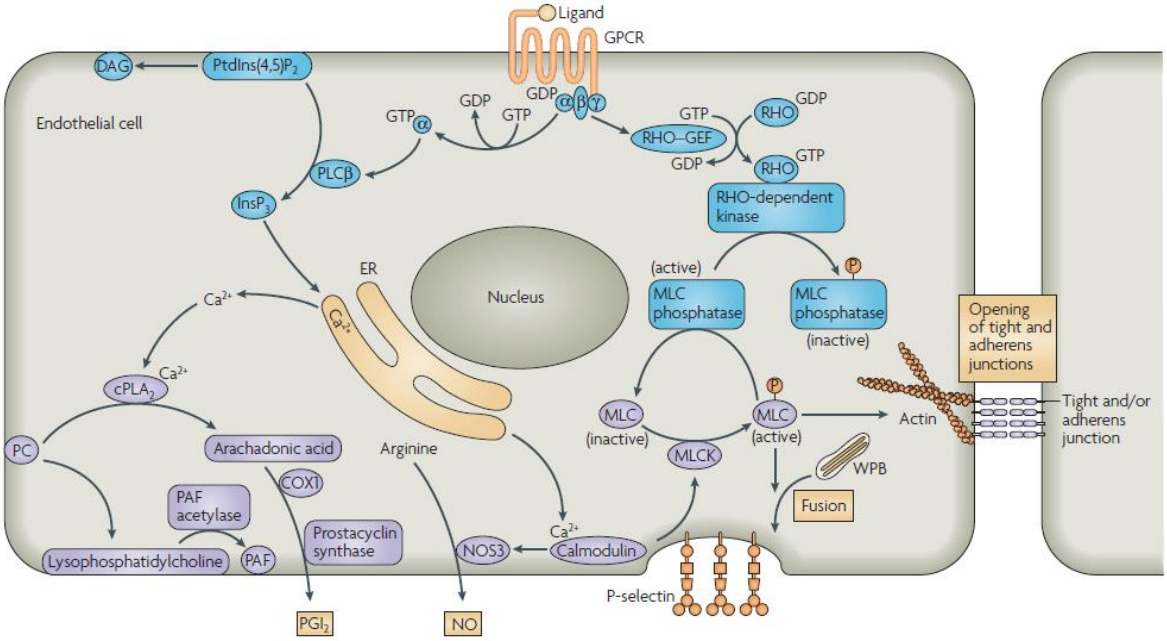


**Figure 3.** Type I endothelial cells activation phatway (Pober & Sessa 2007).

***Type II activation of endothelial cells***. is a delayed response that involves the activation of gene transcription and the de novo synthesis of proteins (Pober & Sessa 2007). including adhesion molecules, cytokines, chemokines, and procoagulant factors (Bach 1994; Goepfert et al. 2000). Typically it is initiated in an inflammatory process that is sustained in the time and requires a more persistent form of endothelial cell activation. Tumour-necrosis factor (TNFα) and interleukin-1 (IL-1), derived principally from activated leukocytes, are the principal mediators involved in this response (Pober & Cotran 1990). The signalling pathway used by TNF in endothelial cells begin when TNF binds to the extracellular domains of TNF receptor 1 (TNFR1) and recruits TNFR-associated via death domain protein (TRADD) to the intracellular death domain of the receptor.

TRADD in turn recruits the serine/theonine kinase receptor interacting protein 1 (RIP1) and TNFR-associated factor 2 (TRAF2), an E3 ubiquitin ligase. This complex, also called as signalosome, initiates various kinase cascades that lead to activation of activator protein 1 (AP1) and the transcription factors nuclear factor-κB (NF-κB) (Aird 2006).

On the other hand, the binding of IL-1 to the type 1 IL-1 receptor (IL-1R1) induces a signalling pathway mediated by a signalling complex comprised of Toll/IL-1 receptor accessory protein (TIRAP), myeloid differentiation primary-response gene 88 (MyD88), IL-1R-associated kinase 1 (IRAK1), IRAK4, and TRAF6, that activates the same transcription factors trigger by TNF (Martin & Wesche 2002). The pro-inflammatory responses induced by these cytokines emerge from new gene transcription mediated by AP1 and NF-κB. Type II activation response require a longer time to be initiated (hours) since these responses require both transcription and translation of new proteins. Type II activation also leads to increase vascular leakage of plasma proteins, increase blood flow and increase leukocyte recruitment at the site of inflammation. Furthermore, due to the induction of COX2, type-II-activated endothelial cells enhance PGI2 synthesis, which, as COX1, can initiate prostaglandin synthesis by converting arachadonic acid to PGH2 in a much throughput higher level. The increase in blood flow, in type-II-activated endothelial cells, still depends on a rise in cytosolic Ca2+ produced in type I activation in order to activate cPLA2 and release arachadonic acid (Zavoico et al. 1989).

TNF and IL-1 induce the leakage of plasma proteins by stimulating venular endothelial cells to reorganize their actin and tubulin cytoskeletons and thereby open up gaps between adjacent cells (Pober et al. 1987; Petrache et al. 2003). The stimulation of vascular endothelial cells to reorganize their actin and tubulin cytoskeletons and thereby open up gaps between adjacent cells, produced by TNF and IL-1, results in a leakage of plasma proteins which in turn is triggered in an NF-κB- and protein-synthesis-dependent manner.

A particularly firm provisional matrix described as an induration (hard swelling), is produced by the sustained leakage of plasma proteins, such as fibrinogen, which is converted into a fibrin-rich clot and conduce to the hardness (Clark et al. 2007).

In type II activation the leukocyte recruitment is much more effective than in type I activation. The synthesis and display of chemokines, such as CXC-chemokine ligand 8 (CXCL8; also known as IL-8) and new leukocyte adhesion molecules, such as E-selectin produce the recruitment of neutrophils. Endothelial cells can also capture chemokines made by other cells and display them on their luminal surface bound to heparin sulphate proteoglycans. CXCL8, similar to PAF, triggers

firm attachment of neutrophils to endothelial cells and induces diapedesis into the tissue. It can only act efficiently on neutrophils that are tethered or rolling on the endothelial cell surface; that is, CXCL8 and E-selectin (Ley & Reutershan 2006).

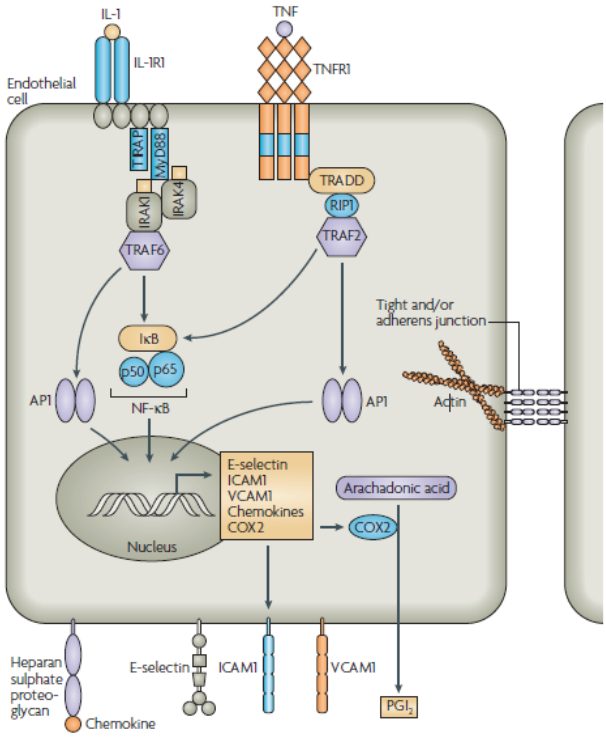


**Figure 4**. Type II endothelial cells activation phatway (Pober & Sessa 2007).

# 2.2 Endothelial cells and angiogenesis

Vascular remodeling is an active process that leads to a reorganization of the vascular structure also determining functional changes. Remodeling often occurs in response to prolonged physiological haemodynamic changes, for example during pregnancy or during physiological processes such as wound healing (Klein et al. 2014). However, even pathological conditions, including phenomena such as atherosclerosis, thrombosis, hypertension, ischemic diseases, congenital vascular lesions (aneurysms, fibromuscular hyperplasia and stenosis) and tumor growth are characterized by an increase in vascular reorganization (Korshunov et al. 2007; Mulvany 1999; Renna et al. 2013). Vascular remodeling during embryonic life occurs mainly through vasculogenesis phenomena, in which the formation of new blood vessels from the differentiation of bone marrow-derived endothelial progenitors called angioblasts. In the adult life, the vascular remodeling takes place mainly through the phenomenon of angiogenesis, that is the formation of new vessels from a pre-existing vascular network. The role of ECs in the angiogenesis is fundamental in fact the sprouting of new vessel segment start from the activation of endothelial

cells due to a pro-angiogenic stimuls such as the growth factor “Vascular Endothelai Growth Factor” VEGF or the fibroblast growth factor (FGF). EC selected to become the “tip cell” migrates and guide the new sprout formation. In particular, the sprouting of new vessel follow a well-defined program: the degradation of basement membrane by metal proteinases (MMPs) secretion, then endothelial cell proliferation and a formation of solid sprouts. New sprouts connect neighboring vessels with a lumen lined by endothelial cells and integrated in the vascular network. Finally the coverage of pericytes and smooth muscle cells render stable the new vessels (Betz et al. 2016).

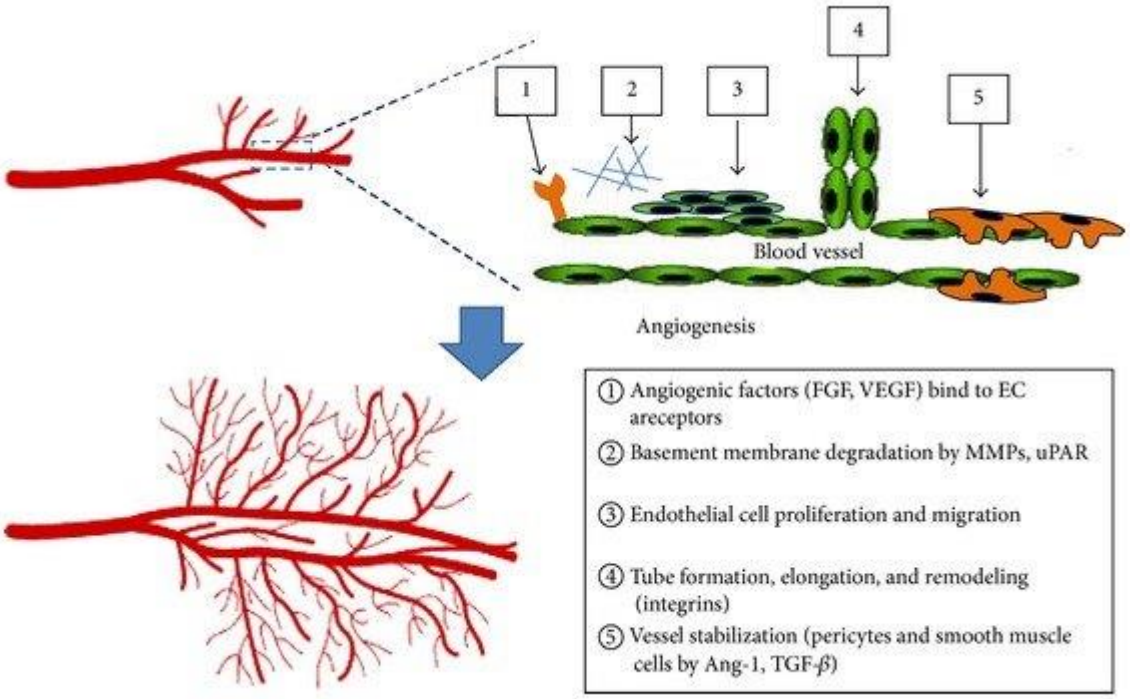


**Figure 5.** “Sprouting angiogenesis” (Yoo & Kwon 2013).

Endothelial cells could made angiogenesis also for a less descripted phenomenon of intussusceptive angiogenesis in which a cylindrical microstructure spans the lumen of small vessels and capillaries. The extension of the intussusceptive pillar appears to be a mechanism for pruning redundant or inefficient vessels, modifying the branch angle of bifurcating vessels and duplicating existing vessels (Mentzer & Konerding 2014). The process begins with the protrusion of opposing capillary walls into vessel lumen, after establishing an interendothelial contact EC bilayer and the basal membranes are perforated centrally and the new formed pillar increases in

girth after being invaded by pericytes and fibroblasts, which produce collagen fibrils (Burri et al. 2004).

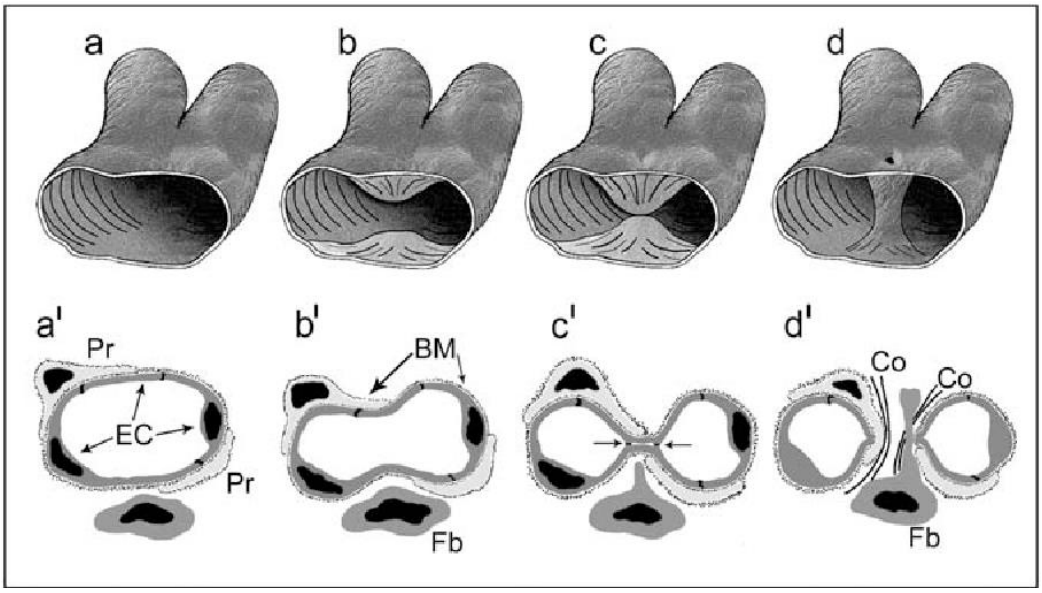


**Figure 6.** Intussusceptive angiogenesis (Burri et al. 2004)

An out of balance of angiogenesis is the basis of many pathological disorders (Ungvari et al. 2018). In particular, excessive angiogenesis is involved in diseases such as cancer, blinding disorders, arthritis and sepsis. On the contrary an insufficient angiogenesis occurs in chronic wounds, coronary heart diseases, stroke. (Zhang et al. 2013)

## 2.3 Secondary metabolites and their role in nature

Plants have populated the majority of the worldwide surface. They are present, by volume and weight, in more proportion than all other forms of life combined owing of rich levels of specialization and the capacity to interact with other organisms (Ramakrishna & Ravishankar 2011). In general plant metabolism is often centered around two major classes. Primary metabolism allows the plant to use water, carbon dioxide and minerals to the production of macromolecules (carbohydrates, lipids and amino acids) required to cellular creation and maintenance.(Kennedy & Wightman 2011).

Secondary metabolites are molecules that play a major role in the adaptation of plants to different environment and stress conditions. At the beginning were considered as compounds without fundamental role in the maintenance of life processes in the plants, but an indication of their necessity in plants survival is reflected in the energy invested in their synthesis, which is usually far in excess of that required to synthesize primary metabolites. Moreover, they are implicated in

several straightforward activities; such as, protecting the plant against different microorganisms (bacteria, fungi, and viruses), acting as allelopathic defenders of growing space against competitor plants and at the same time produce antioxidant and antiproliferative agents. Furthermore, have significant practical applications in medicinal, nutritive and cosmetic purposes.(Kliebenstein 2013).

## 2.4 Bioactive compounds from natural sources and drug discovery

Natural products, especially plants, have been used since ancient time for their therapeutic properties. Certainly, their use goes back to the Sumerian civilization and even more it has been recorded that Hippocrates used approximately 400 different plant species for medicinal purposes. Likewise, in different civilizations, such as Chinese, Ayurveda, and Egyptian, natural products played a prominent role in ancient traditional medicine systems. Over the last century, an impressive number of modern drugs have been derived and developed from natural sources (vincristine from Vinca rosea, morphine from Papaver somniferum, Taxol from T. brevifolia) and many of them based on their use in traditional medicine (Sarker et al. 2006). According to Cragg et al. 1997, 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives, and 60–80% of antibacterial and anticancer drugs were from natural origins. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancers had natural origins. In 2001, eight (simvastatin, pravastatin, amoxycillin, clavulanic acid, azithromycin, ceftriaxone, cyclosporin, and paclitaxel) of the 30 top-selling medicines were natural products or their derivatives, and these eight drugs together totaled US $16 billion in sales. The emergence of ‘‘natural’’ pharmaceutical industry, which is growing rapidly in Europe and North America, as well as in traditional medicine programs being incorporated into the primary health care systems of Mexico, the People’s Republic of China, Nigeria, and other developing countries has done one more time becoming popular the use of natural products in the form of food supplements, nutraceuticals, and complementary and alternative medicine. Due to the extension of the planet, only a small fraction of the world’s biodiversity has been explored for bioactivity to date. As example, so far only 5-10 % of the almost 250,000 species of higher plants that exist in the planet have been investigated. Additionally, the reinvestigation of previously studied plants has continued to produce new bioactive compounds with interesting drug potential. Another valuable source for novel bioactive compounds is represented by marine organisms of which few studies have been reported (Cragg & Newman 2001). Fortunately, the introduction and development of

several new and highly specific in vitro bioassay techniques, chromatographic methods, and spectroscopic techniques, especially nuclear magnetic resonance (NMR), have made much easier to screen, isolate, and identify potential drug lead compounds quickly and precisely (Sarker et al. 2006).

## 2.5 Phytoterapy in veterinary medicine

The use of natural products including animals, minerals, and plants for medicinal purpose originates even before recorded history. In fact, natural products used to treat different diseases is not only restricted to humans but also extended to the treatment of various animal disorders (Antonio et al. 2015). Thus, the search to find more effective and affordable medicines to be used in the treatment of different diseases in animals, as in the human case, is ongoing.

McCorkle 1995 defined the ethnoveterinary research as 'the systematic investigation and application of folk veterinary knowledge, theory and practice. Moreover, ethnoveterinary medicine (EVM), additionally to the use of ethnoveterinary plants to prevent and control disease, includes animal husbandry practices, the use of diagnostic procedures, surgical methods and traditional veterinary theory (Schillhorn van Veen 1997; Van der Merwe et al. 2001).

Despite just very few phytotherapeutic products are registered for animal's treatment and scientific literature relies on few books or manuals regarding veterinary phytotherapy, EVM has attracted considerable interest in various parts of the world as attested by a high number of ethnoveterinary surveys conducted in Africa, America, Asia, and Europe (Ahmad et al. 2015; Benítez et al. 2012), even currently is a major topic in an increasing number of research projects, and the publication of studies on EVM, in certain international journals dealing with veterinary medicine or pharmacology, are more frequent (Akerreta et al. 2010; Lans et al. 2009). EVM is often the only available alternative to rural and suburban populations of the developing countries (McCorkle 1995; Wanzala et al. 2005) in which having farm animals (donkey, mule, pig or chickens) is fairly common, desirable, and probably indispensable for the family economy and in consequence looking after the health of these animals is crucial for the family economy. There are also still rural communities in Europe, especially in the Mediterranean region, where the health care of the members of a family extends to that of their domestic animals.

To reduce the use of conventional drugs, EVM is utilized in organic farms, in which not only herbal drugs, such as plant extracts and essential oils, but also homeopathic products, nutraceuticals and oligoelements, are considered the main drugs to administer in different animal's diseases. Likewise, herbal drugs usually used in human practice to treat respiratory, skin, urinary,

digestive, and cardiovascular affections, and to reduce stress are often utilized in pets, in particular by owners that used such remedies for themselves (Severino et al., 2008).

In addition is important to consider many aspects in the use of medicinal plants in EVM as example seasonal unavailability of plant material, methods of preparation and administration of the plant, management practices to limit the impact of the disease, as well as lack of dosing certainty and standardization of remedies. In consequence, a lack of activity in a laboratory-based in vitro screening system does not automatically correspond to lack of efficacy of a traditional medication. Obviously EVM is possible to be used in common ailments (mild diarrhoea, skin diseases, intestinal worms and wounds) because at the moment orthodox treatments are certainly indispensable in cases such as epidemics of contagious diseases (Martin et al. 2001).

## 2.6 Animal models for translational medicine

The translation of theoretical knowledge and experimental breakthroughs into clinical practice of medicine is also known as Translational Medicine. Obviously, scientific discoveries prove to be complete when they result into an application: usually, a medical discovery follows the path, from the "benchside to the bedside" resulting in the so-called Translational Research (Keramaris et al. 2008). Animal testing places itself exactly in the middle of the process of the Translational Research applied to the study of human biology.

Evidently, the best model to study human biology (physiology, pathology, treatment of diseases) would be the human subject, but it is clear that this option cannot be considered ethical. To overcome this problem, animals have been routinely used as models to characterize pathogenesis of diseases and to discover new drugs, entailing several advantages such as the homogeneity of the population and the wider possibilities to study toxicity of treatments, pathology and physiology. The evaluation of mechanism of action of existing drugs and the discovery of new drugs have been widely carried out using animal models. However, even if the animal is a complex and functional model, effective treatments on animals could be less or not effective or, sometimes, even harmful on humans (Hooijmans & Ritskes-Hoitinga 2013; McGonigle & Ruggeri 2014). The main challenges in Translational Research consist in the biological differences among species, the poor methodological quality of animal experiments, the differences in designing the experiments that involve animals, as well as the deficient reporting of the details concerning the animal experimentation (Hooijmans & Ritskes-Hoitinga 2013).

Scientists are always committing to the continuous challenge faced by the translational research involving animal experiments. In 2002 the LANCET journal reported the article "Systematic

reviews of animal experiments" written by Sandercock and Roberts. This paper strongly recommended the systematic review of the literature concerning well performed animal experiments (high quality methodology) as the base of any new clinical trial (Sandercock & Roberts 2002). It is quite clear that the high-quality methodology (Russell and Burck's Refinement) is essential to improve the outcome of a translational research. The urgent need for guidelines in animal experiments was also underlined in the Kilkenny and colleagues' paper. After a survey on a consistent number of scientific papers concerning animal testing, the authors reported a lack of some of the major information about the experiments that involve animals and recommend an accurate and transparent description of the methods (Kilkenny et al. 2009). In their following articles, they also defined the Animal Research Reporting In Vivo Experiments (ARRIVE) guidelines as a tool for animal-based experimentation (Kilkenny et al. 2010; Hooijmans & Ritskes-Hoitinga 2013; Dothel et al. 2013), available on the web (https://www.nc3rs.org.uk/arrive-guidelines) and actually required by many relevant biological journals for publications of animal experiments.

## 2.7 Use of medicinal plants in Ecuador

The number of vascular plants that exists on Earth varies according different estimations between 260,000 and 320,000 species (Bennett & Prance 2000; Judd et al. 2002). The vast majority of them are plants with flowers or angiosperms, which appeared in the Cretaceous 145 million years ago (Judd et al. 2002). Adaptations of plants for life in firm ground formed the basis for the development of human, who has populated the entire planet and has obtained from plants the sustenance necessary to survive. The more than 17,000 species of vascular plants that exist in Ecuador (Jørgensen & León-Yánez 1999, Ulloa Ulloa & Neill 2005) are the result of a history of adaptations to diverse means, coevolution with other organisms and the dynamics of the earth's surface. This great diversity of Ecuadorian plants comes from own species of the Tropical Andes, of tropical and subtropical zones of America, tropical Asia, Malaysia, Africa, as well as from temperate zones of the northern and southern hemispheres, even from the cold regions of the austral element, as the sub-Antarctic and Antarctic and of cosmopolitan plants (Gentry 1990, Ulloa Ulloa & Jørgensen 1995). The quarter of the Ecuadorian species are endemic (Valencia et al., 2000) and of these, 7% have been reported to be useful in different applications.

The first inhabitants used plants to fed (roots, seeds, stems and fruits) and probably obtained spices or condiments as a food supplement. In addition to treat their ailments, infestations and diseases, through a long process of trial and error, used plants like medicines. They also obtained poisons

to hunt, fish and even, to kill his enemies. They identified what woods burned better as fuel and which ones were optimal for the construction of boats and shelters. They learned the extraction of natural fibers to knit goods for the transport of food and to manufacture textiles. They also learned the properties of stimulating plants as yocó (Paullinia yoco) and the guayusa (Ilex guayusa) Also of those as yaje (Banisteriopsis caapi), San Pedro (Echinopsis pachanoi), wantuk (Brugmansia sanguinea) or vilca (Anadenanthera colubrina), that could lead them to altered states of consciousness, to explore the metaphysical world and communicate with spirits and gods that were part of his worldview. In summary, the immense diversity of plants with which the human being interacted in the different Ecuadorian ecosystems, were fundamental pillar of current cultures.

Traditional knowledge and the use of wild plants is being lost, so the acculturation process is occurring rapidly and silently in Ecuador (Byga & Balslevb 2004, Guerrero 2005). Scientific research is a basic instance that has the power to influence beneficially the interaction of man with the plants at different scales and who can contribute notably to this new social reality, as it provides guidelines for the sustainable use of species and ecosystems and provides objective approaches when carrying out actions or make conservation decisions or modes of exploitation of species.

The use of medicinal plants is immersed in the daily life of its inhabitants. Folk medicine is practiced mainly by inhabitants of rural areas, but also by persons of all social classes. In markets of the Sierra, Coast and Amazon is possible to find a variety of plants with medicinal uses (Ortega 1988, Cerón & Gaybor 1994, Cerón & Montalvo 1994). The causes that accentuate the extensive employment of medicinal plants among Ecuadorians are: the low purchasing power of the majority of the population that does not allow to access to medicines, the lack of an official health system effective and, mainly, because the medical ancestral knowledge ancestral is immense (Estrella 1995, Buitrón 1999). Because of the Spanish conquest that took place in the XVI century and the constant coming and going of diverse people, have been incorporated new medicinal elements that have been amalgamated and have enriched the ancestral indigenous medical knowledge (Varea 1922, Acosta-Solís 1992b, Buitrón 1999). In traditional Ecuadorian medicine, the real and the magical world (populated by spirits, gods and demons) are the both sides of the medal. In this context, the diseases are not consequences of the failures of the organs and their functions, or the invasion of pathogens, but the result of the influences of external and supernatural forces that are behind of all things of the real world, to warn, reward or punish according to their codes, mandates, interests or sympathies (Kohn 1992b, Kothari 1993, Rodríguez 1995). As in other countries, it is important and characteristic the use of psychotropic agents for healing through rituals (Schultes & Hofmann 2000, Angelo & Capriles 2004). However, in most part of the cases, this knowledge is

transmitted only in oral form and that is why it becomes indispensable to improve the research in this field in Ecuador, with the aim to conserve in a better way the species and their habitats, improving the use and trade of medicinal plants and derivative products, in order to benefit the population's health.

# 3 Aim

The present research was performed in the Dipartimento di Scienze Mediche Veterinarie of the University of Bologna, Dr Tubon Usca participate to the research activities thanks to a special scholarship offered by Secretaría de Educación Superior, Ciencia, Tecnología e Innovación (Ecuador)

Principal aims were:

- To isolate and characterize primary cultures of porcine aortic endothelial cells (pAECs).
- To characterize, by high Performance Liquid Chromatography (HPLC), different medicinal plant extracts used in the traditional medicine.
- To analyze by biological tests the anti-inflammatory activity of different medicinal plants on porcine aortic endothelial cells (pAECs) in order to find scientific arguments that support their traditional uses.
- To demonstrate the importance of the study of medicinal plants, especially in countries with high biodiversity, to contribute with the development of the scientific knowledge and improve the quality life and health of the people.

In this framework four medicinal plants, *Cucumis sativus*, *Boswellia serrata, Salvia sagittata* and *Clinopodium tomentosum* were studied.

# 4 Experimental part

## 4.1 Cell isolation characterization and use

Thoracic aortic traits were collected from adult pigs at a local slaughterhouse. After collection, aortas were firstly washed with a sterile sodium chloride physiological solution contained ampicillin and amphotericin B and then with Dulbecco Phosphate Buffer Solution (Gibco-Life technologies Carlsbad CA, USA) contained antibiotics-antimicotics 10X (Gibco-Invitrogen). After that aorta was cleaning by cutting the excess of material adhered to the arterial branches. Then all the arterial side branches were tied, and aorta was canulated with modified syringe cones to set up a closed system. The vessels were gently flushed with a solution of DPBS containing antibiotics-antimicotics 1X (antibiotic-antifungal) to remove residual blood, then was filled with DPBS added with 0.2% collagenase (Sigma Chemical Company, St Louis, MO, USA) and clamped at both ends. After 20 minutes of incubation at 38,5°C, the collagenase solution was collected, and the vessels were washed with DPBS containing 10% of fetal bovine serum (FBS) (Gibco-Life technologies Carlsbad CA, USA). The solutions were pooled and centrifuged at 500 g for 10 minutes. The cellular pellet was resuspended in 1 mL Human Endothelial Serum Free Medium (hESFM) (Gibco-Life technologies Carlsbad CA, USA) supplemented with 10% of FBS and 1% antibiotics-antimicotics (Gibco-Invitrogen). Cell number and viability (85–90%) were determined under a phase-contrast microscope. Approximately 3x105 cells was placed in T-25 tissue culture flasks (T25-Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA) in a 5% CO2 atmosphere at 38.5°C. The cells were maintained in a logarithmic growth phase by routine passages every 2–3 days at a 1:3 split ratio. To confirm their endothelial origin, cultured cells were grown in slide chambers (Falcon, Becton-Dickinson) for 24 hours, they were then fixed with ethanol:acetic acid (2: 1) and stained with 1:200 rabbit polyclonal anti-human factor VIII (Dako A/S, Glostrup, Denmark), 1:100 mouse monoclonal anti-porcine CD31 (Serotec LTD, Oxford, UK), and 1:25 rat monoclonal anti-porcine Caderine (Serotec LTD) antisera. Primary antibodies were added in a humidified chamber for 1 hour at room temperature (RT); fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were then added (1:800 dilution in DPBS). The cells were then counterstained with propidium iodide and examined under an epifluorescence microscope (Eclipse E600 Nikon, Japan) equipped with fluorescein (FITC) and tetramethylrhodamine (TRITC) filters and with a Nikon digital camera.

**Figure 7:** Methodology for production of primary pAEC culture http://www.portaledidatticovet.org/vet02/78-fisiologia/335-colture-cellulari.html

## 4.2 *Cucumis Sativus*

Bernardini C, Zannoni A, Bertocchi M, Tubon I, Fernandez M, Forni M. Water/ethanol extract of Cucumis sativus L. fruit attenuates lipopolysaccharide-induced inflammatory response in endothelial cells. *BMC Complementary and Alternative Medicine*. 2018;18:194.

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# Water/ethanol extract of *Cucumis sativus* L. fruit attenuates lipopolysaccharide-induced inflammatory response in endothelial cells

Chiara Bernardini*, Augusta Zannoni, Martina Bertocchi, Irvin Tubon, Mercedes Fernandez and Monica Forni

**Abstract**

**Background:** It is widely accepted the key role of endothelium in the onset of many chronic and acute vascular and cardiovascular diseases.
In the last decade, traditional compounds utilized in "folk medicine" were considered with increasing interest to discover new bioactive molecules potentially effective in a wide range of diseases including cardiovascular ones. Since ancient times different parts of the *Cucumis sativus* L. plant were utilized in Ayurvedic medicine, among these, fruits were traditionally used to alleviate skin problem such as sunburn irritation and inflammation. The main purpose of the present research was, in a well-defined in vitro model of endothelial cells, to investigate whether a water/ethanol extract of *Cucumis sativus* L. (CSE) fruit can attenuate the damaging effect of pro-inflammatory lipopolysaccharide (LPS).

**Methods:** Cell viability, gene expression of endothelial cell markers, cytokines secretion and in vitro angiogenesis assay were performed on porcine Aortic Endothelial Cells exposed to increasing doses (0.02; 02; 2 mg/ml) of CSE in the presence of pro-inflammatory lipopolysaccharide (LPS 10 μg/ml).

**Results:** CSE reduced LPS-induced cytotoxicity and decreased the cellular detachment, restoring the expression of tight junction ZO-1. The increase of TLR4 expression induced by LPS was counterbalanced by the presence of CSE, while the protective gene Hemeoxygenase (HO)-1 was increased. *Cucumis sativus* L. inhibited the early robust secretion of inflammatory IL-8 and GM-CSFs, furthermore inhibition of inflammatory IL-6 and IL-1α occurred late at 7 and 24 h respectively. On the contrary, the secretion of anti-inflammatory IL-10, together with IL-18 and IFN-γ was increased. Moreover, the in vitro angiogenesis induced by inflammatory LPS was prevented by the presence of *Cucunis sativus* L. extract, at any doses tested.

**Conclusions:** Our results have clearly demonstrated that *Cucumis sativus* L. extract has attenuated lipopolysaccharide-induced inflammatory response in endothelial cells.

**Keywords:** Endothelium, *Cucumis sativus* L., Inflammation, Hemeoxygenase-1, Cytokines, Angiogenesis

## Background

Vascular integrity contributes to the maintenance of the homeostasis of the whole organism [1]. The break of the vascular balance causes many pathological alterations, including cardiovascular diseases (CDVs), that represent the principle cause of death globally [2].

Among vascular cellular components, endothelial cells (EC) establish the inner lining of blood vessels and perform a pivotal role in the maintenance of the vascular integrity [1, 3–5]. Moreover endothelial cells have a key position in the beginning, progression, control and resolution of the vascular dysfunction [6–9]. Several endogenous and exogenous pro-inflammatory stimuli, such as lipopolysaccharide (LPS), induce "EC activation". The phenotype of activated endothelial cells promotes phenomena of vasoconstriction, leukocyte adhesion, coagulation and thrombosis. This change involves the up-regulation of pro-inflammatory genes, including secretion of inflammatory cytokines and chemokines. If the pro-inflammatory

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status is not counterbalanced by the synthesis of protective molecules, the endothelial activation converts into the endothelial dysfunction and then in the vascular disease [10, 11].

In full accordance with the principle of "Replacement", one of the commonly-accepted 3Rs rules (Replacement, Reduction and Refinement) for more ethical use of animals in experimental testing, primary culture of porcine Aortic Endothelial Cells (pAECs) were successfully used in many different in vitro models, preceding the in vivo, confirming swine as a relevant animal model for translational medicine [12–17].

In the last decade, traditional compounds utilized in "folk medicine" have been considered with increasing interest to discover new bioactive molecules potentially effective in a wide range of diseases including cardiovascular ones. Nevertheless, to support the traditional medicine use of these compounds, scientific informations regarding the phytochemical or biological activity are needed. [18, 19].

Cucumber (*Cucumis sativus* L.) is a popular vegetable crop member of the Cucurbitaceae family commonly cultivated for its edible fruits. Since ancient times, different parts of the cucumber plant have been employed in Ayurvedic medicine, among these, fruits are traditionally used to alleviate skin problem such as sunburn's irritation and inflammation [20, 21]. Recently in vitro evidences [22] suggested that a *Cucumis sativus* extract show strong anti-oxidant capacity and ability to stability the membrane of human red blood. Moreover, Patil [23] demonstrated that aqueous extracts of *Cucumis sativus* is efficacious on inflammatory model of ulcerative colitis in in vivo model of Wister rats.

Nowadays no studies have investigated the effect of *Cucumis sativus* L. on vascular endothelial cells. Therefore, to provide new scientific evidence to support traditional medicine use of *Cucumis sativus* L., the main purpose of the present research was to investigate whether a water/ethanol extract of *Cucumis sativus* L. fruit (CSE) can attenuate the deleterious effects of LPS in in vitro model of endothelial cells.

## Methods

### Chemicals and reagents

Human endothelial SFM medium, heat inactivated FBS (Fetal Bovine Serum), antibiotic-antimycotic and Dulbecco's phosphate buffered saline (DPBS) were purchased from Gibco-Life technologies (Carlsbad CA, USA).

RNA isolation was performed with NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG, Düren Germany), iScript cDNA synthesis kit and iTaq Universal SYBR Green Supermix were used for cDNA synthesis and qPCR analysis (Bio-Rad Laboratories Inc., Hercules, CA, USA). All plastic supports were purchased from Falcon, Beckton-Dickinson.

A water/ethanol extract of *Cucumis sativus* L. fruit (CSE), titrated for total iminosugar acids content by HPLC-MS (2 g/100g), was kindly provided by Naturalea (Naturalea SA, Lugano, CH - Cuvrex batch number CE1501).

### Cell culture

Porcine Aortic Endothelial Cells (pAECs) were isolated and maintained as previously described by Bernardini and colleagues [12]. Briefly thoracic aortic traits were collected in a local slaughterhouse from adult pigs. After collection, thoracic aortic traits were washed with DPBS, ligated at the ends, and transferred to the laboratory within 1 h on ice. After ligation of all arterial side branches, aortas were cannulated with modified syringe cones and silicone tubes to set up a closed system. The vessels were repeatedly flushed with DPBS and then filled with a collagenase solution and incubated for 20 min at 37 °C. The cellular sospension were then centrifuged at 800 x *g* for 10 min. The cellular pellet was resuspended in 1 mL human endothelial basal growth medium (Gibco-Invitrogen, Paisley, UK) supplemented with 5% fetal bovine serum (Gibco-Invitrogen) and 1% antibiotics-antimicotics (Gibco-Invitrogen). Cell number and viability (85–90%) were determined using a Burker chamber under a phase-contrast microscope after vital staining with trypan blue dye. Cells were maintained in a logarithmic growth phase by routine passages every 2–3 days at a 1:3 split ratio. To confirm their endothelial origin, cultured cells were checked by immunocitochemistry for endothelial cell markers: CD31 and Caderine. Then cells were expanded till 20th passages. All experiments were performed with cells from the third to the eighth passage. The first seeding after thawing was always performed in T-25 tissue culture flasks ($3 \times 10^5$ cells/flask) and successive experiments were conducted in 24-well plates (qPCR and western blot analysis), in 96-well assay plates (cytotoxicity) and 8-well slide chamber for in vitro angiogenesis assay. Cells were cultured in Human endothelial SFM medium, added with FBS (5%) and antimicrobial/antimycotic solution (1×) in a 5% $CO_2$ atmosphere at 38.5 °C.

### Cytotoxicity

Since the non-toxicity of the extract is a fundamental pre-requisite, we first tested the cytotoxicity of the CSE in a concentration range of 0.0002–2 mg / ml. No toxicity was showed at any doses tested.

pAECs were seeded in a 96 wells plate (approximately $3 \times 10^3$ cells/well) and exposed to increasing doses of *Cucumis sativus* L. (CSE) (0.02; 0.2; 2 mg/ml) in presence of lipopolysaccharide (LPS) (10 μg/ml) (*E. coli* 055:B5, Sigma-Aldrich Co, St Louis, MO, USA) for 24 h. Cytotoxicity was evaluated by trypan Blue exclusion dye

using Countess® II FL Automated Cell Counter (Life Technologies).

### Quantitative real time PCR for ZO-1, TLR4, HO-1

pAECs were seeded in a 24 wells plate (approximately $4 \times 10^4$ cells/well) and exposed to increasing doses (0.02; 0.2; 2 mg/ml) of CSE in presence of LPS (10 μg/ml) for 1, 7 and 24 h. At the end of experimental times, treated or control cells were collected and stored until gene expression analysis.

Total RNA was isolated using the NucleoSpin®RNA Kit, and high quality RNA, with A260/A280 ratio above 2.0 was used for cDNA synthesis. Total RNA (500 ng) was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit in a final volume of 20 μL. Swine primers were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences, expected PCR product lengths and accession numbers in the NCBI database are shown in Table 1.

Quantitative real-time PCR was performed to evaluate gene expression profiles in CFX96 (Bio-Rad) thermal cycler using SYBR green detection system. A master mix of the following reaction components was prepared in nuclease free water to the final concentrations indicated: 0.2 μM forward primer, 0.2 μM reverse primer, 1X iTaq Universal SYBR Green Supermix. One μl of cDNA was added to 19 μl of the master mix. All samples were analyzed in duplicate. The qPCR protocol used was: 10 min at 95 °C, 40 cycles at 95 °C for 15 s and at 61 °C for 30 s, followed by a melting step from 55 °C to 95 °C (80 cycle of 0.5 °C increase/cycle).

The expression level of interest genes was calculated as fold of change using the $2^{-\Delta\Delta CT}$ method [24].

### Western blot for TLR4 and HO-1

pAECs were seeded in a 24 wells plate (approximately $4 \times 10^4$ cells/well) and exposed to increasing doses (0.02; 0.2; 2 mg/ml) of CSE for 24 h. At the end of experimental time, cells were harvested and lysed in SDS solution (Tris–HCl 50 mM pH 6.8; SDS 2%; glycerol 5%). Protein Assay Kit (TP0300, Sigma) was used to determine the protein content of cellular lysates. Aliquots containing 20 μg of proteins were separated on NuPage 4–12% bis-Tris Gel (Gibco-Life-Technologies) for 50 min at 200 V. The proteins were then electrophoretically transferred onto a nitrocellulose membrane by Turbo Blot System (Bio-Rad). The blots were washed in PBS and protein transfer was checked by staining the nitro-cellulose membranes with 0.2% Ponceau Red. Non-specific binding on nitrocellulose membranes was blocked with 5% milk powder in PBS-T20 (Phosphate Buffer Saline-0.1% Tween-20) for 1 h at room temperature. The membranes were then incubated over-night at 4 °C with a 1:500 dilution of anti-HO-1 rabbit polyclonal antibody (SPA 896 StressGen Biotecnologies Corp, Victoria BC, Canada) and 1:1000 anti TLR4 mouse monoclonal antibody (NB100–56566 Novus Biologicals, Littleton, CO, USA). After several washings with PBS-T20, the membranes were incubated with the secondary biotin-conjugate antibody and then with a 1:1000 dilution of an anti-biotin horseradish peroxidase (HRP)-linked antibody.

The western blots were developed using chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instructions. Chemidoc instrument using Quantity One Software (Bio-Rad) acquired the intensity of the luminescent signal of the resultant bands.

In order to normalize the HO-1 and TLR4 data on the housekeeping protein, membranes were stripped (briefly: the membranes were washed 5 min in water, then 5 min in 0.2 M NaOH and then washed again in water) and re-probed for housekeeping α-tubulin (1:500 of anti α-tubulin MA1–19162, Thermo Fisher Scientific, Rockford, IL, USA).

The relative protein content (HO-1 or TLR4/α-tubulin) was expressed as arbitrary units (AUs).

### Multiparametric enzyme-linked immunosorbent assay (ELISA) for cytokines and chemokines

Concentration of 13 cytokines and chemokines (GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α) was measured by quantitative multiparametric ELISA (Enzyme-linked immunosorbent assay). (Porcine Cytokine/Chemokine Magnetic Bead Panel kit, Milliplex Map Kit, EMD Millipore Corporation, Billerica MA USA), following the manufacturer's instructions. The Luminex xMAP bead-based multiplexed immunoassay technology and MAGPIX instrument provided with xPONENT 4.2 software were used.

**Table 1** Primer sequence used for quantitative Real Time PCR analysis

| Genes | Forward (5'-3') | Reverse (5'-3') | Product size (bp) | Accession Number |
|---|---|---|---|---|
| HPRT | GGACAGGACTGAACGGCTTG | GTAATCCAGCAGGTCAGCAAAG | 115 | AF143818 |
| HO-1 | CGCTCCCGAATGAACAC | GCTCCTGCACCTCCTC | 112 | NM_001004027 |
| TLR4 | CAGATACAGAGGGTCATGCTTTC | GGGGATGTTGTCAGGGATTTG | 215 | NM_001113039.1 |
| ZO-1 | AGTGCCGCCTCCTGAGTTTG | CATCCTCATCTTCATCATCTTCTACAG | 147 | AJ318101 |

### Capillary-like tube formation assay

The experiments were carried out using 8-well slide chamber (BD Falcon Bedford, MA USA) coated with undiluted Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix. Extracellular matrix coating was carried out for 3 h in a humidified incubator, at 38.5 °C, 5% $CO_2$. pAECs ($8 \times 10^4$ cells/well) were exposed to increasing doses (0.02; 0.2; 2 mg/ml) of (CSE) in the presence of LPS (10 μg/ml) for 24 h.

At the end of experimental time, images were acquired using a digital camera installed on a Nikon epifluorescence microscope (Nikon, Yokohama, Japan) and analyzed by open software Image J 64.

### Statistical analysis

Each treatment was replicated three times or six times (cytotoxicity) in three independent experiments. The data were analysed by a one-way analysis of variance (ANOVA) followed by the Tukey post hoc comparison Test. Differences of at least $p < 0.05$ were considered significant. Statistical analysis was carried out by using R software (http://www.R-project.org) [25].

## Results

### CSE prevented LPS-induced cell death and ZO-1 reduction

The protective effect of *Cucumis sativus* L. extract on LPS-induced toxicity was evaluated in pAECs. LPS treatment provoked an increased number of round and detached cells after 24 h (Fig. 1a), while CSE reduced the cellular detachment in a dose dependent manner (Fig. 1b-d). Cytotoxicity assay confirmed the ability of CSE to protect cells against LPS-induced cellular death (Fig. 1e). Moreover, we studied the expression of ZO-1, a critical component of tight junction scaffold; LPS induced the downregulation of ZO-1 gene expression, while CSE restored ZO-1 expression to control level at the intermediate and higher doses (Fig. 1f).

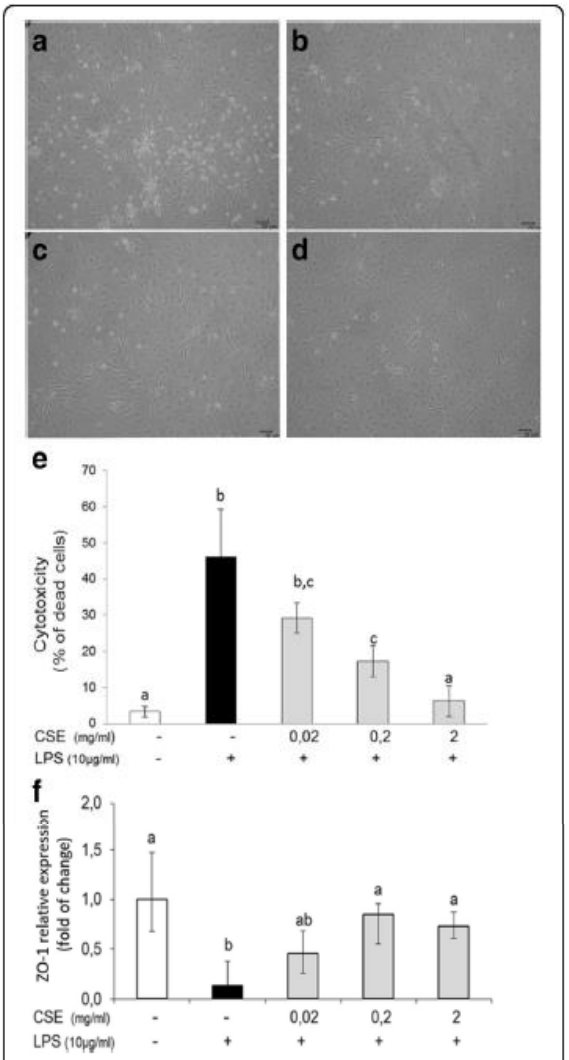


**Fig. 1** Effect of *Cucumis sativus* L. extract (CSE) on LPS-induced pAECs toxicity. **a** Representative images of pAECs morphology under LPS (10 μg/ml) stimulation (**b, c, d**) and in the presence of increasing dose of CSE (0.02; 0.2; 2 mg/ml). **e** Protective effect of CSE on LPS-induced cytotoxicity; data shown are representative of at least three independent experiments and represent the mean ± SEM. **f** CSE restored the LPS-induced decrease of ZO-1 mRNA expression; relative expression was calculated as fold of change in respect to the control cells and error bar represents the range of relative gene expression. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test)

### Effect of CSE on TLR-4 and HO-1 expression

We studied the effect of CSE on the expression of the Toll-like receptor 4 (TLR4) that is the main receptor for LPS recognition. LPS induced a significant increase of TLR4 mRNA after 1 and 7 h of treatment, while CSE inhibited its expression at all doses studied (Fig. 2a). This inhibitory effect was confirmed at protein level by western blot analysis as shown in Fig. 2b and c. Moreover, we studied the effect of CSE on the vascular protective molecule HO-1. LPS induced HO-1 expression in pAECs, additionally CSE increased HO-1 induction at both mRNA (Fig. 3a) and protein level (Fig. 3b and c).

### Effect of CSE on cytokine/chemokines secretion

To assess whether CSE could influence the LPS-induced secretion of inflammatory mediators we evaluated the presence of 13 cytokines/chemokines in the culture medium of pAECs treated with LPS in the presence or absence of CSE (2 mg/ml). LPS-treated endothelial cells released significant level of Il-6, Il-8, IL-10, IL-18, GM-CSF and IFN-γ. CSE significantly influenced these cytokines setting; in particular, the presence of the extract decreased the concentration of GM-CSF, IL-8 and IL-1α with a different kinetic, whereas the concentration

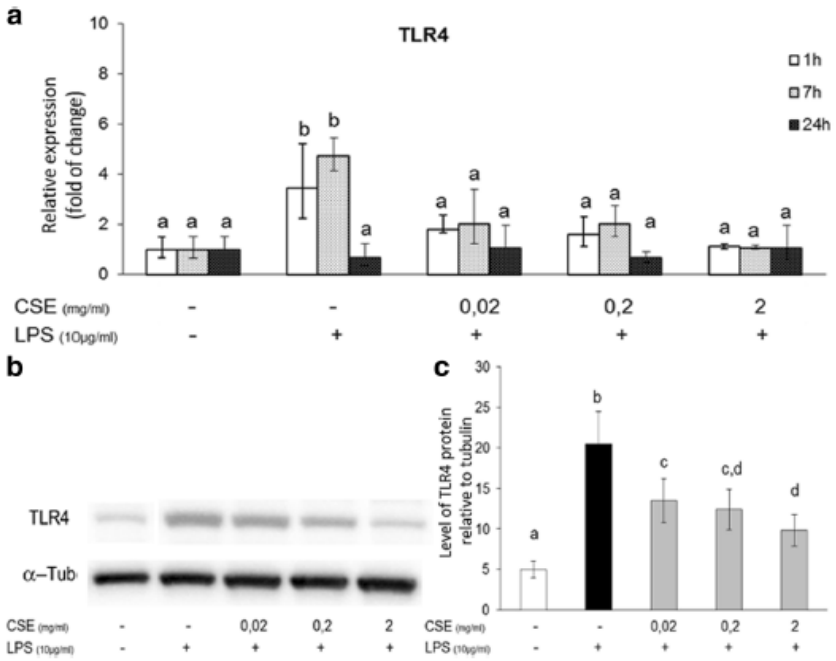


**Fig. 2** Effect of *Cucumis sativus* L. extract (CSE) on LPS induced TLR4 expression. **a** Expression of TLR4 mRNA in pAECs treated with LPS (10 μg/ml) for different time (1, 7, 24 h) in the presence or absence of increasing doses of CSE (0.02; 0.2; 2 mg/ml). mRNA expression of TLR4 is determined by quantitative PCR; relative expression was calculated as fold of change in respect to the control cells and error bar represents the range of relative expression. Different letters above the bars indicate significant differences. **b** Representative Western Blot of TLR4 and relative housekeeping α-tubulin were reported. **c** Expression of TLR4 protein in pAECs treated with LPS (10 μg/ml) in the presence or absence of increasing doses CSE (0.02; 0.2; 2 mg/ml); data shown are representative of at least three independent experiments and represent the mean ± SEM. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test)

of IL-10, IL-18 and IFN-γ was increased at each experimental point, as shown in Table 2. The level of IL-6 showed a precocious increase (1 h) in presence of CSE but after 7 and 24 h, cells treated with plant extract produced less IL-6 than cells only treated with LPS. IL-1β, IL-1ra, IL-2, IL-4, IL-12 and TNF-α were never detected in cell culture medium (data not shown).

### Effect of CSE on LPS-induced angiogenesis

We examined the effect of CSE on in vitro LPS-induced pAECs angiogenesis in an extracellular matrix-based assay. Cells cultured on extracellular matrix in the presence of pro-inflammatory LPS assembled in a complete tube and network formation (Fig. 4a and e), while the in vitro angiogenesis induced by inflammatory LPS was prevented by the presence of *Cucunis sativus* L. extract, at any doses tested (Fig. 4b-e).

## Discussion

It is widely accepted the key role of endothelium in the onset of many chronic and acute vascular and cardiovascular diseases. The shift from the healthy endothelium to the endothelial dysfunction is a complex process involving many different factors that starts with "the activation of endothelial cells".

Recently, great effort is attempted to translate the potential activity of traditional compounds into the modern therapy, in a broad range of pathologies including cardiovascular disease [18]. *Cucumis sativus* L. is a very popular vegetable, native of India but nowadays commercially cultivated all over the world. Furthermore, since ancient time, Ayurvedic medicine has always used its fruits and seeds for their refrigerant, haemostatic tonic properties. It is now evident that fruits contain many interesting phyto-complex [20, 26] that makes it an interesting candidate for anti oxidant [27] and anti-inflammatory action [28] but the study of their effect still requires scientific supports.

The present study demonstrates that the protective effects reported for *Cucumis sativus* L., might be also mediated by its protective activity on the vascular endothelium.

Being the pig an excellent model for translational researches [29], in vitro approaches based on primary cell culture are required to better define the subsequent eventual in vivo activities to respect the 3Rs rules. We used in vitro cultures of porcine Aortic Endothelial Cells

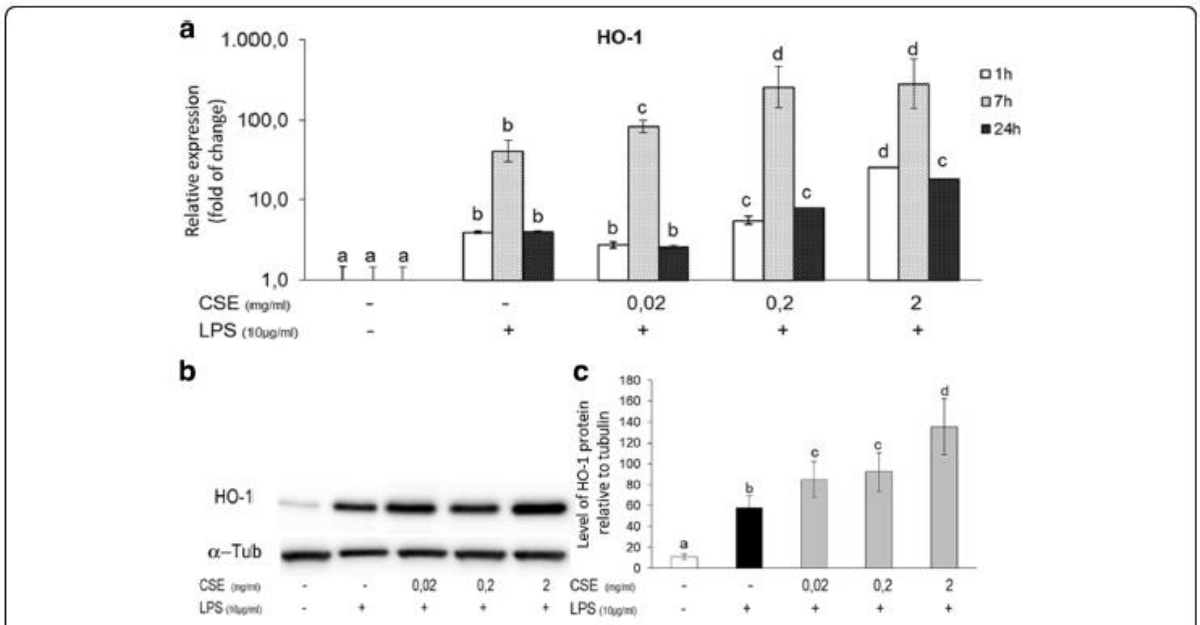


**Fig. 3** Effect of *Cucumis sativus* L. extract (CSE) on LPS induced HO-1 expression. **a** Expression of HO-1 mRNA in pAECs treated with LPS (10 μg/ml) in the presence or absence of increasing doses of *Cucumis sativus* L. extract (0.02, 0.2, 2 mg/ml). mRNA expression of HO-1 as determined by quantitative PCR. Relative expression was calculated as fold of change in respect to the control cells and error bar represents the range of relative gene expression. Different letters above the bars indicate significant differences. **b** Representative Western Blot of HO-1 and relative housekeeping α-tubulin were reported. **c** Expression of HO-1 protein in pAECs treated with LPS (10 μg/ml) in the presence or absence of increasing doses CSE (0.02; 0.2; 2 mg/ml); data shown are representative of at least three independent experiments and represent the mean ± SEM. (AU = Arbitrary Units) Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test)

(pAECs), previously isolated and cultured by us to study vascular endothelial response to different shock, including LPS [12, 13, 30].

In the present research, LPS-induced effects on pAECs is contrasted by the contemporary administration of *Cucumis sativus* L. extract; in fact CSE protected endothelial cells against LPS-induced toxicity, in a dose dependent manner. Furthermore LPS reduced the expression of the tight junction molecule ZO-1, increasing the number of round and detached cells. Instead, *Cucumis sativus* L. extract, restored the ZO-1 expression, contributing to maintain the integrity of cellular tight junction, as confirmed by the reduction of cellular detachment.

It is well demonstrated that the exposure to LPS induces endothelial cell activation through the binding of a receptor complex that includes TLR4 [31–34]. In our model TLR4 expression is increased by LPS making cells more responsive to the stimulation, according to what seen by other researchers [35, 36]. In contrast, the

**Table 2** Levels of cytokines/chemokines in culture medium of pAECs stimulated with LPS (10 μg/ml) in the presence or absence of *Cucumis sativus* L. extract (CSE 2 mg/ml). Data shown are representative of at least three independent experiments and represent the mean ± SEM. Significant differences are indicated by ($p < 0.05$) *, and ($p < 0.001$) by ** *nd* not detectable

| ng/ml | 1 h | | 7 h | | 24 h | |
|---|---|---|---|---|---|---|
| | CSE-/LPS+ | CSE+/LPS+ | CSE-/LPS+ | CSE+/LPS+ | CSE-/LPS+ | CSE+/LPS+ |
| IL-1α | nd | nd | nd | nd | 0.0592 ± 0.0019 | 0.0140 ± 00002** |
| IL-6 | nd | 0.1574 ± 0.0064 | 4.3163 ± 0.0893 | 3.3889 ± 0.0835* | 8.0129 ± 0.3067 | 3.9270 ± 0.0335** |
| IL-8 | 6.3445 ± 0.4821 | 2.3809 ± 0.0434** | > 50 | 24.2300 ± 2.2100 | 31.5600 ± 3.8210* | 25.1500 ± 2.7210 |
| IL-10 | 0.0077 ± 0.0023 | 0.0355 ± 0.0010** | 0.0187 ± 0.0025 | 0.0395 ± 0.0034* | 0.0209 ± 00017 | 0.0400 ± 0.0056** |
| IL-18 | 0.0319 ± 0.0021 | 0.1796 ± 0.0049** | 0.0572 ± 0.0011 | 0.1897 ± 0.0033** | 0.0606 ± 0.0034 | 0.1913 ± 0.0056** |
| GM-CSF | 0.1083 ± 0.0071 | nd | 0.1895 ± 0.0221 | nd | 0.2061 ± 0.0094 | 0.0021 ± 0.0037** |
| IFN-γ | 0.0501 ± 0.0436 | 1.5529 ± 0.0292** | 0.2654 ± 0.0889 | 0.5230 ± 0.0725* | 0.0725 ± 0.0378 | 0.3522 ± 0.0340** |

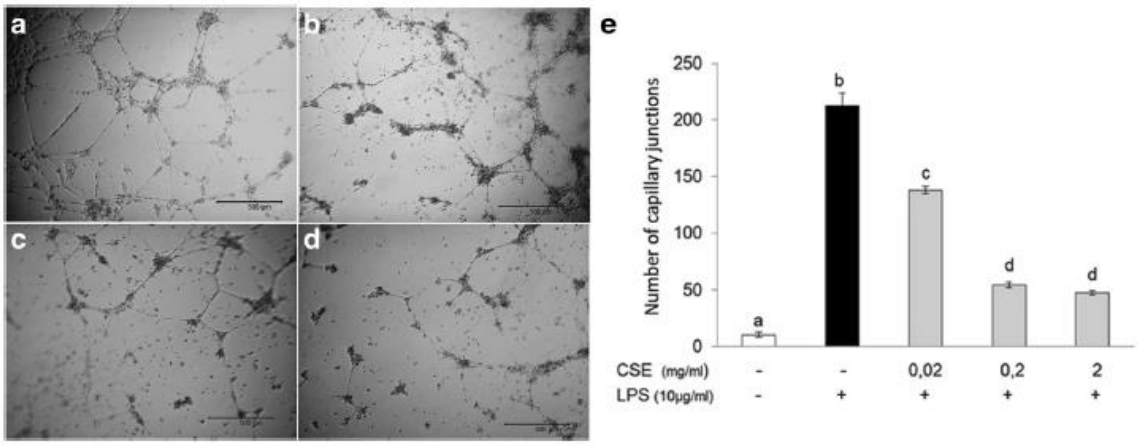


**Fig. 4** Effect of *Cucumis sativus* L. extract on LPS-induced angiogenesis. pAECs were cultured on a extracellular matrix with LPS (10 μg/ml) in the absence (**a**) or presence of increasing doses (0.02; 0.2; 2 mg/ml) of *Cucumis sativus* L. extract (**b, c, d** respectively) for 18 h. LPS induced a capillary like network (**a**, **e**) while CSE reduced LPS-induced angiogenesis at any doses tested (**b-e**). Data shown are representative of at least three independent experiments and represent the mean ± SEM. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test)

presence of *Cucumis sativus* L. extract, inhibited the LPS-induced expression of TLR4.

The inflammatory signalling driven by TLR4 in endothelial cells goes through the activation of NF-κB and the consequently induction or shutdown of several genes including those for cytokines/chemokines synthesis [37].

The presence of CSE in the culture medium contrasted early the robust secretion of inflammatory IL-8 and GM-CSFs; while inhibition of inflammatory IL-6 and IL-1α occurred late at 7 and 24 h respectively. On the contrary, the anti-inflammatory IL-10 is increased together with IL-18 and IFN-γ.

Since the production and function of cytokines overlaps, what is the meaning of our results? Considering that in vivo endothelial cells mainly produce IL-6 and IL-8 and being, among the major functions of these cytokines, the induction of angiogenesis [11], the anti-angiogenic effect of CSE, evidenced by the in vitro-angiogenesis assay, is in agreement with the inhibition of these cytokines. Moreover interleukin 18 (IL-18), firstly described as a novel cytokine that stimulates interferon-γ (IFN-γ) production, possessed potent antitumor effects achieved by the inhibition of angiogenesis in vivo [38], so the increase of IL-18 in our model, could also contribute to a reduction in inflammatory angiogenesis.

Overall, our results demonstrate that the extract of *Cucumis sativus* L. influenced the secretion of cytokines/chemokines trough the reduction of TLR4 expression; moreover, the effect of this modulation inhibited the inflammation-induced angiogenesis. Overall, these important results suggest that *Cucumis sativus* L. extract could be a very interesting candidate in counteracting inflammatory pathologies in which TLR play a crucial modulatory role.

Furthermore, to avoid that the endothelial cell activation results in dysfunction, the induction of protective genes must be strictly regulated. Among protective genes, Hemeoxygenase (HO)-1, the rate-limiting enzyme in the heme catabolism, has been demonstrated to present important beneficial roles in the vasculature [39]; in particular HO-1 exerts antiapoptotic, antioxidants, antithrombotic and anti-atherogenic effects [39]. Our previous reports showed the LPS ability to induce HO-1 expression [12]; in the present research we demonstrated that *Cucumis sativus* L. extract increased the expression of vascular protective HO-1. Moreover, the role of HO-1 in angiogenesis is intriguing in fact HO-1 activity is necessary for VEGF-induced angiogenesis, whereas HO-1 has the opposite effect in the pathological angiogenesis [39]. Therefore, in our model, the increase of HO-1 could exert protective effect including the inhibition of LPS-induced inflammatory angiogenesis.

## Conclusions

Our results demonstrate the efficacy of a water/ethanol extract of *Cucumis sativus* L. fruit to protect vascular endothelial cells against LPS-challenge: decreasing LPS-induced TLR4 expression, influencing cytokines secretion, increasing the expression of protective HO-1. Moreover, the presence of *Cucumis sativus* L. extract inhibited the LPS-induced cellular toxicity and inflammation-induced angiogenesis. These impressive and robust results propose the *Cucumis sativus* L. extract as a promising natural compound in vascular endothelium protection.

**Abbreviations**
CDVs: Cardiovascular diseases; CSE: *Cucumis sativus* L extract; EC: Endothelial cells; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HO-1: Heme oxygenase 1; IFN-γ: Interferon γ; IL-10: Interleukin 10; IL-12: Interleukin 12; IL-18: Interleukin 18; IL-1ra: Interleukin-1 receptor antagonist; IL-1α: Interleukin 1α; IL-1β: Interleukin1β; IL-2: Interleukin 2; IL-4: Interleukin 4; IL-6: Interleukin 6; IL-8: Interleukin 8; LPS: Lipopolysaccharide; pAECs: Porcine aortic endothelail cells; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor α; ZO-1: Zona occludens-1

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**Availability of data and materials**
The datasets used and analysed during the current study available from the corresponding author on reasonable request.

**Authors' contributions**
CB and MFo conceived and designed the research; CB, AZ, MB, IT and MFe made substantial contribution to perform experiments, analyse data and contributed to write the manuscript. MFo supervised the work and revised critically the manuscript. All authors read and approved the final version of the manuscript.

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Not applicable.

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**References**
1. Aird WC. Endothelium and haemostasis. Hamostaseologie. 2015;35:11–6.
2. WHO | Cardiovascular diseases (CVDs). WHO. World Health Organization; 2016. http://www.who.int/cardiovascular_diseases/en/.
3. Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev Am Physiol Soc. 2004;84:869–901.
4. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res Lippincott Williams & Wilkins. 2007;100:158–73.
5. Burger D, Touyz RM. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. J Am Soc Hypertens. 2012;6:85–99.
6. Aird WC. Endothelium in health and disease. Pharmacol Rep. 2008;60:139–43.
7. Cahill PA, Redmond EM. Vascular endothelium – gatekeeper of vessel health. Atherosclerosis. 2016;248:97–109.
8. Chistiakov DA, Orekhov AN, Bobryshev YV. Endothelial barrier and its abnormalities in cardiovascular disease. Front Physiol. 2015;6:365.
9. Regina C, Panatta E, Candi E, Melino G, Amelio I, Balistreri CR, et al. Vascular ageing and endothelial cell senescence: molecular mechanisms of physiology and diseases. Mech Ageing Dev. 2016;159:14–21.
10. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochem Pharmacol. 2009;78:539–52.
11. Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2016;138:984–1010.
12. Bernardini C, Zannoni A, Turba ME, Fantinati P, Tamanini C, Bacci ML, et al. Heat shock protein 70, heat shock protein 32, and vascular endothelial growth factor production and their effects on lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells. Cell Stress Chaperones Springer; 2005;10:340–348.
13. Bernardini C, Galbani P, Zannoni A, Vocale C, Bacci ML, Piana G, et al. Treponema denticola alters cell vitality and induces HO-1 and Hsp70 expression in porcine aortic endothelial cells. Cell Stress Chaperones. 2010;15:509–16.
14. Botelho G, Bernardini C, Zannoni A, Ventrella V, Bacci ML, Biochemistry FMC, Physiology PC. Effect of tributyltin on mammalian endothelial cell integrity. Comp Biochem Physiol Part C Elsevier Inc. 2015;177:79–86.
15. Dao VT-V, Medini S, Bisha M, Balz V, Suvorava T, Bas M, et al. Nitric oxide up-regulates endothelial expression of angiotensin II type 2 receptors. Biochem. Pharmacol. 2016;112:24–36.
16. Dushpanova A, Agostini S, Ciofini E, Cabiati M, Casieri V, Matteucci M, et al. Gene silencing of endothelial von Willebrand factor attenuates angiotensin II-induced endothelin-1 expression in porcine aortic endothelial cells. Sci Rep. 2016;6:30048.
17. Grossini E, Farruggio S, Qoqaiche F, Raina G, Camillo L, Sigaudo L, et al. Monomeric adiponectin increases cell viability in porcine aortic endothelial cells cultured in normal and high glucose conditions: data on kinases activation. Data Br. 2016;8:1381–6.
18. Pan S-Y, Zhou S-F, Gao S-H, Yu Z-L, Zhang S-F, Tang M-K, et al. New perspectives on how to discover drugs from herbal medicines: CAM's outstanding contribution to modern therapeutics. Evidence-Based Complement Altern Med. 2013;2013:1–25.
19. Accardi G, Aiello A, Gambino CM, Virruso C, Caruso C, Candore G. Mediterranean nutraceutical foods: strategy to improve vascular ageing. Mech Ageing Dev. 2016;159:63–70.
20. Mukherjee PK, Nema NK, Maity N, Sarkar BK. Phytochemical and therapeutic potential of cucumber. Fitoterapia. 2013;84:227–36.
21. Park SY, Kim YH, Park G. Cucurbitacins attenuate microglial activation and protect from neuroinflammatory injury through Nrf2/ARE activation and STAT/NF-κB inhibition. Neurosci Lett. 2015;609:129–36.
22. Muruganantham N, Solomon S, Senthamilselvi MM. Anti-oxidant and anti-inflammatory activity of Cucumis sativas (cucumber) flowers. Int J Pharm Sci Res. 2016;7:1740–5.
23. Patil VM, Kandhare AD, Bhise SD. Effect of aqueous extract of Cucumis sativus Linn. Fruit in ulcerative colitis in laboratory animals. Asian Pac J Trop Biomed. 2012;2(5962-9):39–11.
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods Academic Press. 2001;25:402–8.
25. R Core Team. R: a language and environment for statistical computing [Internet]. R Found. Stat. Comput. Vienna, Austria. 2015. Available from: https://www.r-project.org/
26. Chu Y-F, Sun J, Wu X, Liu RH. Antioxidant and antiproliferative activities of common vegetables. J Agric Food Chem. 2002;50:6910–6.
27. Nema NK, Maity N, Sarkar B, Mukherjee PK. Cucumis sativus fruit-potential antioxidant, anti-hyaluronidase, and anti-elastase agent. Arch Dermatol Res Springer-Verlag. 2011;303:247–52.
28. Qiao J, Xu L, He J, Ouyang D, He X. Cucurbitacin E exhibits anti-inflammatory effect in RAW 264.7 cells via suppression of NF-κB nuclear translocation. Inflamm Res SP Birkhäuser Verlag Basel; 2013;62:461–469.
29. Prather RS, Lorson M, Ross JW, Whyte JJ, Walters E. Genetically engineered pig models for human diseases. Annu Rev Anim Biosci Annual Reviews. 2013;1:203–19.
30. Bernardini C, Greco F, Zannoni A, Bacci ML, Seren E, Forni M. Differential expression of nitric oxide synthases in porcine aortic endothelial cells during LPS-induced apoptosis. J Inflamm. 2012;9:47.
31. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. Lab Investig. 2006;86:9–22.
32. Zeuke S, Ulmer AJ, Kusumoto S, Katus HA, Heine H. TLR4-mediated inflammatory activation of human coronary artery endothelial cells by LPS. Cardiovasc Res. 2002;56:126–34.
33. Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, et al. Endothelium-derived toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. J Clin Invest. 2003;111:1011–20.
34. Mai CW, Kang YB, Pichika MR. Should a toll-like receptor 4 (TLR-4) agonist or antagonist be designed to treat cancer? TLR-4: its expression and effects in the ten most common cancers. Onco Targets Ther. 2013;6:1573–87.

35. Heo S-K, Yun H-J, Noh E-K, Park W-H, Park S-D. LPS induces inflammatory responses in human aortic vascular smooth muscle cells via toll-like receptor 4 expression and nitric oxide production. Immunol Lett. 2008;120:57–64.
36. Wang Y, Zhang MX, Meng X, Liu FQ, Yu GS, Zhang C, et al. Atorvastatin suppresses LPS-induced rapid upregulation of toll-like receptor 4 and its signaling pathway in endothelial cells. Am J Physiol Heart Circ Physiol American Physiological Society. 2011;300:H1743–52.
37. Sun R, Zhu Z, Su Q, Li T, Song Q. Toll-like receptor 4 is involved in bacterial endotoxin-induced endothelial cell injury and SOC-mediated calcium regulation. Cell Biol Int. 2012;36:475–81.
38. Zheng J-N, Pei D-S, Mao L-J, Liu X-Y, Sun F-H, Zhang B-F, et al. Oncolytic adenovirus expressing interleukin-18 induces significant antitumor effects against melanoma in mice through inhibition of angiogenesis. Cancer Gene Ther. 2010;17:28–36.
39. Calay D, Mason JC. The multifunctional role and therapeutic potential of HO-1 in the vascular endothelium. Antioxid Redox Signal. 2014;20:1789–809.

## 4.3 *Boswellia serrata*

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*Research Article*

# Anti-Inflammatory Activity of *Boswellia serrata* Extracts: An *In Vitro* Study on Porcine Aortic Endothelial Cells

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This study is aimed at investigating the cytotoxicity, anti-inflammatory, and angiogenic activities of two *Boswellia serrata* extracts on primary culture of porcine aortic endothelial cells (pAECs). Chemical characterization of a dry extract (extract A) and a hydroenzymatic extract (extract G) of *B. serrata* was performed by HPLC using pure boswellic acids (BAs) as standard. In cultured pAECs, extract G improved cell viability, following LPS challenge, in a dose-dependent manner and did not show any toxic effect. On the other hand, extract A was toxic at higher doses and restored pAEC viability after LPS challenge only at lower doses. Pure BAs, used at the same concentrations as those determined in the phytoextracts, did not contrast LPS-induced cytotoxicity. Extract A showed proangiogenic properties at the lowest dose, and the same result was observed using pure AKBA at the corresponding concentration, whereas extract G did not show any effect on the migration capacity of endothelial cells. In conclusion, an anti-inflammatory activity of *B. serrata* extracts on endothelial cells was reported, though cytotoxicity or proliferative stimulation can occur instead of a protective effect, depending on the dose and the formulation.

## 1. Introduction

The endothelium, uniquely positioned at the interface between the vascular wall and the blood, regulates multiple functions such as maintenance of normal vascular tone, modulation of coagulation, and immune responses [1]. It is widely demonstrated that the exposure of endothelial cells to proinflammatory stressors results in the production of molecules correlated with a proadhesive, prothrombotic, and proinflammatory phenotype that contributes to vascular disorders [2, 3], including cardiovascular diseases (CVDs).

Since ancient times, the extracts from the oleo-gum resin of *Boswellia serrata* Roxb. ex Colebr. (family *Burseraceae*), also identified as Indian frankincense or Salai Guggal, have been used in traditional Ayurvedic medicine for the treatment of inflammatory diseases, including osteoarthritis and chronic bowel diseases [4–8].

The oleo-gum resin, obtained by incision of the bark, is composed by essential oil (5–9%), mucopolysaccarides (21–22%), and pure resin (65–85%), containing tetracyclic and pentacyclic triterpene acids, of which boswellic acids (BAs) are the most important bioactive molecules [4, 9, 10]. In particular, 11-keto-$\beta$-boswellic acid (KBA) and 3-O-acetyl-11-keto-$\beta$-boswellic acid (AKBA) were proposed to act as inhibitors of 5-lipoxygenase (5-LO) [11, 12]. Recently, other components of the phytocomplex, such as $\beta$-boswellic acid ($\beta$BA), have been suggested as anti-inflammatory molecules, acting through inhibition of serine protease cathepsin G (catG) and microsomal prostaglandin E synthase (mPGES) [9].

Differences in the relative amount of BAs and other components of the phytocomplex are related to the existence of different species of the genus *Boswellia*, to environmental conditions (e.g., soil composition, season, and air humidity), and to the extraction procedure [13] leading to herbal products of different composition and quality. In a previous study, seven *B. serrata* extracts were compared for their AKBA content and antioxidant power, highlighting wide variations

[14]. In particular, one of the extracts obtained by biolique-faction based on enzyme biocatalysis (hydroenzymatic extract) [15] showed interesting peculiarities. A lower content of AKBA and antioxidant power but higher activity in ex vivo tests on peripheral blood mononuclear cells (PBMCs) was determined in comparison with the dry extract [14]. In recent years, attention has also been focused on the role of other BAs, namely, KBA and $\beta$BA [16, 17], suggesting a possible pharmacological activity also for these BAs. Preliminary data showed wide variability in the concentration of BAs in different extracts [18]; therefore, the present research is aimed at deepening the chemical characterization of the two extracts previously studied, focusing on HPLC quantification of KBA and $\beta$BA. The effect of different formulations will be evaluated in comparison with the individual pure BAs in an interesting *in vitro* model: primary culture of porcine aortic endothelial cells (pAECs). With pig as an excellent model for translational medicine in the cardiovascular field [19, 20], we have previously isolated and cultured endothelial cells from thoracic aortas [21]. These primary cultures maintain a stable phenotype, and they prove to be an excellent model of study for the vascular response to different stressors [22, 23]. Therefore, pAECs were chosen as an ideal *in vitro* model to study the anti-inflammatory and angiogenic properties of the two *B. serrata* extracts in comparison with pure AKBA, KBA, and $\beta$BA, either individually or mixed together.

## 2. Materials and Methods

*2.1. Chemicals and Reagents.* Human endothelial SFM medium, heat-inactivated fetal bovine serum (FBS), antibiotic-antimycotic, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Gibco-Life technologies (Carlsbad CA, USA). Dimethyl sulfoxide (DMSO), trypsin EDTA solution, lipopolysaccharide (LPS) (*E. coli* 055: B5), glycerol, methanol, phosphoric acid, acetonitrile, and AKBA (batch number BCBN2928V and CAS number 67416-61-9) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). KBA and $\beta$BA (batch numbers 15020106 and 15010405 and CAS numbers 17019-92-0 and 631-69-6, resp.) were obtained from PhytoPlan (Heidelberg, Germany). Six out of seven samples (extracts A–F) are dry extracts of *B. serrata* oleo-gum resin. The powder is insoluble in water but soluble in methanol and dimethyl sulfoxide (DMSO). Extract G is an aqueous extract obtained by a process of bioliquefaction based on enzyme biocatalysis [15]. Briefly, the gum resin from *B. serrata* was suspended in water (1 : 10 *w*/*v*) and subjected to enzymatic digestion by xylanase, $\alpha$-amylase, and glucosidase for 24 hours. One ml of hydroenzymatic extract is obtained from 145 mg of *B. serrata* resin (145 mg resin/ml).

*2.2. Qualitative and Quantitative Characterization of B. serrata Extracts.* Qualitative and quantitative analyses of *B. serrata* extracts were performed by a reversed-phase high-performance liquid chromatography (HPLC) method using the HPLC system (Beckman Coulter, Brea, CA, USA), comprising a 116 pump, a 507 automatic autosampler, a UV-Diode Array 168 detector, and integration software 32 Karat as reported by Beghelli et al. [14]. Seven samples (A–G) were analyzed for KBA and $\beta$BA concentrations and were prepared by dissolving extracts in methanol. KBA and $\beta$BA standard stock solutions were prepared by dissolving 5 mg of analytical standard in methanol (5 mL). The calibration curves were obtained by analyzing six serial dilutions (50 ppm, 25 ppm, 10 ppm, 5 ppm, 2.5 ppm, and 1 ppm) of the stock solution and by plotting the peak area measured at 260 nm against KBA concentrations and at 210 nm against $\beta$BA concentrations. The following equations of the curves were obtained:

$$\begin{aligned} \text{KBA} &= 77361x + 44918,\ r^2 = 0.999, \\ \beta\text{BA} &= 26532x + 721.54,\ r^2 = 0.999. \end{aligned} \quad (1)$$

The KBA and $\beta$BA peaks in the samples were identified on the basis of the retention time on the chromatogram at 260 nm and 210 nm, respectively. All measurements were performed in triplicate and data were reported as mean ± SD.

*2.3. Cell Culture and Treatment.* Porcine aortic endothelial cells (pAECs) were isolated and maintained as previously described by Bernardini et al. [21]. All experiments were performed with cells from the third to the eighth passage. The first seeding after thawing was always performed in T-25 tissue culture flasks ($3 \times 10^5$ cells/flask) (T-25, BD Falcon, Franklin Lakes, NJ, USA), and successive experiments were conducted in 24-well plates (scratch test) or 96-well plates (cell viability) with confluent cultures. Cells were cultured in human endothelial SFM medium, added with FBS (5%) and antimicrobial/antimycotic solution (1x) in a 5% $CO_2$ atmosphere at 38.5°C. Extract A was dissolved in DMSO at 10 mg dry extract/ml (stock solution) and then diluted in culture medium to obtain four doses containing 0.1, 1, 10, and 100 $\mu$g of dry extract/ml, respectively. Extract G, which is an aqueous solution, was directly diluted in culture medium to obtain four doses referring to 2.4, 24, 240, and 2400 $\mu$g of resin/ml. These doses were chosen and normalized on the basis of AKBA concentration in extracts as reported in [14]: for both extracts, the lowest dose contained 3.8 ng/ml of AKBA and the highest dose contained 3.8 $\mu$g/ml of AKBA.

Pure analytical grade BAs (KBA, AKBA, and $\beta$BA) were dissolved in methanol (stock solution 1 mg/ml) and then in culture medium to obtain the required concentrations. Two doses were chosen: *low*, corresponding to 3.8 ng/ml AKBA, 3 ng/ml KBA, and 8 ng/ml $\beta$BA, and *high*, corresponding to 380 ng/ml AKBA, 300 ng/ml KBA, and 800 ng/ml $\beta$BA. For each treatment, the same concentration of the specific vehicle was used as control.

*2.4. Effect of B. serrata Extracts on pAEC Viability.* pAECs were seeded in a 96-well plate ($6 \times 10^3$ cells/well) and exposed to four increasing doses of *B. serrata* extracts for 24 h. Cell viability was measured using tetrazolium salt (MTT assay). The formazan absorbance was measured at a wavelength of 570 nm, using Infinite® F50/Robotic absorbance microplate readers from TECAN (Life Sciences). The background absorbance of multiwell plates at 690 nm was also measured and subtracted from the 570 nm measurements.

*2.5. Effect of B. serrata Extracts on LPS-Induced pAEC Death.* pAECs seeded in a 96-well plate ($6 \times 10^3$ cells/cm$^2$) were exposed to lipopolysaccharide (LPS) (25 $\mu$g/ml) for 24 h either in the presence or in the absence of extracts A and G or pure BAs at the concentrations reported above. Cell viability was evaluated by MTT assay.

*2.6. Effect of B. serrata Extracts on pAEC Migration Capacity.* pAECs were seeded in a 24-well plate ($4 \times 10^4$ cells/well). When cells reached confluence, a wound was induced scratching the surface by a pipette tip, then the detached cells were removed by washing with DPBS. Complete medium containing low and high doses of extract A (0.1 $\mu$g dry extract/ml and 10 $\mu$g dry extract/ml) and extract G (2.4 $\mu$g resin/ml and 240 $\mu$g resin/ml) and pure BAs at low (3 ng/ml KBA, 3.8 ng/ml AKBA, and 8 ng/ml $\beta$BA) and high (300 ng/ml KBA, 380 ng/ml AKBA, and 800 ng/ml $\beta$BA) concentrations were added. Microscopic phase-contrast pictures and three measurements of the damaged areas were taken immediately after the scratches (T0) and after 6 h (T1) and 24 h (T2). Images were acquired using a Nikon epifluorescence microscope equipped with digital camera (Nikon, Yokohama, Japan).

*2.7. Statistical Analysis.* Each treatment was replicated three times (migration capacity) or eight times (cell viability and LPS challenge). Data were analyzed with a one-way analysis of variance (ANOVA) followed by the Tukey post hoc comparison test or Student's *t*-test. Differences of at least $p < 0.05$ were considered significant. Statistical analysis was carried out using R software (http://www.R-project.org).

## 3. Results

*3.1. KBA and $\beta$BA Quantification by HPLC-DAD Analysis.* Representative chromatograms of KBA, AKBA, and $\beta$BA analytical standards as well as extracts A and G analyzed at 210 and 260 nm are reported in Figure 1.

Both extracts presented two major peaks at 260 nm: the first one, at Rt of 13.2 min, identified as KBA by the use of the analytical standard, and the second one, at Rt of 26 min, previously identified as AKBA. Other components of the *B. serrata* phytocomplex were only visualized at 210 nm, and the peak at Rt of 49 min was identified as $\beta$BA by the use of the analytical standard. KBA, AKBA, and $\beta$BA concentrations, calculated based on the peak area and the calibration curve, are shown in Table 1.

Quantitative and qualitative differences were present. The concentrations of BAs in extract G were two orders of magnitude lower than in extract A, and the chromatogram of extract G was characterized by a major number of peaks resolved at 210 nm. Data on KBA and $\beta$BA concentrations in other additional five dry extracts (B–F) are reported in Table S1 in the Supplementary Material.

*3.2. Effect of B. serrata Extracts on pAEC Viability.* Extract A was cytotoxic at higher concentrations, resulting in a reduction in cell viability of 12 and 47%, respectively, while lower concentrations did not affect cell viability (Figure 2(a)). Extract G did not show any toxic effect on pAECs (Figure 2(b)). In the presence of pure BAs, a significant ($p < 0.05$) cytotoxic effect was detected at the concentrations studied (Figure 2(c)). Only AKBA presented a dose-dependent effect.

*3.3. Effect of B. serrata Extracts on LPS-Induced pAEC Death.* LPS challenge determined a significant 30% reduction of cell viability. Extract A significantly ($p < 0.05$) reduced the cytotoxicity induced by LPS at the lower concentrations (Figure 3(a)). The highest concentration elicited a significant exacerbation of LPS cytotoxicity resulting in 70% reduction of cell viability, while the lowest concentration showed a significant proliferative effect, resulting in a 40% increase in cell viability. Extract G significantly ($p < 0.05$) restored pAEC viability after LPS treatment at all the concentrations analyzed (Figure 3(b)), without a dose-dependent effect. None of pure BAs, individually or mixed together, was able to contrast LPS cytotoxicity (Figure 3(c)).

*3.4. Effect of B. serrata Extracts on pAEC Migration Capacity.* Extract A reduced the damaged area at T1 (6 h) and restored completely the monolayer at T2 (24 h) at the lower concentration, while at 10 $\mu$g dry extract/ml no significant effect on cell proliferation was measured (Figure 4(a)). The incubation with extract G did not determine the recovery of the damage (Figure 4(b)). Pure BAs showed a significant wound-healing effect at the end of the incubation at the lower concentration (Figure 4(c)). In particular, AKBA at 3.8 ng/ml completely restored the monolayer.

## 4. Discussion

The gum resin obtained from *B. serrata*, used in Ayurvedic medicine for the treatment of a variety of diseases, is considered a promising natural source of anti-inflammatory molecules, in particular BAs [4, 9].

The quantification of these active molecules is a prerequisite for testing any biological effect of a phytoextract from *B. serrata*. Therefore, the first aim of this study was to better characterize the BA profile through the quantification of KBA and $\beta$BA in addition to AKBA. The concentrations of BAs determined in extract A are in the range of those reported by other authors [24–26]. AKBA and KBA are used as markers to ensure the quality of *B. serrata* dry extracts, but their concentrations show wide variability in commercial products, which in general claim 65% of BAs. In general, BAs represent only a percentage of total organic acids, whose concentrations are determined by unspecific titration methods and, as a consequence, the claimed content of 65% BAs is absolutely unrealistic as recently pointed out also by other authors [24, 25]. Very low percentages of KBA and $\beta$BA were found in extract G compared to extract A. This aqueous extract was also characterized by low AKBA and low polyphenol concentrations [14], confirming again the importance of the extraction procedure on the phytocomplex composition.

To evaluate the possible biological effects of these different formulations, extracts A and G, normalized on the basis of AKBA content, were used for *in vitro* analyses to assess

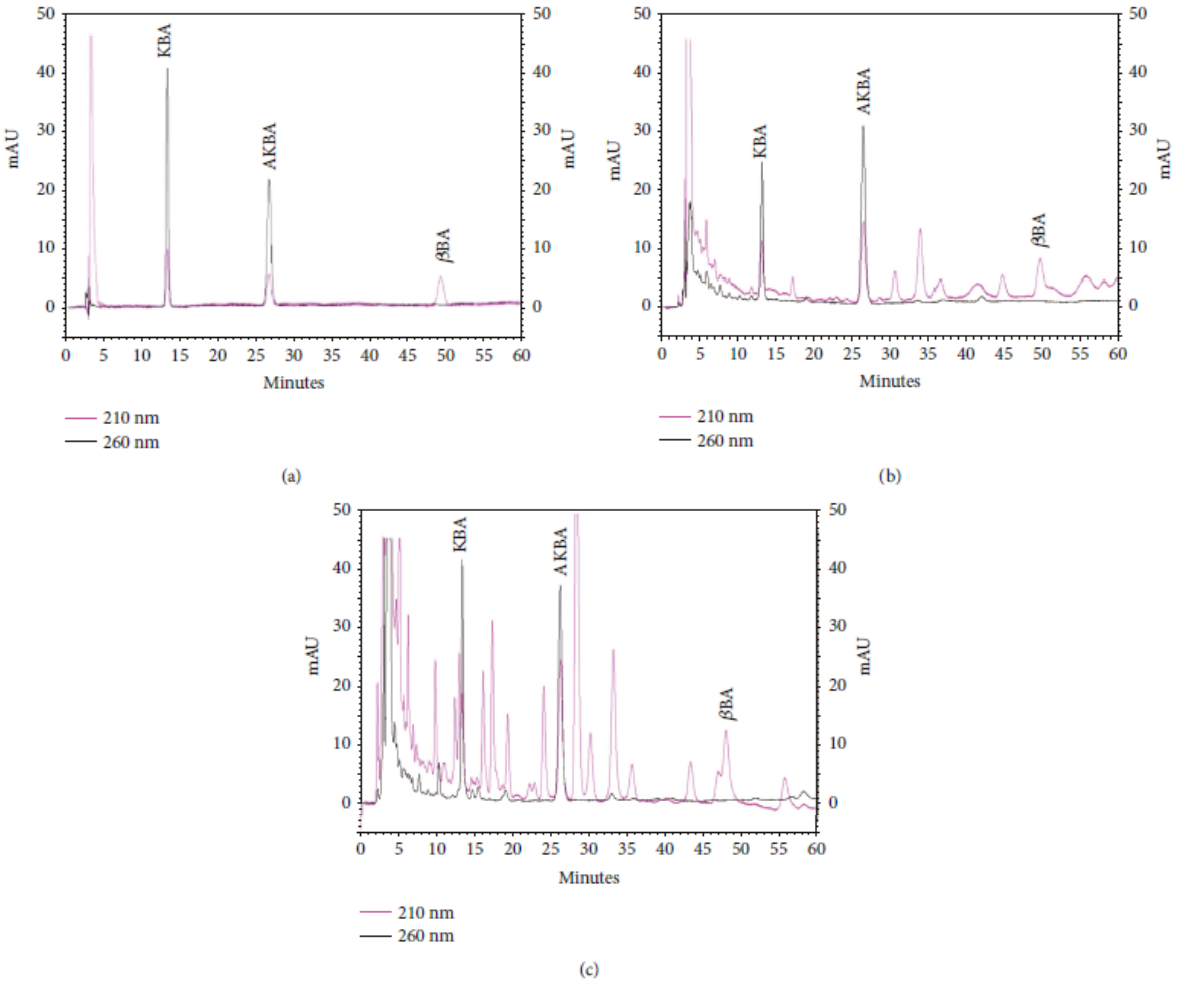


FIGURE 1: Representative chromatograms of pure analytical grade BAs (KBA, AKBA, and $\beta$BA) (25 ppm each) (a), extract A (b), and extract G (c) at 210 (pink chromatogram) and 260 nm (black chromatogram).

TABLE 1: KBA, $\beta$BA, and AKBA quantification in *Boswellia serrata* extracts. Data are reported as mean ± SD ($n = 3$). Concentration is expressed in mg/g of dry extract (extract A) or mg/ml of hydroenzymatic extract (extract G). For each BA, significant differences between extracts are indicated by $^{*}(p < 0.05$, Student's $t$-test) and by $^{**}(p < 0.001$, Student's $t$-test).

| Extract | KBA[§] | $\beta$BA | AKBA[§] |
|---|---|---|---|
| A | 15.86 ± 0.56** | 33.53 ± 7.23* | 38.30 ± 1.01** |
| G | 0.19 ± 0.02 | 0.50 ± 0.03 | 0.29 ± 0.04 |

[§]Data of AKBA concentrations are reported in Beghelli et al. [14].

cytotoxicity, anti-inflammatory activity, and angiogenic properties in comparison with pure BAs. Cytotoxic effects of *B. serrata* dry extracts and BAs were reported in several studies in different cancer cell lines, such as leukemia cells, prostate cancer cells, and gastrointestinal cancer cells [7, 27–30]. As regards the biochemical mechanism of cell death, Liu et al. [31] reported that BAs are able to induce apoptosis in Hep-G2 cells through the activation of caspase-8, while Bhushan et al. [32] found that a triterpendiol derived from BAs induced apoptosis in HL-60 cells through the activation of Bcl-2 and caspase-3.

The anti-inflammatory activity of *Boswellia* extracts was demonstrated in microvascular endothelial cells by preventing TNF$\alpha$-induced expression and activity of MMP-3, MMP-10, and MMP-12 [33]. Moreover, previous studies have shown that *B. serrata* extracts and BAs antagonize the inflammatory effect of LPS in human and mouse macrophages, monocytes, and PBMCs [34–36]. Our results demonstrated for the first time the protective effect of *B. serrata* extracts against LPS inflammatory stimulus in endothelial cells. In particular, extract G was the most effective, restoring completely cell viability at all the doses studied without any cytotoxicity. On the contrary, increasing concentrations of extract A lead to opposite results ranging

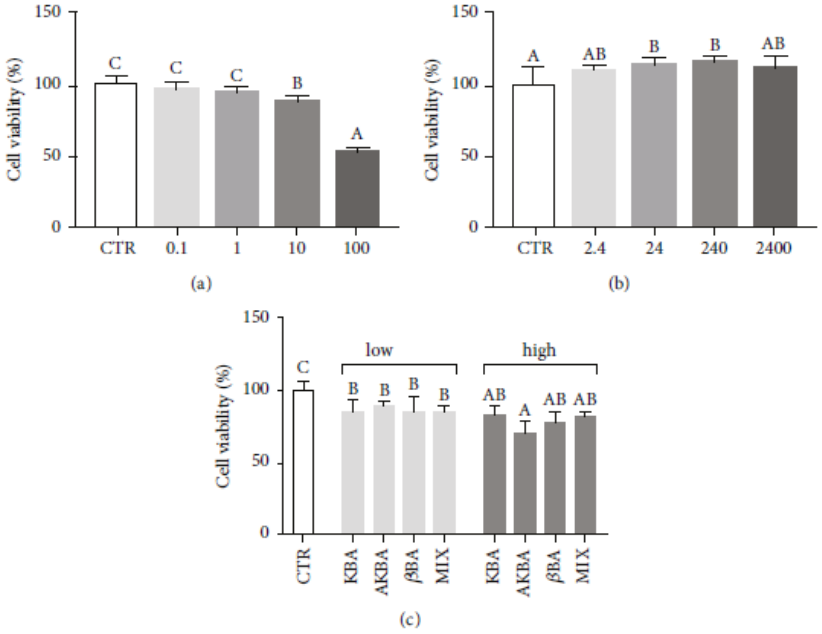


Figure 2: Effect of increasing doses of *B. serrata* extract A (0.1, 1, 10, and 100 $\mu$g dry extract/ml) (a), extract G (2.4, 24, 240, and 2400 $\mu$g resin/ml) (b), and pure BAs (*low*, corresponding to 3.8 ng/ml AKBA, 3 ng/ml KBA, and 8 ng/ml $\beta$BA, and *high*, corresponding to 380 ng/ml AKBA, 300 ng/ml KBA, and 800 ng/ml $\beta$BA) (c) on pAECs. Cell viability was measured by MTT assay. Data are reported as mean ± SD of 8 independent replicates. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test).

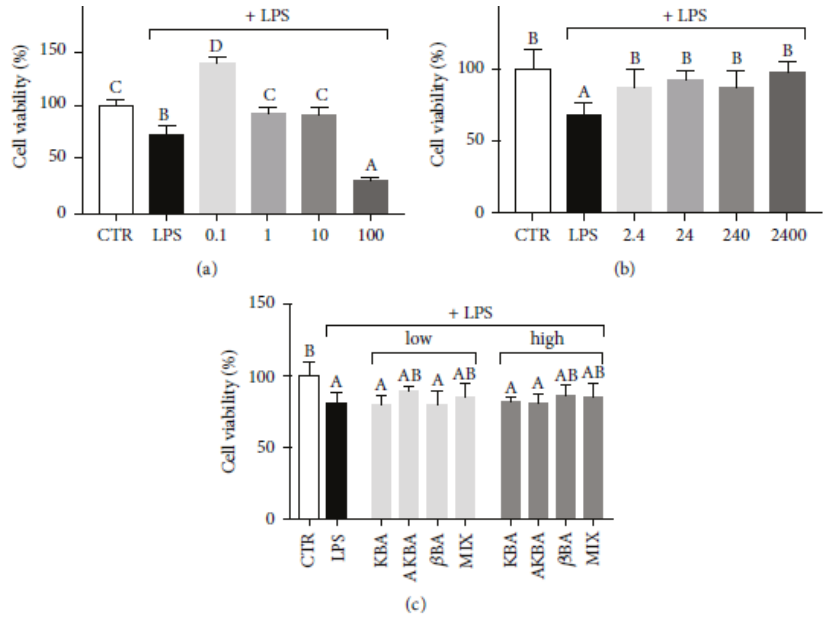


Figure 3: Effect of increasing doses of *B. serrata* extract A (0.1, 1, 10, and 100 $\mu$g dry extract/ml) (a), extract G (2.4, 24, 240, and 2400 $\mu$g resin/ml) (b), and pure BAs (*low*, corresponding to 3.8 ng/ml AKBA, 3 ng/ml KBA, and 8 ng/ml $\beta$BA, and *high*, corresponding to 380 ng/ml AKBA, 300 ng/ml KBA, and 800 ng/ml $\beta$BA) (c) on pAEC viability, in the presence of LPS (25 $\mu$g/ml), measured by MTT assay. Data are reported as mean ± SD of 8 independent replicates. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test).

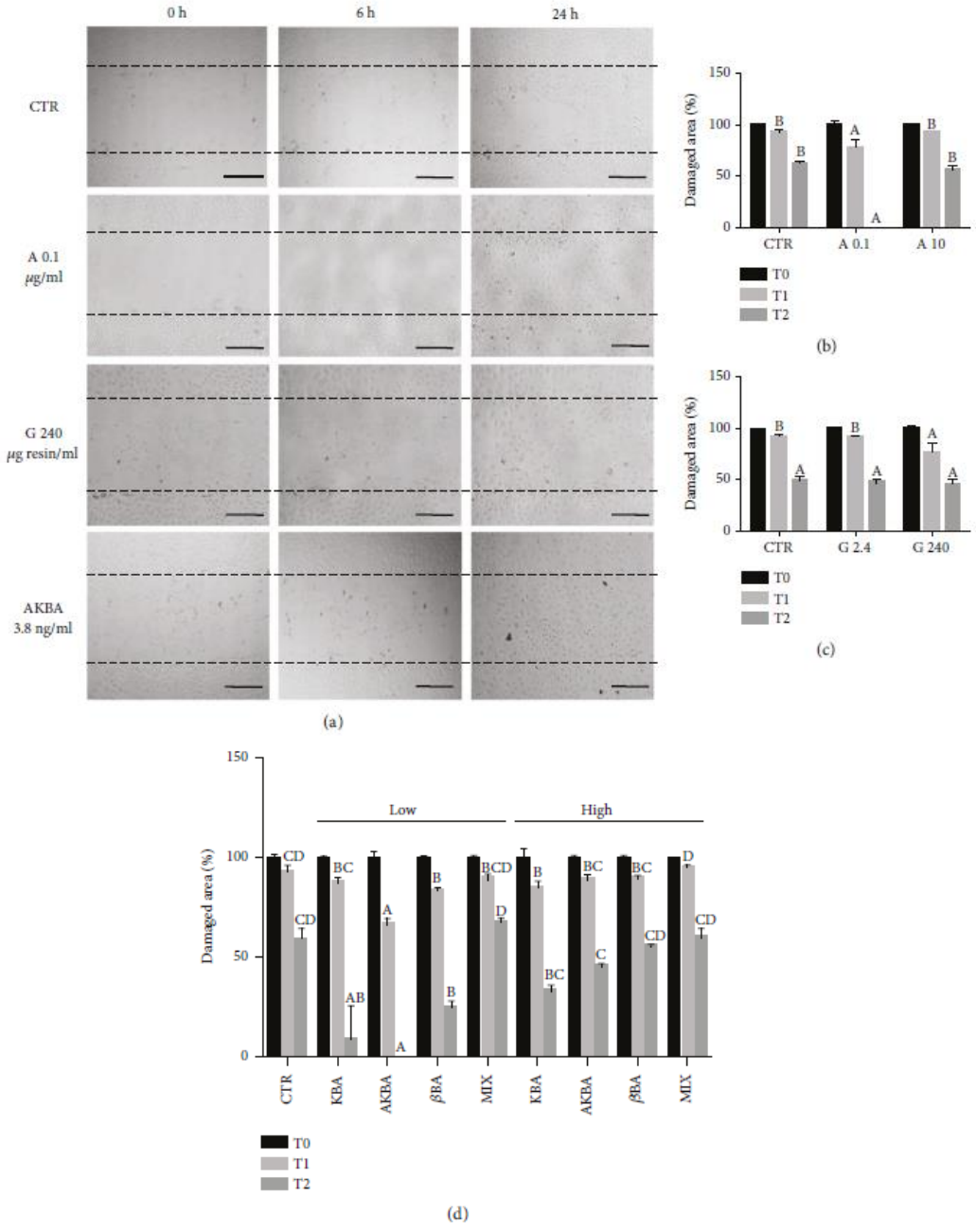


FIGURE 4: Effect of *B. serrata* extracts on pAEC migration capacity. Cells were scratch wounded and then treated with extracts A and G and pure BAs. Photographs were recorded at 0 h (T0), 6 h (T1), and 24 h (T2) after scratching. (a) Representative microscopic phase-contrast pictures showing the size of the scratch wound in different treatment groups compared with control. Scale bar, 200 $\mu$m. The extent of the damaged area (%) is reported for treatment with extract A (0.1 and 10 $\mu$g dry extract/ml) (b), extract G (2.4 and 240 $\mu$g resin/ml) (c), and pure BAs (*low*, corresponding to 3 ng/ml KBA, 3.8 ng/ml AKBA, and 8 ng/ml $\beta$BA, and *high*, corresponding to 300 ng/ml KBA, 380 ng/ml AKBA, and 800 ng/ml $\beta$BA) (d). Data are reported as mean of 3 replicates ± SD. Inside each experimental time (T1 and T2), different letters above the bars indicate significant differences among treatments ($p < 0.05$, ANOVA post hoc Tukey's test).

from hyperproliferative effect (the lowest dose) to cytotoxic effect (the highest dose). Interestingly, in our model the use of pure KBA, $\beta$BA, and AKBA, either individually or mixed together, failed to protect endothelial cells from LPS toxicity and are only partially in accord with data reported by Henkel et al. [35]. In a cell-free assay, those authors suggested a direct molecular interaction between LPS and BAs lacking the keto moiety, in particular $\beta$BA, underlying the anti-inflammatory effect of *Boswellia* extracts.

Our results support the hypothesis that the anti-inflammatory effect of *Boswellia* extracts is not strictly dependent on the presence of the most studied BAs, but it can be related to other bioactive molecules. Other triterpenes, as incensole, could be considered interesting candidates for the pharmacological properties of frankincense, accordingly to suggestions previously reported by other authors [9, 37, 38]. Beyond these bioactive terpenes, the gum resin does contain polysaccharides. These molecules are likely to be minor components in dry extract A, whereas they can be more concentrated in extract G, due to the different polarity of the extraction medium. A water-soluble fraction extracted from the gum resin of *B. serrata* containing galactose, arabinose, and D-glucuronic acid was suggested to act as a potent enhancer of humoral and cell-mediated immune response [39], while the potential anti-inflammatory activity of these polysaccharides has not yet been explored. We cannot exclude that the polysaccharide fraction present in extract G can develop additional modulatory effects on pAECs.

The migration ability of endothelial cells is critical in the physiological and pathological angiogenesis [40]. Our results obtained with an *in vitro* model of physiological angiogenesis showed proangiogenic activity of extract A at the lowest concentration, in agreement with a proliferative effect of the same dose recorded in LPS challenge. In addition, incubation with pure AKBA at the same concentration as that measured in extract A determined the same proangiogenic effect, indicating a possible involvement of this BA in promoting angiogenesis. In contrast, incubation in the presence of extract G containing the same concentrations of AKBA did not show any effect on endothelial cell migration capacity, indicating one more time the existence of complex molecular interactions, which can modify the biological effect of the phytoextract. Contrasting results are also reported in literature. Lulli et al. [41] observed that AKBA reduced proliferation, migration, and tube formation in human retinal microvascular endothelial cells (HRMECs) stimulated with exogenous vascular endothelial growth factor (VEGF). On the other hand, Wang et al. [17] reported that $\beta$-BA can attenuate endothelial cell injury in a blood stasis model and protect human umbilical vein endothelial cells (HUEVCs) against cell death induced by oxygen and glucose deprivation. Different regulation pathways could be involved in the repairing activity of *Boswellia* extracts, and further investigations will be necessary to explain why different formulations determine different effects on endothelial cells pathophysiology.

How extracts of *B. serrata* gum resin should modulate the cardiovascular system has been scarcely investigated, so far. Kokkiripati et al. [42] reported that antioxidant and antithrombotic activities of extracts from *B. serrata* gum resin determined the inhibition of human monocytic cell activation and platelet aggregation. However, recently Siemoneit et al. [43] pointed out the complex agonizing and antagonizing effects of BAs on human platelet aggregation and prompted for careful evaluation of *B. serrata* extract safety in cardiovascular disease-risk patients.

In conclusion, our results demonstrate that different formulations (e.g., dry and hydroenzymatic extracts) obtained from the same botanical species show significantly different biological effects on endothelial cells. The anti-inflammatory activity of *B. serrata* extracts on endothelial cells suggests a potential pharmaceutical application for cardiovascular health, though cytotoxicity or proliferative stimulation can occur instead of a protective effect, depending on the dose and the formulation. This aspect should be carefully considered when these herbal products are used in human and animal phytotherapy.

## Disclosure

Preliminary data has been presented as a poster at the 21th Congress Phytopharm 2017, Graz, Austria, 2–5 July 2017.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Acknowledgments

The authors greatly acknowledge Phenbiox Srl (Calderara di Reno, Bologna, Italy) and Dr. Maurizio Scozzoli for providing the *Boswellia serrata* hydroenzymatic and dry extracts, respectively, and Dr. Alberto Altafini for technical assistance. The authors are grateful to Università di Bologna and Fondazione Cassa di Risparmio di Imola for their financial support.

## Supplementary Materials

Table S1: the concentrations of the two boswellic acids analyzed and discussed in the manuscript in five additional dry extracts of *Boswellia serrata*. The table has been added to emphasize the variability of boswellic acid concentration in different dry extracts obtained from the same botanical species. *(Supplementary Materials)*

## References

[1] W. C. Aird, "Endothelium and haemostasis," *Hämostaseologie*, vol. 35, no. 1, pp. 11–16, 2015.

[2] T. Aki, N. Egashira, M. Hama et al., "Characteristics of gabexate mesilate–induced cell injury in porcine aorta endothelial cells," *Society*, vol. 106, no. 3, pp. 415–422, 2008.

[3] S. Jamwal and S. Sharma, "Vascular endothelium dysfunction: a conservative target in metabolic disorders," *Inflammation Research*, vol. 67, no. 5, pp. 391–405, 2018.

[4] H. P. T. Ammon, "Boswellic acids in chronic inflammatory diseases," *Planta Medica*, vol. 72, no. 12, pp. 1100–1116, 2006.

[5] H. P. T. Ammon, "Boswellic acids and their role in chronic inflammatory diseases," in *Anti-Inflammatory Nutraceuticals and Chronic Diseases*, S. C. Gupta, S. Prasad, and B. B. Aggarwal, Eds., pp. 291–327, Cham: Springer International Publishing, Switzerland, 2016.

[6] I. Gupta, A. Parihar, P. Malhotra et al., "Effects of gum resin of *Boswellia serrata* in patients with chronic colitis," *Planta Medica*, vol. 67, no. 5, pp. 391–395, 2001.

[7] M. A. Khan, R. Ali, R. Parveen, A. K. Najmi, and S. Ahmad, "Pharmacological evidences for cytotoxic and antitumor properties of Boswellic acids from *Boswellia serrata*," *Journal of Ethnopharmacology*, vol. 191, pp. 315–323, 2016.

[8] K. Sengupta, K. V. Alluri, A. Satish et al., "A double blind, randomized, placebo controlled study of the efficacy and safety of 5-Loxin® for treatment of osteoarthritis of the knee," *Arthritis Research & Therapy*, vol. 10, no. 4, article R85, 2008.

[9] M. Abdel-Tawab, O. Werz, and M. Schubert-Zsilavecz, "*Boswellia serrata*," *Clinical Pharmacokinetics*, vol. 50, no. 6, pp. 349–369, 2011.

[10] A. R. M. Al-Yasiry and B. Kiczorowska, "Frankincense - therapeutic properties," *Postępy Higieny i Medycyny Doświadczalnej*, vol. 70, pp. 380–391, 2016.

[11] H. P. T. Ammon, "Modulation of the immune system by *Boswellia serrata* extracts and boswellic acids," *Phytomedicine*, vol. 17, no. 11, pp. 862–867, 2010.

[12] H. Hussain, A. Al-Harrasi, R. Csuk et al., "Therapeutic potential of boswellic acids: a patent review (1990-2015)," *Expert Opinion on Therapeutic Patents*, vol. 27, no. 1, pp. 81–90, 2017.

[13] M. Gupta, P. K. Rout, L. N. Misra et al., "Chemical composition and bioactivity of *Boswellia serrata* Roxb. essential oil in relation to geographical variation," *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology*, vol. 151, no. 4, pp. 623–629, 2017.

[14] D. Beghelli, G. Isani, P. Roncada et al., "Antioxidant and ex vivo immune system regulatory properties of *Boswellia serrata* extracts," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 7468064, 10 pages, 2017.

[15] L. Setti and D. Zanichelli, "Bioliquefaction as a biorefinery's approach for the production of natural bioactive compounds for functional cosmetics," in *Waste Recovery: Strategies, Techniques and Applications in Europe*, L. Morselli, F. Passarini, and I. Vassura, Eds., pp. 122–128, Franco Angeli, Milan, Italy, 2009.

[16] Y. Ding, M. Chen, M. Wang, Y. Li, and A. Wen, "Posttreatment with 11-keto-$\beta$-boswellic acid ameliorates cerebral ischemia–reperfusion injury: Nrf2/HO-1 pathway as a potential mechanism," *Molecular Neurobiology*, vol. 52, no. 3, pp. 1430–1439, 2015.

[17] M. Wang, M. Chen, Y. Ding et al., "Pretreatment with $\beta$-boswellic acid improves blood stasis induced endothelial dysfunction: role of eNOS activation," *Scientific Reports*, vol. 5, no. 1, article 15357, 2015.

[18] M. Bertocchi, F. Medici, C. Bernardini et al., "Characterization of *Boswellia serrata* extracts and evaluation of their effects on porcine aortic endothelial cells," in *Reviews on Clinical Pharmacology and Drug Therapy (The 21th International Congress Phytopharm 2017)*, vol. 15, p. 42, Graz, Austria, July 2017.

[19] C. Zaragoza, C. Gomez-Guerrero, J. L. Martin-Ventura et al., "Animal models of cardiovascular diseases," *Journal of Biomedicine & Biotechnology*, vol. 2011, Article ID 497841, 13 pages, 2011.

[20] C. Perleberg, A. Kind, and A. Schnieke, "Genetically engineered pigs as models for human disease," *Disease Models & Mechanisms*, vol. 11, no. 1, article dmm030783, 2018.

[21] C. Bernardini, A. Zannoni, M. E. Turba et al., "Heat shock protein 70, heat shock protein 32, and vascular endothelial growth factor production and their effects on lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells," *Cell Stress & Chaperones*, vol. 10, no. 4, pp. 340–348, 2005.

[22] C. Bernardini, A. Zannoni, M. L. Bacci, and M. Forni, "Protective effect of carbon monoxide pre-conditioning on LPS-induced endothelial cell stress," *Cell Stress and Chaperones*, vol. 15, no. 2, pp. 219–224, 2010.

[23] G. Botelho, C. Bernardini, A. Zannoni, V. Ventrella, M. L. Bacci, and M. Forni, "Effect of tributyltin on mammalian endothelial cell integrity," *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 176-177, pp. 79–86, 2015.

[24] G. Mannino, A. Occhipinti, and M. Maffei, "Quantitative determination of 3-*O*-acetyl-11-keto-$\beta$-boswellic acid (AKBA) and other boswellic acids in *Boswellia sacra* Flueck (syn. *B. carteri* Birdw) and *Boswellia serrata* Roxb," *Molecules*, vol. 21, no. 10, p. 1329, 2016.

[25] J. Meins, C. Artaria, A. Riva, P. Morazzoni, M. Schubert-Zsilavecz, and M. Abdel-Tawab, "Survey on the quality of the top-selling European and American botanical dietary supplements containing boswellic acids," *Planta Medica*, vol. 82, no. 6, pp. 573–579, 2016.

[26] E. Ranzato, S. Martinotti, A. Volante, A. Tava, M. A. Masini, and B. Burlando, "The major *Boswellia serrata* active 3-acetyl-11-keto-$\beta$-boswellic acid strengthens interleukin-1$\alpha$ upregulation of matrix metalloproteinase-9 via JNK MAP kinase activation," *Phytomedicine*, vol. 36, pp. 176–182, 2017.

[27] Y. Shao, C.-T. Ho, C.-K. Chin, V. Badmaev, W. Ma, and M.-T. Huang, "Inhibitory activity of boswellic acids from *Boswellia serrata* against human leukemia HL-60 cells in culture," *Planta Medica*, vol. 64, no. 4, pp. 328–331, 1998.

[28] M. Lu, L. Xia, H. Hua, and Y. Jing, "Acetyl-keto-B-boswellic acid induces apoptosis through a death receptor 5-mediated pathway in prostate cancer cells," *Cancer Research*, vol. 68, no. 4, pp. 1180–1186, 2008.

[29] J.-J. Liu, B. Huang, and S. C. Hooi, "Acetyl-keto-$\beta$-boswellic acid inhibits cellular proliferation through a p21-dependent pathway in colon cancer cells," *British Journal of Pharmacology*, vol. 148, no. 8, pp. 1099–1107, 2006.

[30] B. Park, S. Prasad, V. Yadav, B. Sung, and B. B. Aggarwal, "Boswellic acid suppresses growth and metastasis of human pancreatic tumors in an orthotopic nude mouse model through modulation of multiple targets," *PLoS One*, vol. 6, no. 10, article e26943, 2011.

[31] J.-J. Liu, A. Nilsson, S. Oredsson, V. Badmaev, and R.-D. Duan, "Keto- and acetyl-keto-boswellic acids inhibit proliferation and induce apoptosis in Hep G2 cells via a caspase-8 dependent pathway," *International Journal of Molecular Medicine*, vol. 10, no. 4, pp. 501–505, 2002.

[32] S. Bhushan, A. Kumar, F. Malik et al., "A triterpenediol from *Boswellia serrata* induces apoptosis through both the intrinsic and extrinsic apoptotic pathways in human leukemia HL-60 cells," *Apoptosis*, vol. 12, no. 10, pp. 1911–1926, 2007.

[33] S. Roy, S. Khanna, A. V. Krishnaraju et al., "Regulation of vascular responses to inflammation: inducible matrix metalloproteinase-3 expression in human microvascular endothelial cells is sensitive to antiinflammatory Boswellia,"

*Antioxidants & Redox Signaling*, vol. 8, no. 3-4, pp. 653–660, 2006.

[34] C. Cuaz-Perolin, L. Billiet, E. Bauge et al., "Antiinflammatory and antiatherogenic effects of the NF-κB inhibitor acetyl-11-keto-β-boswellic acid in LPS-challenged ApoE−/− mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 2, pp. 272–277, 2008.

[35] A. Henkel, N. Kather, B. Mönch, H. Northoff, J. Jauch, and O. Werz, "Boswellic acids from frankincense inhibit lipopolysaccharide functionality through direct molecular interference," *Biochemical Pharmacology*, vol. 83, no. 1, pp. 115–121, 2012.

[36] T. Syrovets, B. Buchele, C. Krauss, Y. Laumonnier, and T. Simmet, "Acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF-α induction in monocytes by direct interaction with IκB kinases," *Journal of Immunology*, vol. 174, no. 1, pp. 498–506, 2005.

[37] A. Moussaieff, E. Shohami, Y. Kashman et al., "Incensole acetate, a novel anti-inflammatory compound isolated from Boswellia resin, inhibits nuclear factor-κB activation," *Molecular Pharmacology*, vol. 72, no. 6, pp. 1657–1664, 2007.

[38] B. Gayathri, N. Manjula, K. S. Vinaykumar, B. S. Lakshmi, and A. Balakrishnan, "Pure compound from *Boswellia serrata* extract exhibits anti-inflammatory property in human PBMCs and mouse macrophages through inhibition of TNFα, IL-1β, NO and MAP kinases," *International Immunopharmacology*, vol. 7, no. 4, pp. 473–482, 2007.

[39] A. Gupta, A. Khajuria, J. Singh, S. Singh, K. A. Suri, and G. N. Qazi, "Immunological adjuvant effect of *Boswellia serrata* (BOS 2000) on specific antibody and cellular response to ovalbumin in mice," *International Immunopharmacology*, vol. 11, no. 8, pp. 968–975, 2011.

[40] A. M. Mahecha and H. Wang, "The influence of vascular endothelial growth factor-A and matrix metalloproteinase-2 and -9 in angiogenesis, metastasis, and prognosis of endometrial cancer," *OncoTargets and Therapy*, vol. 10, pp. 4617–4624, 2017.

[41] M. Lulli, M. Cammalleri, I. Fornaciari, G. Casini, and M. Dal Monte, "Acetyl-11-keto-β-boswellic acid reduces retinal angiogenesis in a mouse model of oxygen-induced retinopathy," *Experimental Eye Research*, vol. 135, pp. 67–80, 2015.

[42] P. K. Kokkiripati, L. M. Bhakshu, S. Marri et al., "Gum resin of *Boswellia serrata* inhibited human monocytic (THP-1) cell activation and platelet aggregation," *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 893–901, 2011.

[43] U. Siemoneit, L. Tausch, D. Poeckel et al., "Defined structure-activity relationships of boswellic acids determine modulation of $Ca^{2+}$ mobilization and aggregation of human platelets by *Boswellia serrata* extracts," *Planta Medica*, vol. 83, no. 12/13, pp. 1020–1027, 2017.

# 4.4 *Salvia sagittata*

Submitted to Oxidative Medicine and Cellular Longevity

**_In vitro_ anti-inflammatory effect of _Salvia sagittata_ ethanolic extract on primary cultures of porcine aortic endothelial cells.**

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## Abstract

The aim of the present research was to study the effects of an ethanolic extract of *Salvia sagittata* Ruiz & Pav (SSEE), an endemic Ecuadorian plant traditionally used to treat inflammation and different intestinal affections, on primary cultures of porcine aortic endothelial cells (pAECs). pAECs were cultured in the presence of different concentrations (1-200 µg/mL) of SSEE for 24 h and cytotoxicity was evaluated by the MTT assay. SSEE did not negatively affect cellular viability at any concentration tested. Cell cycle was analyzed and no significant change was observed. Then, the anti-inflammatory effects of SSEE on pAECs were analyzed using Lipopolysaccharide (LPS) as the inflammatory stimulus. Different markers involved in the inflammatory process, such as cytokines and protective molecules, were evaluated by the MTT assay, Real-Time quantitative PCR (RT-qPCR) and Western blot. SSEE showed the ability to restore pAECs physiological conditions reducing IL-6 and increasing HO-1 protein levels. The phytochemical composition of SSEE was also evaluated by HPLC-DAD and spectrophotometric assays. The presence of different phenolic acids and flavonoids was revealed, with rosmarinic acid as the most abundant component. SSEE possess an interesting antioxidant activity, as assessed through both Oxygen Radical Absorbance Capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

In conclusion, results suggest that SSEE is endowed with an *in vitro* anti-inflammatory effect. This represents the initial step in finding a possible scientific support for the traditional therapeutic use of this plant.

## 1. Introduction

In the last years, researches aimed to scientifically define the effects of natural products have been growing, not only due to the increasing popularity of the plant-based Traditional Medicine, but also because it provides the primary health-care needs for the majority of the population in developing countries [1]. Moreover, a huge number of medicinal plants, not still investigated, are available worldwide. Currently, more than 20.000 plant species are used to treat several diseases and are considered as potential reservoirs for new drugs [2]. Recent studies suggest that the historical ethnopharmacological uses of plant-based medicines can represent a useful preliminary screening tool in the field of drug discovery [3].

Ecuador is considered one of the countries with the largest biodiversity in the world. The flora of mainland Ecuador is extremely rich: an estimated total of 17.000 species have so far been recorded [4,5], and more than 3000 medicinal plants are used in different native communities living on the highlands of Ecuadorian Andes [6]. However, in most cases, the preparation, doses and route of administration of these herbal remedies are only transferred orally from generation to generation, while scientific information regarding their phytochemical or biological activity is insufficient or lacking at all [7].

*Salvia* L. (sage) is widely known as the largest genus in the Lamiaceae family, and to date, approximately 980 species have been recognized, most of which are restricted to the New World [8]. Some species of this genus have been used since ancient times as medicinal plants all around the world [9-11]. In addition, chemical constituents of various sage plants were described and comprise different terpenoids, several phenolic compounds, such as simple phenolics and caffeic acid derivatives, flavonoids, as well as phenolic diterpenoids [12].

*Salvia sagittata* Ruiz & Pav is an herbaceous perennial plant distributed in Ecuador and Peru. It has yellow-green arrow-shaped leaves and very sticky inflorescences in the apical part of the plant, formed by brilliant blue flowers with a prominent lower lip. Its leaves are commonly prepared either in infusion to counteract different affections such as spasms, diarrhea, flatulence, fever, influenza, gastritis, stomach pain, cuts and bumps, or heated with brandy and applied topically to treat rheumatisms and articular pain [13-15]. Despite the ethnobotanical information in favor of

multiple beneficial health effects of *S. sagittata,* scientific evidences from *in vivo* or *in vitro* studies are still lacking.

In order to test the possible biological activity of *S. sagittata* ethanolic extracts (SSEE), we used endothelial cells as model system, given their fundamental role in different physiological processes. These cells are normally in dynamic equilibrium with their environment, preventing thrombus formation by the expression and secretion of anti-coagulant, anti-adhesive, and anti-inflammatory molecules. Nevertheless, in pathologic processes, such as inflammation, infection or genetic alterations, endothelial cells change their phenotype from a resting to an active function that modulates the complement and coagulation cascades, thrombus formation, inflammation and innate and adaptive immunity [16,17]. Endothelial cells are also recognized as key regulators of the inflammatory response controlling adhesion and migration of inflammatory cells as well as resolution of inflammation [18].

Tests were carried out on a primary culture instead of cell line model. Despite their viability and unlimited expansion, cell lines do not preserve various of the important markers and functions shown *in vivo* [19,20]. On the contrary, primary cells preserve most of these functions. In this context, primary cultures of porcine aortic endothelial cells have been used as a suitable *in vitro* model of human ones [21-23], due to the biological similarities between swine and human at anatomic [24], proteomic [25] and genomic level [26].

In this context, we decided to evaluate SSEE for its phytochemical, antioxidant and anti-inflammatory characteristics as related to biological activities in primary cultures of porcine aortic endothelial cells stimulated with bacterial lipopolysaccharide.

## 2. Materials and methods

### *2.1 Chemicals and reagents*

Human Endothelial Serum Free Medium (hESFM), heat inactivated fetal bovine serum (FBS), antibiotic-antimycotic, dulbecco's phosphate buffered saline (DPBS), phosphate buffered saline (PBS) were purchased from Gibco-Life technologies (Carlsbad CA, USA). Propidium iodide (PI) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). RNaseA/T1 was purchased from Thermo Scientific (Waltham, MA, USA). Trizol reagent was purchased from Thermo Fisher Scientific (Rockford, IL, USA), RNA isolation was performed with NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), iScript cDNA synthesis kit and iTaq Universal SYBR Green Supermix were used for cDNA synthesis and qPCR analysis, respectively (Bio-Rad Laboratories Inc., Hercules, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemicals Co, St. Louis, Mo., USA. Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxyl-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein, gallic acid, rutin, phenolic acids (4-hydroxybenzoic, caffeic, chlorogenic, ferulic, gallic, *p*-coumaric, synapic, syringic, trans-cinnamic, rosmarinic acids), quercetin, quercetin-3-O-glucoside, quercetin-3-O-ramnoside, quercetin-3-O-galactoside, kaempferol, kaempferol-3-O-rutinoside, hesperetin, hesperidin pure standards (>99.5% purity) in powder form, and HPLC-grade solvents were purchased from Sigma-Aldrich Italia (Milan, Italy). All standards were prepared as stock solutions at 1 mg/mL in methanol and stored in the dark at -18 °C for less than three months.

### *2.2 Preparation of plant extract*

*Salvia sagittata* Ruiz & Pav (SS) plants were collected, according to previous authorization of Ministry of the Environment (Nro. 003-IC-DPACH-MAE-2018-F), in Riobamba Ecuador on May 2016. The plants were identified and certified by Escuela Superior Politecnica de Chimborazo Herbarium, Riobamba, Ecuador and a voucher specimen was deposited (N. 3342). Dried leaves (300 g) were ground and extracted with 96% ethanol for 48 h at room temperature. After filtration, the solvent was evaporated using a rotary vacuum evaporator (Büchi Ch-9230 Flawil, Switzerland) and dried in vacuum at 40 °C to obtain EtOH extract with a yield of 6.18%. For experiments, the dry extract was dissolved in ethanol. The stock solution (20 mg/mL) was further used for HPLC analysis or diluted in culture media and membrane filtered by a 0.2 µm Millipore filter (Millipore, Darmstadt, Germany).

### *2.3 Cell culture and treatments*

Porcine aortic endothelial cells (pAECs) were isolated and maintained as previously described by Bernardini et al. [27]. For the experiments, cells from the third to the tenth passage were used. Cells were seeded and routinely cultured in T25 tissue culture flasks ($4x10^5$ cells/flask) (T 25-Falcon, Beckton-Dickinson, Franklin Lakes, NJ, USA). Successive experiments were conducted in 96-well plates (cell viability and anti-inflammatory test) or 24-well plates (qPCR and Western blot) (both by Beckton-Dickinson) at confluent cultures. Cells were cultured in hESFM, added with 5% FBS and 1x antimicrobial/antimycotic solution in a 5% $CO_2$ atmosphere at 38.5°C.

The SSEE stock solution was diluted in culture medium to obtain desired concentrations (1 – 200 µg/mL) for cell exposure. Ethanol (1%) was used as solvent control.

### *2.4 Cell viability*

Viability was determined using MTT assay. Briefly, pAECs were seeded in 96-well culture plates at a density of $3 \times 10^3$ cells/well and incubated for 24 h. Then, media were replaced with hESFM containing 5% FBS and increasing SSEE doses (1, 10, 50, 100 and 200 µg/mL), and incubated for another 24 hours at 38.5°C. Next, MTT solution (5 mg/mL in PBS) was added to a final concentration of 0.5 mg/mL and then incubated for 2 hours at 38.5°C, followed by the addition of 0.1 mL MTT solubilisation solution. The formazan Abs was determined at 570 nm, using Infinite® F50/Robotic absorbance microplate readers from TECAN Life Sciences (Männedorf, Switzerland).

### *2.5 Cell cycle*

After defrosting and growing to 80% of pAECs confluence, flask media were replaced with hESFM containing 5% FBS and increasing SSEE doses (1, 10, 50, 100 µg/mL). After 24 hours, pAECs were harvested, washed once in 5 ml of PBS and 1 mL /$10^6$cells of 70% ice-cold ethanol was added drop-by-drop with continuous vortexing. The single cell suspension was fixed at 4°C for 24 hours. Then, the cells were washed with 10 ml of PBS and cellular pellet was treated with 1 mL/$10^6$cells of staining solution containing PBS, 50 µg/mL of PI and 100 µg/mL RNaseA/T1 for 20 min in the dark at r.t. Cell distribution in cell cycle phases was analyzed by MACSQuant® Analyzer10 and Flowlogic software (Miltenyi Biotec, Bergisch Gladbach, Germany). Dean-Jett-Fox Univariate Model was used for this analysis.

### *2.6 In vitro tube formation assay*

The experiments were carried out using a 8-slide-chamber glass (BD Falcon Bedford, MA, USA) coated with undiluted Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix. Firstly, extracellular matrix coating was carried out 1 hour before the seeding in a humidified incubator, at 38.5°C and 5% $CO_2$. Then pAECs ($8x10^4$ cells/well) were seeded with increasing SSEE doses (1, 10, 25, 50 and 100 µg/mL) for 18 h.

At the end of experimental time, images were acquired using a digital camera installed on a Nikon contrast phase microscope (Nikon, Yokohama, Japan) and analyzed by open software Image J 64.

### *2.7 Cell viability after LPS treatment*

Briefly, pAECs were seeded in 96-well culture plates at a density of $3{\times}10^3$ cells/well and incubated for 24 h, then exposed to different SSEE concentrations (1, 10 and 100 µg/mL) in presence of LPS (25 µg/mL) and incubated for another 24 hours at 38.5°C.

MTT solution was added to a final concentration of 0.5 mg/mL and then incubated for 2 hours at 38.5°C followed by the addition of 0.1 mL of dimethyl sulfoxide to dissolve the MTT-formazan. The amount of MTT-formazan was then determined by measuring Abs at 570 nm.

### *2.8 Quantitative real time PCR for IL-6, IL-8, HO-1*

pAECs were seeded in a 24 wells plate (approximately $4x10^4$ cells/well), incubated until confluence and then exposed to different concentrations of SSEE (1, 10 and 100 µg/mL) in absence or presence of LPS (25 µg/mL). RNA extraction was performed using Trizol reagent and NucleoSpin RNA II kit. After 24 h, cells were harvested and lysed using 1 mL Trizol reagent and mixed by vortex (3 min) then 200 uL of chloroform was added to the suspension and mixed well. After incubation at room temperature (10 min), samples were centrifuged (12000 g for 10 min) and the aqueous phase was recovered. An equal volume of absolute ethanol (99%) was added and the resulting solution was applied to the NucleoSpin RNA Column. RNA was then purified according to the manufacturer's instructions. After spectrophotometric quantification, total RNA (250 ng) was reverse-transcripted to cDNA using the iScript cDNA Synthesis Kit in a final volume of 20 µL.

Swine primers were designed using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences, expected PCR product lengths and accession numbers in the NCBI database are shown in Table 1.

To evaluate gene expression profiles, quantitative real-time PCR (qPCR) was performed in CFX96 thermal cycler (Bio-Rad) using a multiplex real time reaction for reference genes (GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine guanine phosphoribosyl transferase; β-Actin), and using Taq-Man probes and SYBR green detection for target genes (IL-6, interleukin-6; IL-8, interleukin-8; HO-1, Heme Oxigenase-1). All amplification reactions were performed in 20 µL and analyzed in duplicates (10 µL/well). Multiplex PCR contained: 10 µL of iTaqMan Probes Supermix (Bio-RAD), 1 µL of forward and reverse primers (5 µM each) of each reference gene, 0.8 µL of iTaq-Man Probes (5 µM) of each reference gene, 2 µL cDNA and 2.6 µL of water. The following temperature profiling was used: initial denaturation at 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconnds.

The SYBR Green reaction contained: 10 µL of IQSYBR Green Supermix (Bio-RAD), 0.8 µL of forward and reverse primers (5 µM each) of each target gene, 2 µL cDNA and 7.2 µL of water. The real-time program included an initial denaturation period of 1.5 min at 95 °C, 40 cycles at 95

°C for 15 s, and 60 °C for 30 s, followed by a melting step with ramping from 55 °C to 95 °C at a rate of 0.5°C/10 s.

The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis.

The relative expressions of the studied genes were normalized based on the geometric mean of the three reference genes. The relative mRNA expression of tested genes was evaluated as fold of increase using the $2^{-\Delta\Delta CT}$ method [28] referred to pAECs cultured under standard condition (control).

**Table. 1** Primer sequences used for quantitative real-time polymerase chain reaction analysis.

| Gene | Sequence (5'-3') | PCR product (bp) | Gene Bank Accession Number | Reference |
|---|---|---|---|---|
| HO-1 | For: CGCTCCCGAATGAACAC<br>Rev: GCTCCTGCACCTCCTC | 112 | NM_001004027 | Bernardini et al., 2010 |
| Il-8 | For: AGGACCAGAGCCAGGAAGAGAC<br>Rev: TGGAAAGGTGTGGAATGCGTATTTATG | 203 | AB057440.1 | Present Study |
| Il-6 | For: AGCAAGGAGGTACTGGCAGAAAACAAC<br>Rev: GTGGTGATTCTCATCAAGCAGGTCTCC | 110 | AF518322.1 | Zannoni et al., 2012 |
| GAPDH | For: ACATGGCCTCCAAGGAGTAAGA<br>Rev: GATCGAGTTGGGGCTGTGACT<br>Probe: HEX-CCACCAACCCCAGCAAGAGCACGC-BHQ1 | 106 | NM_001206359 | Duvigneau et al., 2005 |
| HPRT | For: ATCATTATGCCGAGGATTTGGAAA<br>Rev: TGGCCTCCCATCTCTTTCATC<br>Probe: Tx-Red-CGAGCAAGCCGTTCAGTCCTGTCC-BQ2 | 102 | NM_001032376 | Present study |
| β-ACT | For: CTCGATCATGAAGTGCGACGT<br>Rev: GTGATCTCCTTCTGCATCCTGTC<br>Probe: FAM-ATCAGGAAGGACCTCTACGCCAACACGG-BHQ1 | 114 | KU672525.1 | Duvigneau et al., 2005 |

### *2.9 Western Blot for HO-1*

pAECs, treated in the same manner as mentioned above, were washed twice with ice-cold PBS, harvested and lysed in SDS solution (Tris-HCl 50 mM pH 6.8; SDS 2%; glycerol 5%). After quantitative determination of protein content by a Protein Assay Kit (TP0300, Sigma), aliquots containing 20 μg of proteins were separated on NuPage 4-12% bisTris Gel for 45 min at 200 V and electro-transferred onto a nitrocellulose membrane. Blots were washed in PBS and protein transfer was checked by staining the nitrocellulose membranes with 0.2% Ponceau Red. After blocking nonspecific binding with 5% non-fat milk in PBS-T20 (PBS-0.1% Tween-20) at r.t. for 1 h, membranes were incubated with a 1:1000 dilution of anti-HO-1 rabbit polyclonal antibody (SPA 896 StressGen Biotecnologies Corp, Victoria BC, Canada) overnight at 4 °C.

After several washings with PBS-T20, membranes were incubated with the secondary biotin-conjugate antibody and then with a 1:1000 dilution of an antibiotin horseradish peroxidase (HRP)-linked antibody. The Western Blots were developed using chemiluminescent substrate (Clarity Western Substrate, BioRad) according to the manufacturer's instructions. The intensity of the luminescent signal of the resultant bands was acquired by Fluor-S TM Multimager using Quantity One Software (Bio-Rad).

In order to normalise the HO-1 data on the housekeeping protein, membranes were stripped and re-probed for housekeeping β-tubulin (1:500 sc-5274 Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). The relative protein content (HO-1/β-tubulin) was expressed as arbitrary units (AUs).

### *2.10 Antioxidant activity assays*

Antioxidant activity (AA) of SSEE was measured by the ORAC and DPPH assays. ORAC assay was performed in an automated plate reader (Victor 3, Perkin Elmer, Turku, Finland) with 96-well plates, according to Ou et al. [29] with some modifications. All reagents were freshly prepared before the assay. In each well, 210 µl of fluorescein (10 nM) and 35 µl of sample, blank (10 mM phosphate buffer, pH 7.4), or standard (Trolox in the range 1-50 µM) were placed. The plate was heated to 37°C for 10 min, and then 35 µl of AAPH (240 mM) were added, immediately before beginning fluorescence (FL) measurement. Relative FL intensity was monitored at 1.5 min intervals until it was less than 5% of the value of the initial reading. Final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as mmol Trolox equivalents per g of extract or per g of plant material (D.W.).

DPPH assay was done according to the method of Brand-Williams et al. [30] with some modifications. A stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored at -20°C until needed. The working solution was prepared by mixing 10 mL stock solution with 45 mL methanol to obtain an Abs of 1.1±0.02 units at 515 nm. 150 µL of SSEE were allowed to react with 2850 µL of the DPPH solution for 24 h in the dark. Abs measurements were carried out at 515 nm. Results were determined from the regression equation of Trolox calibration curve in the range 25-500 µM, and expressed as mmol TE per g of extract or per g of plant material (D.W.).

### *2.11 Total Phenol content and Total Flavonoid content*

Total Phenol Content (TPC) was determined using the Folin–Ciocalteu method [31]. 50 µL of diluted extract were mixed with 250 µL of a ten-fold-diluted Folin–Ciocalteu phenol reagent. After 1 min, 800 µL of 30% sodium carbonate solution were added to the mixture, shaken thoroughly and diluted to 1.6 mL by adding 500 µL of distilled water. The mixture was allowed to stand for 40 min at r.t. and the blue colour formed is measured at 700 nm using a UV-VIS spectrophotometer (V-630 Jasco, Jasco Europe S.r.l., Cremella, Italy). A calibration curve of gallic acid (ranging from 5 to 500 µg/mL) was prepared and results, determined from regression equation of the calibration curve, were expressed as mg of Gallic Acid Equivalents (GAE) per g of extract or per g of plant material (D.W.).

Total Flavonoid content (TFC) was determined according to Zhishen et al. [32] with some modifications. 500 µL of extract were diluted to 5 mL with distilled water, 300 µL of 5% $NaNO_2$ were added and the mixture was mixed well. After 5 min, 3 mL of a 10% $AlCl_3$ solution were added. After 6 min, 2 mL of 1 M NaOH solution were added, and the total volume was made up to 10 mL with distilled water. Absorbance was measured against a blank at 510 nm. Rutin was used as standard for the calibration curve. TFC was calculated using the regression equation based on calibration curve, and results were expressed as mmol of Rutin Equivalents (RE) per g of extract or per g of plant material (D.W.).

### *2.12 HPLC-DAD determination of phenolic acids and flavonoids*

20 µL of SSEE were injected into the HPLC system (Jasco Italy; PU-4180 pump, MD-4015 PDA detector, AS-4050 autosampler). The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm x 3 mm I.D., 3.5 µm). The chromatographic method for the analysis of phenolic acids was adapted from Mattila and Kumpulainen [33]. Gradient elution was carried out with a mixture of acidic phosphate buffer (50 mM, pH 2.5) and acetonitrile flowing at 0.7 mL/min. Signals at 254, 280 and 329 nm were used for analyte quantitation. The recovery values of phenolic acids in spiked samples ranged from 78.8 to 92.2% (RSD < 9.8%, n = 6). The chromatographic method for the analysis of flavonoids was adapted from Wojdyło et al. [34]. Gradient elution was carried out with a mixture of 4.5% formic acid and acetonitrile. Runs were monitored at 280 nm for flavan-3-ols and 360 nm for flavonol glycosides. Retention times and spectra were compared with those of pure standards. Calibration curves were constructed for all standards at concentrations ranging from 1.0 to 100.0 ppm ($r^2 \leq$ 0.9998). Results are expressed as mg/g extract or per g of plant material (D.W.).

## 3. Statistical analysis

Each treatment was replicated three or eight times (viability and anti-inflammatory test) in three independent experiments. Data were analysed by a one-way analysis of variance (ANOVA) followed by the *post hoc* Tuckey comparison Test. Differences of at least $p < 0.05$ were considered significant. Statistical analysis was carried out using Graph Pad Prism 7 software.

## 4. Results and discussion

### 4.1 *Effect of SSEE on pAECs viability and angiogenesis*

A large number of plants of the Ecuadorian flora are used for medicinal purposes. Nevertheless, scientific evidences supporting their use are still scarce. For this reason, a study was planned on the anti-angiogenic and antioxidant activity, and on phytochemical composition of *Salvia sagittata*, an endemic plant used in Ecuadorian Traditional Medicine. This choice was corroborated by the fact that several *Salvia* species were demonstrated to possess a protective effect against different external agents [35, 36].

Based on the traditional uses of the plant, it was decided to focus the biological tests on anti-inflammatory activity. A preliminary screening aimed at verifying safety of SSEE was carried out [37]. Treatment of pAECs with SSEE for 24 hours did not negatively affect cell viability at any concentration tested (Fig. 1a). Cells possessed a standard cell cycle for diploid cells (data not shown). Thus, SSEE does not seem to induce any cytotoxicity effect on pAECs. These results are in agreement with other researches in which different *Salvia* species did not affect cell viability [38]. After this preliminary assay, pAECs' angiogenesis was examined by an *in vitro* extracellular matrix-based assay. As a result, the capacity of cells to assemble a tube network formation was reduced after treatment with SSEE at 1, 10, 25 and 50 µg/mL (Fig. 1b and Fig. 1c).

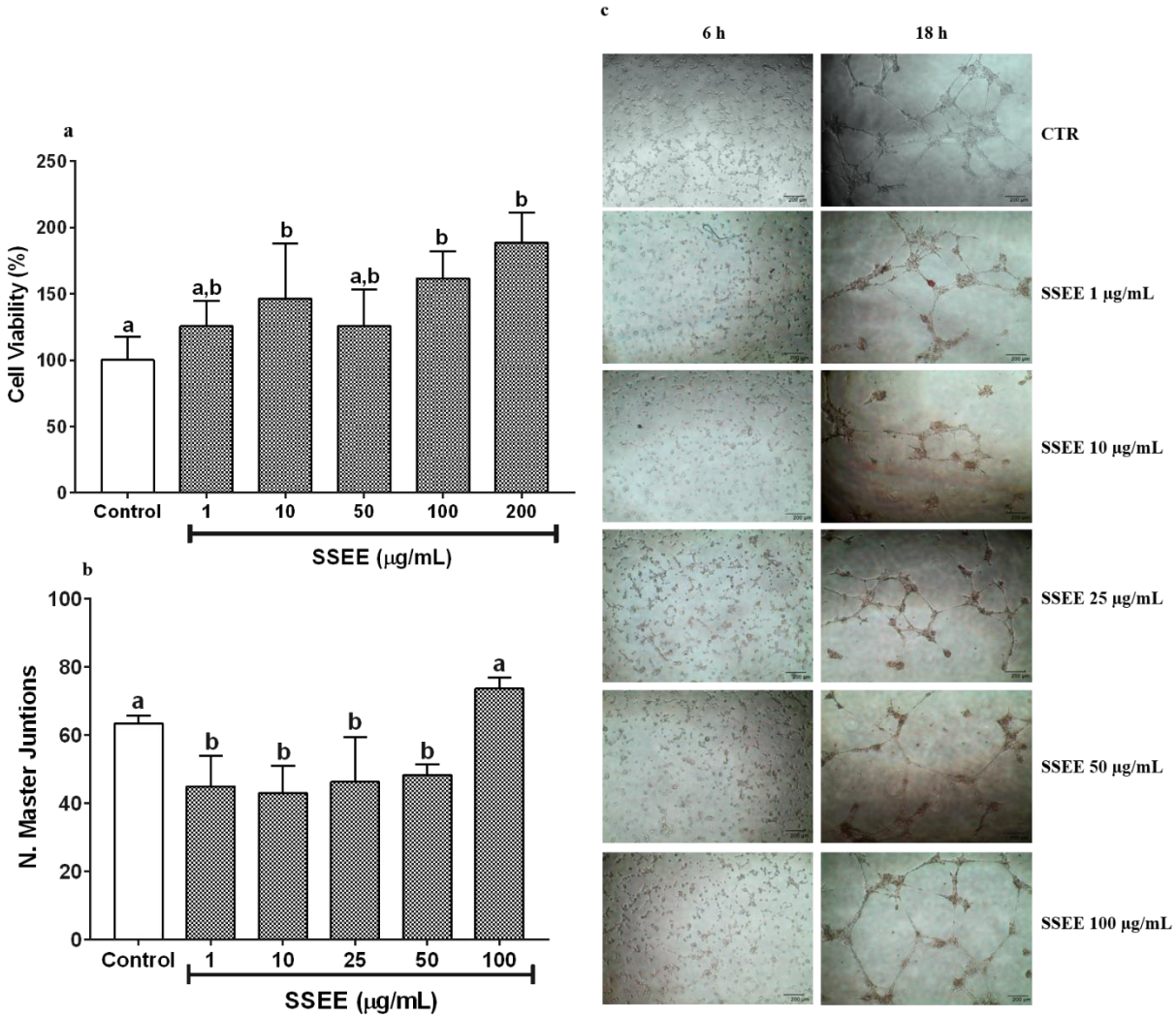


**Figure 1.** Effects of *S*SEE on pAECs physiology. Cells were treated with different concentrations of SSEE for a) 24 hours for cells viability and b) 18 h for angiogenesis. Cells network formation were recorded at 6 h and 18 h after treatment (c). Data shown are representative of 8 (a) or 3 (b) replicates in at least three independent experiments. Each bar represents mean ± S.D. Different letters above the bars indicate significant differences ($p < 0.05$).

### *4.2 Effect of SSEE on LPS-induced cell death and cytokine expression*

Inflammation and endothelial cells are closely related. In fact, in an inflammatory process, endothelial cells trigger the transcription of genes such as TNFR or TLR4 that activates the NF-kB pathway and induces the expression of adhesive receptors (VCAM- 1, E-selectin, ICAM- 1), procoagulant proteins (TF, PAI-1), cytokines, chemokines and protective proteins [17]. Therefore, the anti-inflammatory activity of SSEE on pAECs was evaluated by an endothelial LPS inflammatory model [39, 40]. Firstly, it was decided to assess a possible SSEE protective

effect against LPS damage through a MTT assay. LPS exerted an evident cytotoxic effect, producing a significant 20% reduction of pAECs viability (Fig. 2a) SSEE significantly reduced LPS-induced cytotoxicity, restoring the basal levels at 100 µg/mL concentration (Fig. 2a).

Given the well documented relationship between the inflammation process and the oxidative stress [41], the *in-vitro* antioxidant activity (AA) of the extract was evaluated by two assays, based on two different mechanisms: the ORAC assay, which is based on hydrogen-atom transfer (HAT) mechanism, and the DPPH assays, which is an electron transfer assay. As it can be seen from Table 2, the AA values of the extract were rather similar in the two assays, being 0.10 and 0.11 mmol TE/g DW, respectively. Even though it is extremely difficult to compare and to interpret data on AA of plant extracts, due to the wide number of factors affecting the activity (extract preparation procedure, test method used, etc.), the values of the extract resulting from both DPPH and ORAC assays were very close to those reported for other crude plant extracts prepared in a similar way [42].

**Table 2.** Antioxidant Activity (AA), TPC, TFC, phenolic acids and flavonoid content in SSEE. Data are the mean ± S.E. of three technical determinations

| **Assays or Compounds** | | **Concentration referred to:** | |
|---|---|---|---|
| | | **Ethanolic Plant Extract** | **Plant Dry Weight** |
| **AA** | **ORAC mmol TE/g** | 1.85±0.15 | 0.11±0.09 |
| | **DPPH mmol TE/g** | 1.57±0.11 | 0.097±0.007 |
| **TPC mg/g** | | 164.95±8.57 | 10.19±0.53 |
| **TFC mg/g** | | 109.13±5.23 | 6.74±0.32 |
| **RA mg/g** | | 84.76±9.32 | 5.23±0.57 |
| **ESP mg/g** | | 0.2±0.02 | 0.012±0.001 |
| **Q-3-O-Glu mg/g** | | 0.7±0.0 | 0.043±0.004 |
| **CHA mg/g** | | 1.32±0.16 | 0.08±0.09 |
| **CA mg/g** | | 0.17±0.02 | 0.010±0.001 |
| **SA mg/g** | | 0.02±0.003 | 0.001±0.0001 |

Antioxidant activity and protective effect of SSEE against LPS might confirm its anti-inflammatory activity and justify one the traditional *Salvia sagittata* uses. Nevertheless, to gain insight into the molecular mechanisms involved in mediating these responses, it was decided to investigate how SSEE could influence some of the main inflammatory markers and protective molecules expressed by endothelial cells at both transcription and proteomic level.

IL-6 and IL-8 are stress-responsive proinflammatory chemokines that play a pivotal role in the pathogenesis of different acute inflammatory conditions [43, 44]. They can be synthesized by different cell types; IL-6 activates the differentiation of cytotoxic T cells and monocyte and induces angiogenesis and increases vascular permeability [45, 46], while IL-8 is a potent chemoattractant and activator of leukocytes and fibroblasts and is closely associated with endothelial permeability, inflammatory recruitment, and release of pro-inflammatory mediators [47].

As can be seen in Fig. 2b, 2 c, LPS induced a significant increase of both IL-6 and IL-8 mRNA expression after 24 h of treatment. SSEE at all tested concentrations was able to revert the LPS-induced increase of IL-6 gene expression (Fig. 2b), and to revert that of IL-8 in the 1-10 µg/mL range (Fig. 2c).

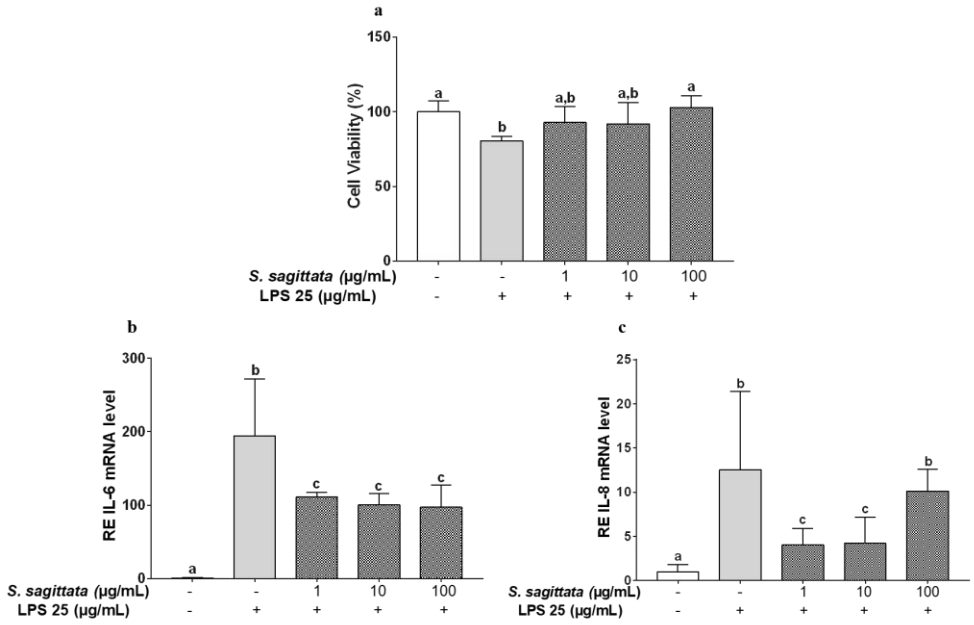


**Figure 2.** Effects of SSEE on LPS-induced pAECs damage. a) Effect of SSEE on LPS-induced cytotoxicity. Each bar represents mean ± S.D. Effect of SSEE on b) IL-6 and c) IL-8 mRNA expression. Relative expression (RE) was calculated as fold of change in respect to the control cells and error bars represent the range of relative gene expression. Data shown are representative of at least three

independent experiments. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test).

This is in agreement with other *in vivo* and *in vitro* reports on other species of *Salvia* genus. Yue et al. [48] have reported that *S. miltiorrhiza* induced a reduction of cytokine expression, thus alleviating liver inflammation. In a similar way, Gao et al. [49] have reported the reduction of nitric oxide, tumor necrosis factor (TNF-α) and IL-6 secretion in RAW264.7 macrophages by a novel compound isolated from S. *miltiorrhiza* in a LPS inflammatory model.

### *4.3 Effect of SSEE on HO-1 expression*

In an inflammatory process, to avoid endothelial dysfunction, there is a tight balance between inflammatory and protective molecules. Recent findings indicated that Heme oxygenase-1 (HO-1), initially studied for its ability to degrade heme, is a key regulator molecule of endothelial cell function providing an important cellular defense mechanism against tissue injury [50, 51]. Moreover, during chronic inflammation, HO-1 performs a double function inhibiting leukocyte infiltration and promoting VEGF-driven non-inflammatory angiogenesis that facilitates tissue repair [52]. For this reason, HO-1 gene expression and protein level were evaluated by RT-PCR and Western-blot, respectively. Compared to control, SSEE treatment at the highest tested level increased HO-1 gene expression (Figure 3a); as concerns protein level, a clearer dose-dependent response was observed (Figure 3b). Although LPS itself did induce an increase in HO-1 levels [27], upon SSEE treatment the protein level was even higher, and this suggest a possible correlation with its antinflammatory properties towards interleukins.

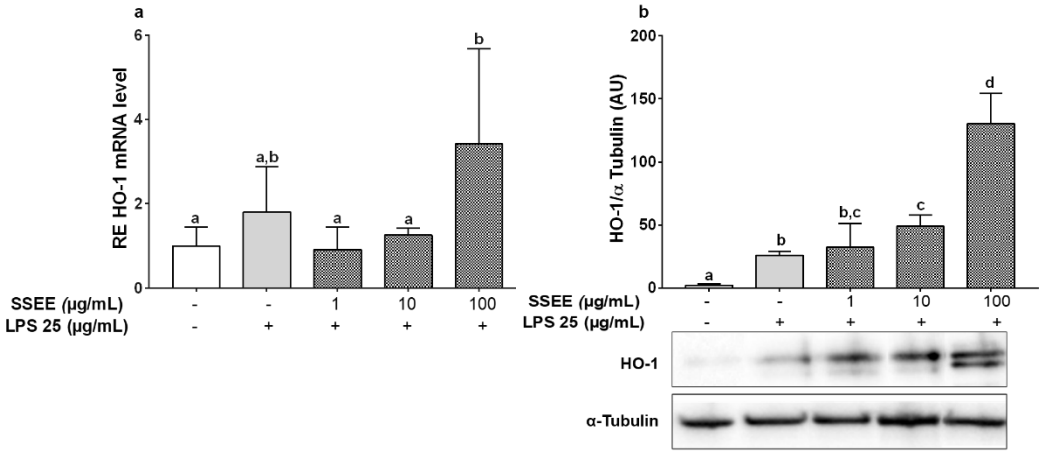


**Figure 3.** Effects of *SSEE* on HO-1 expression in LPS-induced pAECs damage. a) Expression of HO-1 mRNA relative expression was calculated as fold of change in respect to the control cells and error bar

represents the range of relative expression. b) Representative Western Blot of HO-1 and relative housekeeping α-tubulin. Data shown are representative of three replicates in at least three independent experiments. Each bar represents mean ± S.D. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test).

### *4.4 Phytochemical investigation of SSEE*

Phytochemical investigation of the polyphenolic composition was done through both spectrophotometric assays and HPLC-DAD (Tab. 2). TPC and TFC suggest that SSEE was rich in polyphenols, and flavonoids represented more than 65% of polyphenol structures. TPC through the Folin-Ciocalteau procedure was investigated in a wide array of medicinal plants, and values ranging from 9 to 183 mg GAE/g DW in plants belonging to different botanical families were reported. Considering the slight differences in the extract preparation and the different species investigated by these authors, the TPC content of *Salvia sagittata* extract (10.19 mg GAE/g DW) turned out to be very close to that reported by Kähkönen et al. [53] for *Thymus vulgaris* methanolic extract (9 mg GAE/g DW).

HPLC-DAD analysis showed that the major phenolic acid-derivative compound in the extract was rosmarinic acid (RA), reaching about 50% of TPC. Chlorogenic acid (CHA), cinnamic acid (CA) and syringic acid (SA) were also found, although at much lower levels (Tab. 2), together with hesperetin and quercetin-3-*O*-glucoside, which were minor components. These results are in agreement with a recent phytochemical screening carried out on three plant extracts belonging to *Lamiaceae* family by Cocan et al. [54], which demonstrates that rosmarinic acid represents the major phenolic acid compound of an ethanolic extract from *Salvia officinalis* (L.) leaves.

## 5. Conclusions

To our knowledge, this is the first phytochemical and study, of the *in vitro* anti-inflammatory and antioxidant activities of the ethanolic extract obtained from leaves of *Salvia sagittata.* Considering that rosmarinic acid, which is known to show anti-inflammatory and anti-angiogenic activities [55, 56], is the main compound in SSEE, our data suggest that probably Rosmarinic acid is responsible of the majority of the reported protective effects and justify its use in traditional medicine.

**Disclosure**

Preliminary data has been presented as an oral presentation at the I Euroindoamerican Congress, 2018, Madrid, Spain, 29 May–1 June 2018.

**Conflict of interest**

The authors declare no conflict of interest.

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**References**

[1] WHO, "WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues," 2007.

[2] A. Shakeri, A. Sahebkar, and B. Javadi, "Melissa officinalis L. - A review of its traditional uses, phytochemistry and pharmacology," *J. Ethnopharmacol.*, vol. 188, pp. 204–228, 2016.

[3] C. Gyllenhaal, M.R. Kadushin, B. Southavong,.*et al.*, "Ethnobotanical approach versus random approach in the search for new bioactive compounds: Support of a hypothesis," *Pharm. Biol.*, vol. 50, no. 1, pp. 30–41, 2012.

[4] G. Harling, "Current Scandinavian Botanical Research in Ecuador," *Rep. Bot. Inst. Univ. Aarhus*, vol. 15, pp. 9–10, 1986.

[5] C. Ulloa Ulloa and D. Neill, *Cinco años de adiciones a la flora del Ecuador 1999-2004*, Editorial UTPL. Loja, 2005.

[6] P. Jørgensen and S. León-Yánez, *Catalogue of the Vascular Plants of Ecuador*, Missouri Botanical Garden Press. USA, 1999.

[7] P. Naranjo and R. Escaleras, *La Medicina Tradicional en el Ecuador*, Corporacio. Quito, 1995.

[8] J. B. Walker, K. J. Sytsma, J. Treutlein, and M. Wink, "Salvia (Lamiaceae) is not monophyletic: Implications for the systematics, radiation, and ecological specializations of Salvia and tribe Mentheae," *Am. J. Bot.*, vol. 91, no. 7, pp. 1115–1125, 2004.

[9] V. Cardile, A. Russo, C. Formisano, *et al.*, "Essential oils of Salvia bracteata and Salvia rubifolia from Lebanon: Chemical composition, antimicrobial activity and inhibitory effect on human melanoma cells," *J. Ethnopharmacol.*, vol. 126, no. 2, pp. 265–272, 2009.

[10] G. Tel, M. Öztürk, M. E. Duru, M. Harmandar, and G. Topçu, "Chemical composition of the essential oil and hexane extract of Salvia chionantha and their antioxidant and anticholinesterase activities," *Food Chem. Toxicol.*, vol. 48, no. 11, pp. 3189–3193, 2010.

[11] L. Liu, W. Chen, and Y. Li, "Ethnopharmacology, phytochemistry, and pharmacology of Chinese Salvia species: A review," *J. Memb. Sci.*, 2018.

[12] Y. B. Wu, Z.Y. Ni,Q.W. Shi, *et al.*, "Constituents from Salvia species and their biological activities," *Chem. Rev.*, vol. 112, no. 11, pp. 5967–6026, 2012.

[13] H. De-la-Cruz, G. Vilcapoma, and P. A. Zevallos, "Ethnobotanical study of medicinal plants used by the Andean people of Canta, Lima, Peru," *J. Ethnopharmacol.*, vol. 111, no. 2, pp. 284–294, 2007.

[14] E. C. Fernandez, Y. E. Sandi, and L. Kokoska, "Ethnobotanical inventory of medicinal plants used in the Bustillo Province of the Potosi Department, Bolivia," *Fitoterapia*, vol. 74, no. 4, pp. 407–

416, 2003.

[15] M. Gonzales De La Cruz, S. Baldeón Malpartida, H. Beltrán Santiago, V. Jullian, and G. Bourdy, "Hot and cold: Medicinal plant uses in Quechua speaking communities in the high Andes (Callejón de Huaylas, Ancash, Perú)," *J. Ethnopharmacol.*, vol. 155, no. 2, pp. 1093–1117, 2014.

[16] J. S. Pober and W. C. Sessa, "Evolving functions of endothelial cells in inflammation," *Nat. Rev. Immunol.*, vol. 7, no. 10, pp. 803–815, 2007.

[17] L. T. Roumenina, J. Rayes, M. Frimat, and V. Fremeaux-Bacchi, "Endothelial cells: source, barrier, and target of defensive mediators," *Immunol. Rev.*, vol. 274, no. 1, pp. 307–329, 2016.

[18] A. Kadl and N. Leitinger, "The Role of Endothelial Cells in the Resolution of Acute Inflammation," *Antioxid. Redox Signal.*, vol. 7, no. 11–12, pp. 1744–1754, 2005.

[19] C. Pan, C. Kumar, S. Bohl, U. Klingmueller, and M. Mann, "Comparative Proteomic Phenotyping of Cell Lines and Primary Cells to Assess Preservation of Cell Type-specific Functions," *Mol. Cell. Proteomics*, vol. 8, no. 3, pp. 443–450, 2009.

[20] C. S. Alge, S. M. Hauck, S. G. Priglinger, A. Kampik, and M. Ueffing, "Differential protein profiling of primary versus immortalized human RPE cells identifies expression patterns associated with cytoskeletal remodeling and cell survival," *J. Proteome Res.*, vol. 5, no. 4, pp. 862–878, 2006.

[21] C. Bernardini, F. Greco, A. Zannoni, M. L. Bacci, E. Seren, and M. Forni, "Differential expression of nitric oxide synthases in porcine aortic endothelial cells during LPS-induced apoptosis," *J. Inflamm.*, vol. 9, no. 1, p. 47, 2012.

[22] C. Bernardini, A. Zannoni, M. L. Bacci, and M. Forni, "Protective effect of carbon monoxide pre-conditioning on LPS-induced endothelial cell stress.," *Cell Stress Chaperones*, vol. 15, no. 2, pp. 219–24, 2010.

[23] G. Botelho, C. Bernardini, A. Zannoni, V. Ventrella, M. L. Bacci, and M. Forni, "Effect of tributyltin on mammalian endothelial cell integrity," *Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol.*, vol. 176–177, pp. 79–86, 2015.

[24] H. Niemann and W. A. Kues, "Application of transgenesis in livestock for agriculture and biomedicine," *Anim. Reprod. Sci.*, vol. 79, no. 3–4, pp. 291–317, 2003.

[25] A. M. de Almeida and E. Bendixen, "Pig proteomics: A review of a species in the crossroad between biomedical and food sciences," *J. Proteomics*, vol. 75, no. 14, pp. 4296–4314, 2012.

[26] A. L. Archibald *et al.*, "Pig genome sequence - analysis and publication strategy," *BMC Genomics*, vol. 11, no. 1, pp. 3–7, 2010.

[27] C. Bernardini, A. Zannoni, M.E. Turba,*et al.*, "Heat shock protein 70, heat shock protein 32, and vascular endothelial growth factor production and their effects on lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells.," *Cell Stress Chaperones*, vol. 10, no. 4, pp. 340–8, 2005.

[28] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time

quantitative PCR and the 2-ΔΔCT method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[29] B. Ou, M. Hampsch-Woodill, and R. L. Prior, "Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe," *J. Agric. Food Chem.*, vol. 49, no. 10, pp. 4619–4626, 2001.

[30] Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a Free Radical Method to Evaluate Antioxidant Activity," *Food Sci. Technol.*, vol. 28, pp. 25–30, 1995.

[31] V. L. Singleton and J. A. Rossi, "Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents," *Am. J. Enol. Vitic.*, vol. 16, no. 3, p. 144 LP-158, Jan. 1965.

[32] J. Zhishen, T. Mengcheng, and W. Jianming, "The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals," *Food Chem.*, vol. 64, no. 4, pp. 555–559, Mar. 1999.

[33] P. Mattila and J. Kumpulainen, "Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection," *J. Agric. Food Chem.*, vol. 50, no. 13, pp. 3660–3667, 2002.

[34] A. Wojdyło, P. Nowicka, P. Laskowski, and J. Oszmiański, "Evaluation of sour cherry (Prunus cerasus L.) fruits for their polyphenol content, antioxidant properties, and nutritional components," *J. Agric. Food Chem.*, vol. 62, no. 51, pp. 12332–12345, 2014.

[35] L. N. Gao, K. Yan, Y. L. Cui, G. W. Fan, and Y. F. Wang, "Protective effect of Salvia miltiorrhiza and Carthamus tinctorius extract against lipopolysaccharide-induced liver injury," *World J. Gastroenterol.*, vol. 21, no. 30, pp. 9079–9092, 2015.

[36] F. Shaerzadeh *et al.*, "Antioxidant and antiglycating activities of Salvia sahendica and its protective effect against oxidative stress in neuron-like PC12 cells," *J. Nat. Med.*, vol. 65, no. 3–4, pp. 455–465, 2011.

[37] [38] C. Bernardini, P. Gaibani, A. Zannoni *et al.*, "Treponema denticola alters cell vitality and induces HO-1 and Hsp70 expression in porcine aortic endothelial cells," *Cell Stress Chaperones*, vol. 15, no. 5, pp. 509–516, 2010.

[39] A. Zannoni, M, Giunti, C. Bernardini *et al.*, "Procalcitonin gene expression after LPS stimulation in the porcine animal model," *Res. Vet. Sci.*, vol. 93, no. 2, pp. 921–927, Oct. 2012.

[40] J. C. Duvigneau, R. T. Hartl, S. Groiss, and M. Gemeiner, "Quantitative simultaneous multiplex real-time PCR for the detection of porcine cytokines," *J. Immunol. Methods*, vol. 306, no. 1–2, pp. 16–27, Nov. 2005.

[41] Singh, and I. Jialal, "Oxidative stress and atherosclerosis," Pathophysiology, vol. 13, no. 3, pp. 129–142, 2006.

[42] P. Stratil, B. Klejdus, and V. Kubáň, "Determination of total content of phenolic compounds and their antioxidant activity in vegetables - Evaluation of spectrophotometric methods," *J. Agric. Food Chem.*, vol. 54, no. 3, pp. 607–616, 2006.

[43] D. G. Remick, "Interleukin-8," *Crit. Care Med.*, vol. 33, no. 12 SUPPL., pp. 466–467, 2005.

[44] J. Scheller, A. Chalaris, D. Schmidt-Arras, and S. Rose-John, "The pro- and anti-inflammatory properties of the cytokine interleukin-6," *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1813, no. 5, pp. 878–888, 2011.

[45] S. A. Jones, "Directing Transition from Innate to Acquired Immunity: Defining a Role for IL-6," *J. Immunol.*, vol. 175, no. 6, pp. 3463–3468, 2005.

[46] J. Scheller, C. Garbers, and S. Rose-John, "Interleukin-6: From basic biology to selective blockade of pro-inflammatory activities," *Semin. Immunol.*, vol. 26, no. 1, pp. 2–12, 2014.

[47] H. Laursen *et al.*, "Immunohistochemical detection of interleukin-8 in inflamed porcine tissues," *Vet. Immunol. Immunopathol.*, vol. 159, no. 1–2, pp. 97–102, 2014.

[48] S. Yue, B. Hu, Z. Wang, *et al.*, "Salvia miltiorrhiza compounds protect the liver from acute injury by regulation of p38 and NFκB signaling in Kupffer cells," *Pharm. Biol.*, vol. 52, no. 10, pp. 1278–1285, 2014.

[49] H. Gao *et al.*, "Tanshinones and diethyl blechnics with anti-inflammatory and anti-cancer activities from Salvia miltiorrhiza Bunge (Danshen)," *Sci. Rep.*, vol. 6, no. April, pp. 1–13, 2016.

[50] C. F. Chang, X. M. Liu, K. J. Peyton, and W. Durante, "Heme oxygenase-1 counteracts contrast media-induced endothelial cell dysfunction," *Biochem. Pharmacol.*, vol. 87, no. 2, pp. 303–311, 2014.

[51] G. Li Volti, J. Wang, F. Traganos, A. Kappas, and N. G. Abraham, "Differential effect of heme oxygenase-1 in endothelial and smooth muscle cell cycle progression," *Biochem. Biophys. Res. Commun.*, vol. 296, no. 5, pp. 1077–1082, 2002.

[52] D. Calay and J. C. Mason, "The Multifunctional Role and Therapeutic Potential of HO-1 in the Vascular Endothelium," *Antioxid. Redox Signal.*, vol. 20, no. 11, pp. 1789–1809, 2014.

[53] M.P. Kähkönen., A.I. Hopia, H.J. Vuorela *et al.*, "Antioxidant Activity of Plant Extracts Containing Phenolic Compounds," *J. Agric. Food Chem*. no. 47, pp. 3954-3962, 1999.

[54] I. Cocan, E Alexa, C. Danciu, *et al.*, "Phytochemical screening and biological activity of lamiaceae family plant extracts," *Exp. Ther. Med.*, vol. 15, no. 2, pp. 1863–1870, 2018.

[55] W. Cao, C. Hu, L. Wu, L. Xu, and W. Jiang, "Rosmarinic acid inhibits inflammation and angiogenesis of hepatocellular carcinoma by suppression of NF-kB signaling in H22 tumor-bearing mice," *J. Pharmacol. Sci.*, vol. 132, no. 2, pp. 131–137, 2016.

[56] Z. Liang *et al.*, "Rosmarinic acid attenuates airway inflammation and hyperresponsiveness in a murine model of asthma," *Molecules*, vol. 21, no. 6, 2016.

# 4.5 *Clinopodium tomentosum*

Manuscript in preparation

## 1. INTRODUCTION

Medicinal plants are presently in demand and their acceptance is increasing progressively, probably because according the World Health Organization (WHO) approximately 80% of the world's population use them as a primary source of healthcare (Jamshidi-Kia et al. 2017; Fabricant & Farnsworth 2001). In Ecuador, considered a megadiverse country for its high number of vegetal and animal species, have so far been recorded an estimated of 17.000 plant species of which more than 3000 are medicinal plants with promising potential and are widely used in several healing cultures (Harling, 1986., Jørgensen et al., 2006, Ulloa Ulloa and Neill., 2005). Nevertheless, in the most part of the cases, medicinal plants remain untested and their use also not monitored whereby information about dose, route of administration and potential adverse effects are limited and identification of the safest and most effective therapies as well as the promotion of their rational use it is nonexistent (Naranjo and Escaleras, 1995).

Lamiaceae family own popular aromatic plants growing in many regions of the world. Most of them has been studied for their biological and medical applications (Uritu et al. 2018; Vladimir-Knezevic et al. 2014). In Ecuador, lamiaceae family present more than 50 medicinal species that are used to treat different affections (De La Torre et al., 2008). Clinopodium genus is widely distributed in southern and southeastern Europe, Asia and south and north America (Burk et al., 2009; Estrada-Reyes et al., 2010) whereby many species of the genus are used as medicinal plants. Previous phytochemical studies on Clinopodium genus have revealed the presence of several compounds including flavonoids (Estrada-Reyes et al., 2010), phenylpropanoids (Zeng et al. 2016), diterpenes and triterpenoid saponins (Zhu et al. 2017), as well as volatile and fatty oils, which exhibit diverse biological activities (Cassani et al., 2013; Chen et al., 2015; Sarikurkcu et al., 2015). Ecuador possess 7 types of Clinopodium distributed in the central region used, not only, for their medicinal properties. Clinopodium tomentosum (Kunth) Govaerts, commonly known as "Pumin", is a sub-shrub with small orange-yellow colored flowers, that grows between 2000 and 4000 m a.s.l. In the traditional medicine, local people use the aerial parts of the plant to treat respiratory affections, inflammation and gastrointestinal disorders (De La Torre et al., 2008). Despite the traditional uses of the plant, at our knowledge, only the phytochemical composition of the essential oil (Benzo et al. 2007) and the phenolic compounds (Saltos et al., 2014) have been reported.

Vascular endothelium is the largest organ in the body that is indispensable for the regulation and maintenance of the homeostasis of the whole organism (Hadi et al. 2005). Different kind of cells forming vascular tone, among them, endothelial cells (EC) constitute a barrier between the blood and the interstitium that control vascular permeability, adhesion molecule expression, platelet activation, fibrinolysis, inflammation, angiogenesis, and thromboresistance (Deanfield et al. 2007; Roumenina et al. 2016). Since endothelial cells perform multiple functions and the increasing number of publications that report the use of primary cultures in biological research, Bernardini et al., 2005 have isolated and characterized primary culture of porcine aortic endothelial cells which have been used to evaluate the effects of different external agents in the endothelium (Botelho et al. 2015; Bernardini et al. 2018; Bertocchi et al. 2018).

Angiogenesis is the process of forming new vessels from pre-existing vasculature and is tightly controlled by a balance of pro-angiogenic and inhibitor factors (Fan et al., 2006). Different cellular types (endothelial cells, fibroblasts, macrophages) and the surrounding extracellular matrix interplays a fundamental role in this complex process (Tonnesen et al., 2000). The physiological angiogenesis is essential for normal development and wound-healing/reproductive function, nevertheless its abnormal regulation has been implicated in the pathogenesis of several diseases (Carmeliet et al, 2000, 2003). Insufficient angiogenesis caused by the inadequate production of angiogenesis growth factors and/or excessive amounts, and not only, of angiogenesis inhibitors could produce different coronary artery diseases (Libby & Theroux 2005). Excessive angiogenesis is critical for the initiation, growth, and metastasis of different tumors (Huang & Bao 2004).

Despite, the traditional use of *Clinopodium tomentosum*, no studies regarding the biological effects *in vivo* or *in vitro* have been reported. Thus, the present research was performed with the purpose to evaluate the effects of an ethanolic extract of *Clinopodium tomentosum* on vascular endothelium using porcine aortic endothelial cells. Additionally, the phytochemical analysis and its antioxidant activity were reported.

## 2. MATERIALS AND METHODS

### *2.1 Chemicals and reagents*

Human Endothelial Serum Free Medium (hESFM), heat inactivated fetal bovine serum (FBS), antibiotic-antimycotic and Dulbecco's phosphate buffered saline (DPBS) phosphate buffered saline (PBS) were purchased from Gibco-Life technologies (Carlsbad CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemicals Co., St. Louis, Mo. Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl

(DPPH), 6-hydroxyl-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), gallic acid, rutin, phenolic acids (4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, p-coumaric acid, synapic acid, syringic acid, trans-cinnamic acid, rosmarinic acid, quercetin, quercetin-3-O-glucoside, quercetin-3-O-ramnoside, quercetin-3-O-galactoside, kaempferol, kaempferol-3-O-rutinoside, hesperetin, hesperidin) pure standards (>99.5% purity) in powder form, and HPLC-grade solvents were purchased from Sigma-Aldrich Italia (Milan, Italy). All standards were prepared as stock solutions at 1 mg/mL in methanol and stored in the dark at -18 °C for less than three months.

### *2.2 Preparation of plant extract*

*Clinopodium tomentosum* (Kunth) Govaerts (CT) plants were collected, according to previous authorization of Ministry of the Environment (Nro. 003-IC-DPACH-MAE-2018-F), in Riobamba Ecuador on May 2016. The plants were identified and certified by Escuela Superior Politecnica de Chimborazo Herbarium, Riobamba, Ecuador and a voucher specimen was deposited (N. 702). Dried leaves (300 g) were ground and extracted with 96% ethanol for 48 h at room temperature. After filtration, the solvent was evaporated using a rotary vacuum evaporator (Büchi Ch-9230 Flawil, Switzerland) and dried in vacuum at 40 °C to obtain EtOH extract with a yield of 6.18%. For experiments, the dry extract was dissolved in ethanol. The stock solution (20 mg/mL) was further used for HPLC analysis or diluted in culture media and membrane filtered by a 0.2 µm Millipore filter (Millipore, Darmstadt, Germany).

### *2.3 Total Phenol content and Total Flavonoid content*

Total Phenol content (TPC) was determined using the Folin–Ciocalteu method (Singleton and Rossi, 1965). 50 µL of diluted extract were mixed with 250 µL of a ten-fold-diluted Folin–Ciocalteu phenol reagent. After 1 min, 800 µL of 30% sodium carbonate solution were added to the mixture, shaken thoroughly and diluted to 1.6 ml by adding 500 µL of distilled water. The mixture was allowed to stand for 40 min at r.t. and the blue color formed is measured at 700 nm using a UV-VIS spectrophotometer (V-630 Jasco, Jasco Europe S.r.l., Cremella, Italy). A calibration curve of gallic acid (ranging from 5 to 500 µg/ml) was prepared and the results, determined from regression equation of the calibration curve, were expressed as mg Gallic Acid Equivalents (GAE) per g. of extract.

Total Flavonoid content (TFC) was determined according to Zhishen et al. (1999) with some modifications. 500 µL of extract were placed in a 10 mL volumetric flask, and diluted to 5 mL

with distilled water. 300 µL of 5% $NaNO_2$ were added and the mixture was mixed well. After 5 min, 3 mL of a 10% AlCl3 solution were added. After 6 min, 2 mL of 1 M NaOH solution were added, and the total volume was made up to 10 mL with distilled water. The mixture was mixed well again, and the absorbance was measured against a blank at 510 nm with a UV-visible spectrophotometer. Rutin was used as the standard for the calibration curve. The flavonoid content was calculated using the regression equation based on the calibration curve, and results are expressed as mmol Rutin Equivalents (RE) /g. extract.

### *2.4 HPLC determination of phenolic acids and flavonoids*

20 µL of ethanolic extract were injected into the HPLC system (Jasco, Tokyo, Japan; PU-4180 pump, MD-4015 PDA detector, AS-4050 autosampler). The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm x 3 mm I.D., 3.5 µm). The chromatographic method for the analysis of phenolic acids was adapted from Mattila and Kumpulainen, (2002). Gradient elution was carried out with a mixture of acidic phosphate buffer (50 mM, pH 2.5) and acetonitrile flowing at 0.7 mL/min. The signals at 254, 280 and 329 nm were used for analyte quantitation. The recovery values of phenolic acids in spiked samples ranged from 78.8 to 92.2% (RSD < 9.8%, n = 6). The chromatographic method for the analysis of flavonoids was adapted from Wojdyło et al., (2014). Gradient elution was carried out with a mixture of 4.5% formic acid and acetonitrile. The runs were monitored at the following wavelengths: flavan-3-ols at 280 nm, flavonol glycosides at 360 nm. PDA spectra were measured over the wavelength range of 200−600 nm in steps of 2 nm. Retention times and spectra were compared with those of pure standards. Calibration curves were constructed for all standards at concentrations ranging from 1.0 to 100.0 ppm ($r^2 \leq 0.9998$). Results are expressed as mg/g extract.

### *2.5 Antioxidant activity assays*

Antioxidant activity (AA) of the extract was measured by the ORAC and DPPH assays. The ORAC assay was performed in an automated plate reader (Victor 3, Perkin Elmer) with 96-well plates, according to Ou et al., (2001) with some modifications. All reagents were freshly prepared before the assay. In each well, 210 µl of Fuoresceine (10 nM) and 35 µl of sample, blank (10 mM phosphate buffer, pH=7.4), or standard (Trolox in the range 1-50 µM) were placed. The plate was heated to 37 °C for 10 min, and then 35 µl of AAPH (240 mM) were added, immediately before measuring the fluorescence.

Measurements were then taken every 1.5 min until the relative fluorescence intensity (FI %) was less than 5% of the value of the initial reading. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as mmole Trolox equivalents per g. of extract.

The DPPH assay was done according to the method of Brand-Williams et al., (1995) with some modifications. A stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored at -20°C until needed. The working solution was prepared by mixing 10 mL stock solution with 45mL methanol to obtain an Abs of 1.1 ±0.02 units at 515 nm using an UV-VIS spectrophotometer. 150 µL of ethanolic extract were allowed to react with 2850 µL of the DPPH solution for 24 h in the dark. The absorbance was then taken at 515 nm. Results were determined from the regression equation of the calibration curve of Trolox in the range 25-500 µM, and expressed as mmol TE/g extract.

### *2.6 Cell culture and treatments*

Porcine aortic endothelial cells (pAECs) were isolated and maintained as previously described by Bernardini et al., 2005. For the experiments, cells from the third to the tenth passage were used. Cells were seeded and routinely cultured in T25 tissue culture flasks ($4x10^5$ cells/flask) (T 25-Falcon, Beckton-Dickinson, Franklin Lakes, NJ, USA). Successive experiments were conducted in 96-well plates (cell viability and anti-inflammatory test), 24-well plates (wounding migration assay) and 8-slide chambers (tube formation assay) (Beckton-Dickinson) at confluent cultures. Cells were cultured in hESFM, added with 5%FBS and 1x antimicrobial/antimycotic solution in a 5% $CO_2$ atmosphere at 38.5°C.

The dry extract was dissolved in ethanol (98%) to obtain a stock solution (20 mg/mL) and then in culture medium to obtain desired concentrations (1 – 200 µg/mL) for cell exposure. Ethanol (1%) was used as control group.

### *2.7 Cell viability*

Viability was determined using MTT assay. Briefly, pAECs were seeded in 96-well culture plates at a density of $3 \times 10^3$ cells/well and incubated for 24 h. Then, media were replaced with hESFM containing 5% FBS and increasing CTEE doses (1, 10, 50, 100 and 200 µg/mL), and incubated for another 24 hours at 38.5°C. Next, MTT solution (5 mg/mL in PBS) was added to a final concentration of 0.5 mg/mL and then incubated for 2 hours at 38.5°C, followed by the addition of 0.1 mL MTT solubilisation solution. The formazan Abs was determined at 570 nm,

using Infinite® F50/Robotic absorbance microplate readers from TECAN Life Sciences (Männedorf, Switzerland).

### *2.8 In vitro wounding migration assay*

pAECs (approximately $4x10^4$ cells/well) were seeded in 24-well culture plate and incubated at 38,5°C and 5% CO2 until confluence. Then, cells were scratched with a 200 µL pipette tip along the diameter of the well and medium was aspirated and washed twice with PBS, to remove detached cell, and incubated in hESFM with 1% FBS (to minimize cells proliferation) and increasing doses (1, 10, 25, 50 and 100 µg/mL) of CTEE. These culture conditions minimized pAECs proliferation. Wound diameters were photographed at 24 hours. Wound closure was determined with optical microscopy at 40× magnification. Images were acquired using a Nikon epifluorescence microscope equipped with digital camera (Nikon, Yokohama, Japan)

### *2.9 In vitro tube formation assay*

The experiments were carried out using 8 slide chambers (BD Falcon Bedford, MA USA) coated with undiluted Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix. Firstly, extracellular matrix coating was carried out in 8 wells glass slide 1 hour before the seeding in a humidified incubator, at 38.5°C, 5% $CO_2$. Then pAECs ($8x10^4$ cells/well) were seeding with increasing doses (1, 10, 25, 50 and 100 µg/mL) of CTEE for 18 h. At the end of experimental time, images were acquired using a digital camera installed on a Nikon contrast phase microscope (Nikon, Yokohama, Japan) and analyzed by open software Image J 64.

### *2.10 Cell viability after LPS injury*

Briefly, pAECs were seeded in 96-well culture plates at a density of $12×10^3$ cells/well and incubated for 24 h. Then exposed to different concentrations of CTEE (25, 50 and 100 µg/mL) in presence of LPS (25 µg/mL) and incubated for another 24 hours at 38.5°C. Cell viability was evaluated by MTT assay.

## 3. STATISTICAL ANALYSIS

Each treatment was replicated three times or eight times (viability and anti-inflammatory test) in three independent experiments. The data were analysed by a one-way analysis of variance (ANOVA) followed by the *post hoc* Tuckey comparison Test. Differences of at least $p < 0.05$ were considered significant. Statistical analysis was carried out using GraphPad Prism 7 software.

## 4. RESULTS

### *4.1 Phytochemical analysis*

The yield of the extract was 5.35% (w/w). As shown in Table 1, the phytochemical analysis of CTEE revealed the presence of polyphenol (140.15±0.12 mg GAE/g) and flavonoids (97.38±0.62 mg RE/g) as active constituents of the extract. HPLC-DAD analysis showed high quantity of phenolic compounds such as rosmarinic acid (37.03±0.58 mg/g), chlorogenic acid (1.66±0.35 mg/g) and cynnamic acid (0.653±0.03 mg/g) among others, as well as, flavonoids such as hesperidin (16.76±0.89 mg/g), hesperetin (4.14±0.14 mg/g), kaempferol (3.97±0.25 mg/g) and rutin (1.95±0.06 mg/g). Moreover, CTEE showed high antioxidant activity that was measured by DPPH (0.93±0.11 mmol TE/g) and ORAC (4.14±0.24 mmol TE/g) assays.

**Table 1.** Antioxidant Activity (AA), TPC, TFC, phenolic acids and flavonoid content in CTEE. Data are the mean ± S.E. of three technical determinations

| Assays or Compounds | | Ethanolic Plant Extract |
|---|---|---|
| **AA** | **ORAC mmol TE/g** | 4.14±0.24 |
| | **DPPH** mmol TE/g | 0.93±0.11 |
| **TPC mg/g** | | 140.15±0.12 |
| **TFC mg/g** | | 97.38±0.62 |
| **RA mg/g** | | 37.03±0.58 |
| **CHA mg/g** | | 1.66±0.35 |
| **CA mg/g** | | 0.653±0.03 |
| **ESP mg/g** | | 16.76±0.89 |
| **ESPE mg/g** | | 4.14±0.14 |
| **KAM mg/g** | | 3.97±0.25 |
| **RU mg/g** | | 1.95±0.06 |

### *4.2 Effect of CTEE on pAECs viability*

pAECs viability, after 24 hours of treatment with CTEE, were not affected at any concentration tested while a proliferative effect was observed at 100 and 200 µg/mL.

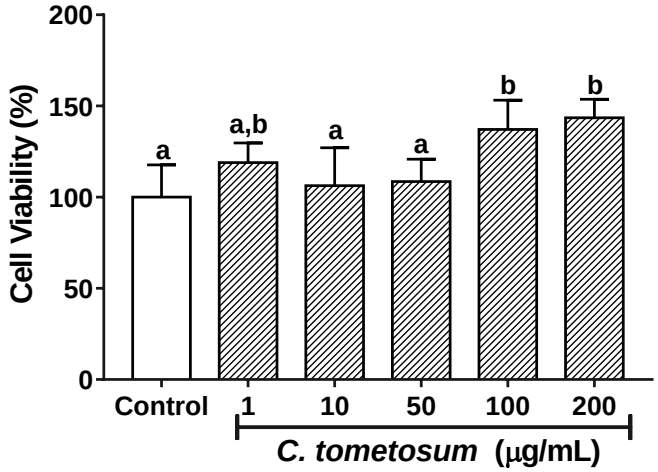


**Figure 1.** Effect of *Clinopodium tomentosum* ethanolic extract on pAECs cell viability. Cells were treated with different concentrations (1, 10, 25, 50, 100 and 200 µg/mL) of CTEE and cell viability was measured by MTT assay. Data shown are representative of 8 independent replicates. Each column represent mean ± S.D. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test)

### *4.3 Effect of CTEE on pAECs wounding capacity and network formation.*

The migration ability of pAECs in a wounded edge was tested by scratch test. Treatment with CTEE markedly increased pAECs migration compared with the control, in fact migration was significantly increased by about 59% at the concentration of 50 µg/mL (Figure 2). Then, pAECs ability to form a capillary network was tested in a Geltrex-based assay. CTEE at 25 and 50 µg/mL showed a significant increase in number of master junction on pAECs compared with the control group (Figure 3).

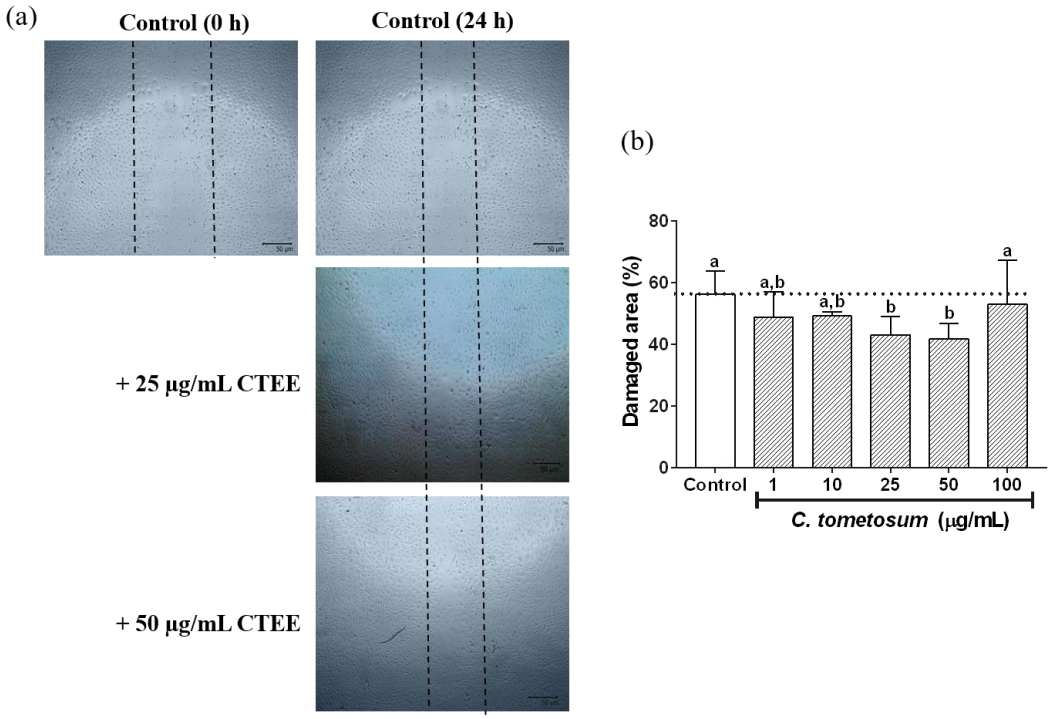


**Figure 2.** Effect of *Clinopodium tomentosum* ethanolic extract on pAEC migration capacity. Migration was observed using a phase-contrast microscope, at a 40× magnification, and the closure area, after treatment with different concentrations of CTEE compared with control group, was calculated. (a) CTEE at 25 and 50 µg/mL reduced the scratched damaged. (b) Data shown are representative of at least three independent experiments after 24h. Each column represent mean ± S.D. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test).

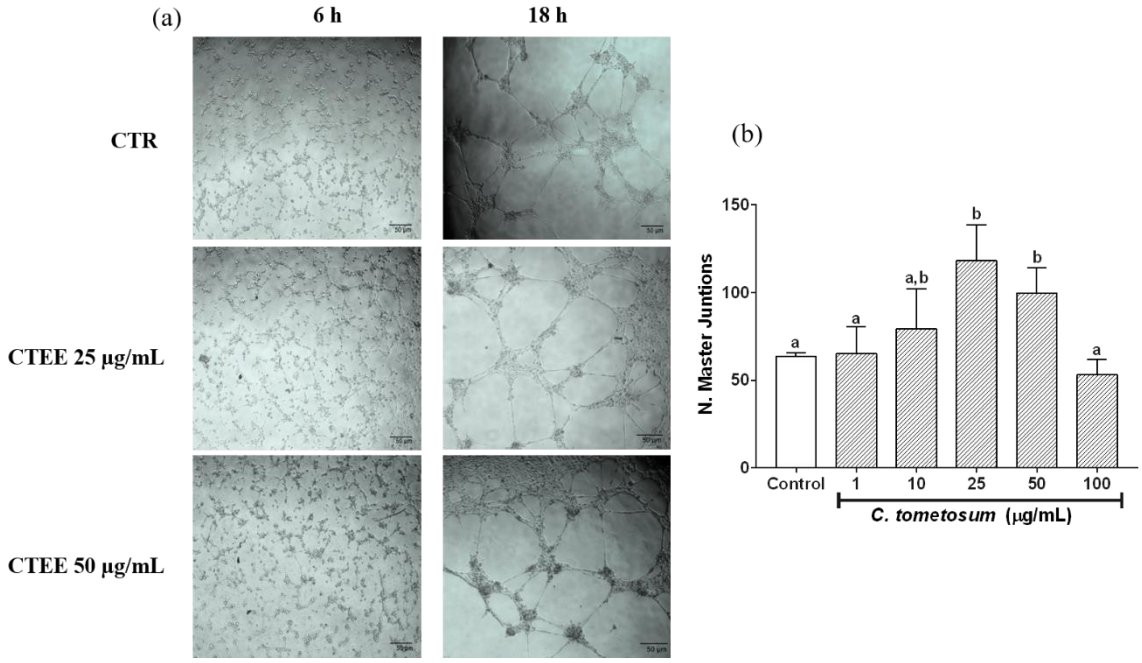

**Figure 3.** Effect of *Clinopodium tomentosum* ethanolic extract on pAECs angiogenesis. pAECs were cultured on a extracellular matrix for 18 h with different concentrations (1, 10, 25, 50, 100 µg/mL) of plant extract. Photographs were recorded at 6 h and 18 h after treatment. (a) CTEE at 25 and 50 µg/mL induce a capillary like network compared with control group. (b). Data shown are representative of at least three independent experiments after 18h. Each column represent mean ± S.D. Different letters above the bars indicate significant differences ($p < 0.02$ ANOVA post hoc Tukey's test)

### *4.4 Effect of CTEE on LPS-induced cell death*

LPS produce an evident cytotoxicity on pAECs, reducing nearly the 25% of their viability. Treatment with CTEE significantly reduced the cytotoxicity induced by LPS restoring to physiological levels at all the concentrations tested (Figure 4).

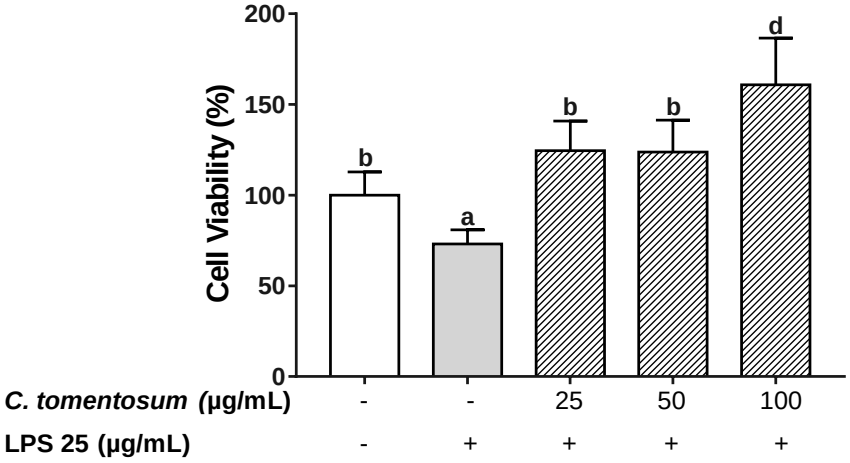


**Figure 4.** Effect of Clinopodium tomentosum ethanolic extract on LPS-induced pAECs damage. Cell viability were measured by MTT assay after treatement with LPS (25 µg/mLa) and different concentrations of CTEE (25, 50, 100 µg/mL). Data shown are representative of 8 independent replicates. Each column represent mean ± S.D. Different letters above the bars indicate significant differences ($p < 0.05$ ANOVA post hoc Tukey's test)

## 5. DISCUSSION

Despite the commonly use of medicinal plants in Ecuador, there is a lack of scientific information that support their uses. Especially in the case of *Clinopodium tomentosum,* in spite of being an endemic plant, few studies have been reported so far. Therefore, our study evaluated the phytochemical composition, antioxidant capacity as well as the biological effects of CTEE on pAECs physiology.

Previously studies have demonstrated that *in vitro* primary culture of porcine aortic endothelial cells (pAECs), could be used as a great model to asses cells physiology, cytotoxicity, angiogenic

and anti-inflammatory activities. (Botelho et al. 2015; Bernardini et al. 2018; Bertocchi et al. 2018).

Ideally to ensure the safety of any natural product or medicinal plants a vitality test on cells should be done. We demonstrated that treatment with CTEE, not only, not influence on pAECs viability but also induce cell proliferation (Fig. 1). In the same line, Burk et al. 2009 reported a similar trend when tested the effects of *Clinopodium vulgare L* in cell viability of RAW264.7 macrophages. Additionally, other authors have confirmed the protective effects *in vivo* and *in vitro* of different *Clinopodium* species (Dzhambazov et al., 2002; Estrada-Reyes et al., 2010; Mohanty et al., 2017).

Endothelial cells perform a pivotal role in the maintenance of the vascular integrity preventing many pathological alterations, including cardiovascular diseases (CDVs) (Roumenina et al. 2016). In addition, endothelial cells migration ability is critical in the physiological and pathological angiogenesis.

Hence the effects of CTEE on pAECs angiogenesis were tested. In our research pAECs migration and tube formation were improved after 24 hours of treatment (Fig. 2). No date regarding the angiogenic activity of CTEE in endothelial cells has been reported until now. Nevertheless, Zeng et al. 2016 has shown that phenolic compounds of *Clinopodium chinensis* exerted a strong protective effect in vascular endothelial cells injury. Moreover, Chen et al. 2015 have demostrated *in vivo* and *in vitro* that total flavonoids from *Clinopodium chinensis* protect against doxorubicin-induced cardiotoxicity by suppressing the phosphorylation of JNK, p38, ERK and overexpression of p53. Likewise, Zhang et al. 2018 following the previosly research has demostrated that pretreatment of total flavonoids from *Clinopodium chinensis* protect against ischemic myocardial injury *in vivo* and *in vitro* by the phosphorylation of AKT via the activation of the Nrf2/HO-1 signaling pathway.

Since CTEE has been used as anti-inflammatory agent in the traditional medicine, we decided to analyze also its effects against LPS-induce inflammation on pAECs by an MTT assay. After 24 hours of treatment, the inflammatory damage produced by LPS was reduced reaching its maximum effect at 100 µg/mL (Fig. 3). Similar results were reported by Burk et al. 2009, in which the aqueous extract of Clinopodium vulgare reduced the LPS-inflammatory effect on 264.7 murine macrophages by suppressing the activation of NF-kB pathway.

Based in the phytochemical composition and the major compounds reported in CTEE, is not possible to confer its biological effects, showed on pAECs, to a single molecule. However different

studies confirm the protective and anti-inflammatory effect of rosmaric acid (Cao et al. 2016; Han et al. 2017; Rocha et al. 2015) as well as the protective role of hespedirin in cardiovascular diseases and its angiogenic effects in diabetic foot ulcer (Roohbakhsh et al. 2015; Wang et al. 2018; Li et al. 2018).

The present study is the first to provide scientific information that support the use *Clinopodium tomentosum* in ecuadorian traditional medicine and our experiments demonstrated the angiogenic and anti-inflammatory effects of CTEE on pAECs, probably the biological properties could be due to its phytochemical compositon and the antioxidant activity. Nevertheless, further research should be performed to elucidate the possible the pathway by which exert its effects.

## 6. Conclusion

To our knowledge, this is the first phytochemical and *in vitro* study the angiogenic, anti-inflammatory and antioxidant activities of the ethanolic extract obtained from leaves of *Clinopodium tomentosum.*

## References


Benzo, M. et al., 2007. Determination of the threshold odor concentration of main odorants in essential oils using gas chromatography-olfactometry incremental dilution technique. *Journal of Chromatography A*, 1150(1–2), pp.131–135.

Bernardini, C. et al., 2005. Heat shock protein 70, heat shock protein 32, and vascular endothelial growth factor production and their effects on lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells. *Cell stress & chaperones*, 10(4), pp.340–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16333987%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1283877.

Bernardini, C. et al., 2018. Water/ethanol extract of Cucumis sativus L. fruit attenuates lipopolysaccharide-induced inflammatory response in endothelial cells. *BMC Complementary and Alternative Medicine*, 18(1), pp.1–9.

Bertocchi, M. et al., 2018. Anti-Inflammatory Activity of *Boswellia serrata* Extracts: An *In Vitro* Study on Porcine Aortic Endothelial Cells. *Oxidative Medicine and Cellular Longevity*, 2018, pp.1–9. Available at: https://www.hindawi.com/journals/omcl/2018/2504305/ [Accessed August 10, 2018].

Botelho, G. et al., 2015. Effect of tributyltin on mammalian endothelial cell integrity. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, 176–177, pp.79–86. Available at: http://dx.doi.org/10.1016/j.cbpc.2015.07.012.

Brand-Williams, Cuvelier, M.E. & Berset, C., 1995. Use of a Free Radical Method to Evaluate Antioxidant Activity. *Food Sci. Technol.*, 28, pp.25–30.

Burk, D.R. et al., 2009. Suppression of lipopolysaccharide-induced inflammatory responses in RAW 264.7 murine macrophages by aqueous extract of Clinopodium vulgare L. (Lamiaceae). *Journal of Ethnopharmacology*, 126(3), pp.397–405.

Cao, W. et al., 2016. Rosmarinic acid inhibits inflammation and angiogenesis of hepatocellular carcinoma by suppression of NF-??B signaling in H22 tumor-bearing mice. *Journal of Pharmacological*

*Sciences*, 132(2), pp.131–137. Available at: http://dx.doi.org/10.1016/j.jphs.2016.09.003.

Carmeliet, P. & Jain, R.K., 2000. Angiogenesis in cancer and other diseases. *Nature*, 407, pp.249–257.

Cassani, J. et al., 2013. Anxiolytic-like and antinociceptive effects of 2(S)-neoponcirin in mice. *Molecules*, 18(7), pp.7584–7599.

Chen, R.C. et al., 2015. Total flavonoids from clinopodium Chinense (Benth.) O. Ktze protect against doxorubicin-induced cardiotoxicity in vitro and in vivo. *Evidence-based Complementary and Alternative Medicine*, 2015.

Deanfield, J.E., Halcox, J.P. & Rabelink, T.J., 2007. Endothelial function and dysfunction: Testing and clinical relevance. *Circulation*, 115(10), pp.1285–1295.

Dzhambazov, B. et al., 2002. In Vitro Screening for Antitumour Activity of Clinopodium vulgare L. (Lamiaceae) Extracts. *Biological & Pharmaceutical Bulletin*, 25(4), pp.499–504. Available at: http://joi.jlc.jst.go.jp/JST.JSTAGE/bpb/25.499?from=CrossRef.

Estrada-Reyes, R. et al., 2010. Depressant effects of Clinopodium mexicanum Benth. Govaerts (Lamiaceae) on the central nervous system. *Journal of Ethnopharmacology*, 130(1), pp.1–8.

Fabricant, D.S. & Farnsworth, N.R., 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives*, 109(SUPPL. 1), pp.69–75.

Fan, T. et al., 2006. Angiogenesis : from plants to blood vessels. , 27(6).

Hadi, H.A.R., Carr, C.S. & Al Suwaidi, J., 2005. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vascular health and risk management*, 1(3), pp.183–98. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1993955&tool=pmcentrez&rendertype=abstract.

Han, J. et al., 2017. Rosmarinic acid protects against inflammation and cardiomyocyte apoptosis during myocardial ischemia/reperfusion injury by activating peroxisome proliferator-activated receptor gamma. *Frontiers in Pharmacology*, 8(JUL), pp.1–11.

Huang, Z. & Bao, S.D., 2004. Roles of main pro- and anti-angiogenic factors in tumor angiogenesis. *World Journal of Gastroenterology*, 10(4), pp.463–470.

Jamshidi-Kia, F., Lorigooini, Z. & Amini-Khoei, H., 2017. Medicinal plants: Past history and future perspective. *Journal of Herbmed Pharmacology*, 7(1), pp.1–7. Available at: http://herbmedpharmacol.com/Abstract/jhp-1198.

De La Torre, L. et al., 2008. *Enciclopedia de las Plantas Útiles del Ecuador*,

Li, W. et al., 2018. Hesperidin, a plant flavonoid accelerated the cutaneous wound healing in streptozotocin-induced diabetic rats: Role of TGF-ß/Smads and Ang-1/Tie-2 signaling pathways. *EXCLI Journal*, 17(Dm), pp.399–419. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5962903/.

Libby, P. & Theroux, P., 2005. Pathophysiology of coronary artery disease. *Circulation*, 111(25), pp.3481–3488.

Mattila, P. & Kumpulainen, J., 2002. Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *Journal of Agricultural and Food Chemistry*, 50(13), pp.3660–3667.

Mohanty, S. et al., 2017. Extract of Clinopodium bolivianum protects against E. coli invasion of uroepithelial cells. *Journal of ethnopharmacology*, 198(October 2016), pp.214–220. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0378874116312284.

Ou, B., Hampsch-Woodill, M. & Prior, R.L., 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*, 49(10), pp.4619–4626.

Rocha, J. et al., 2015. Anti-inflammatory Effect of Rosmarinic Acid and an Extract of *Rosmarinus officinalis* in Rat Models of Local and Systemic Inflammation. *Basic & Clinical Pharmacology & Toxicology*, 116(5), pp.398–413. Available at: http://doi.wiley.com/10.1111/bcpt.12335 [Accessed August 21, 2018].

Roohbakhsh, A. et al., 2015. Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases. *Life Sciences*, 124, pp.64–74.

Roumenina, L.T. et al., 2016. Endothelial cells: source, barrier, and target of defensive mediators. *Immunological Reviews*, 274(1), pp.307–329.

Saltos, M.B.V. et al., 2014. Phenolic Compounds from *Clinopodium tomentosum* (Kunth) Govaerts (Lamiaceae). *Journal of the Brazilian Chemical Society*, 25(11), pp.2121–2124. Available at: http://www.gnresearch.org/doi/10.5935/0103-5053.20140181 [Accessed August 10, 2018].

Sarikurkcu, C. et al., 2015. Phenolic composition, antioxidant and enzyme inhibitory activities of acetone, methanol and water extracts of Clinopodium vulgare L. subsp. vulgare L. *Industrial Crops and Products*, 76, pp.961–966. Available at: http://dx.doi.org/10.1016/j.indcrop.2015.08.011.

Tonnesen, M.G., Feng, X. & Clark, R.A.F., 2000. Angiogenesis in wound healing. *Journal of Investigative Dermatology Symposium Proceedings*, 5(1), pp.40–46. Available at: http://dx.doi.org/10.1046/j.1087-0024.2000.00014.x.

Uritu, C.M. et al., 2018. Medicinal plants of the family Lamiaceae in pain therapy: A review. *Pain Research and Management*, 2018.

Vladimir-Knezevic, S. et al., 2014. Acetylcholinesterase inhibitory, antioxidant and phytochemical properties of selected medicinal plants of the lamiaceae family. *Molecules*, 19(1), pp.767–782.

Wang, L. et al., 2018. Hesperidin enhances angiogenesis via modulating expression of growth and inflammatory factor in diabetic foot ulcer in rats. *European Journal of Inflammation*, 16, p.205873921877525. Available at: http://journals.sagepub.com/doi/10.1177/2058739218775255.

Wojdyło, A. et al., 2014. Evaluation of sour cherry (Prunus cerasus L.) fruits for their polyphenol content, antioxidant properties, and nutritional components. *Journal of Agricultural and Food Chemistry*, 62(51), pp.12332–12345. Available at: https://pubs.acs.org/doi/pdf/10.1021/jf504023z [Accessed June 11, 2018].

Zeng, B. et al., 2016. Phenolic Compounds from Clinopodium chinense (Benth.) O. Kuntze and Their Inhibitory Effects on ??-Glucosidase and Vascular Endothelial Cells Injury. *Chemistry and Biodiversity*, 13(5), pp.596–601.

Zhang, H.J. et al., 2018. Protective effects of total flavonoids from Clinopodium chinense (Benth.) O. Ktze on myocardial injury in vivo and in vitro via regulation of Akt/Nrf2/HO-1 pathway. *Phytomedicine*, 40(February 2017), pp.88–97.

Zhu, Y. Di et al., 2017. Triterpenoid saponins from Clinopodium chinense (Benth.) O. Kuntze and their biological activity. *Archives of Pharmacal Research*, pp.1–14.

# 5 Conclusions

This thesis work reported the results of the phytochemical and biological studies of *Cucumis sativus* L, *Boswellia serrata* Roxb. ex Colebr, *Salvia sagittata* Ruiz & Pav C and *Clinopodium tomentosum* (Kunth) Harley. These medicinal plants have demonstrated their anti-inflammatory activity *in vitro* on primary cultures of porcine aortic endothelial cells.

In the case of the endemic Ecuadorian medicinal plants, it is the first report of the phytochemical characterization and, on the other ands, of the evidence-based demonstration of at least one of their traditional uses.

Moreover the studies confirm primary culture of porcine endothelial cells as an interesting screening model to explore the huge variety of medicinal plants traditionally used in Ecuador.

# 6 Bibliografy (not included in specific chapters)


Acosta-Solís, M. 1992b. Vademécum de plantas medicinales del Ecuador. FESO; Abya-Yala, Quito.

Ahmad, K., Ahmad, M. & Weckerle, C., 2015. Ethnoveterinary medicinal plant knowledge and practice among the tribal communities of Thakht-e-Sulaiman hills, west Pakistan. Journal of ethnopharmacology, 170, pp.275–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26002769.

Aird, W., 2006. *The Endothelium: A Comprehensive Reference*, Taylor & Francis Group. Available at: https://books.google.com.ec/books?id=RTkZPQAACAAJ.

Aird, W.C., 2008. Endothelium in health and disease. *Pharmacological reports : PR*, 60(1), pp.139–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18276995.

Akerreta, S., Calvo, M.I. & Cavero, R.Y., 2010. Ethnoveterinary knowledge in Navarra (Iberian Peninsula). *Journal of Ethnopharmacology*, 130(2), pp.369–378. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20573568 [Accessed October 29, 2018].

Angelo, D. & J.M. Capriles. 2004. La importancia de las plantas psicotrópicas para la economía de intercambio y relaciones de interacción en el altiplano sur andino. Chungará 36: 1023–1035.

Antonio, R. et al., 2015. Investigation of urban ethnoveterinary in three veterinary clinics at east zone of São Paulo city, Brazil. *Journal of Ethnopharmacology*, 173, pp.183–190. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2620564.

Bach, F.H., 1994. The endothelial cell: an obstacle to xenografting. *Pathologie-biologie*, 42(3), pp.213–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8090568.

Benítez, G., González-Tejero, M.R. & Molero-Mesa, J., 2012. Knowledge of ethnoveterinary medicine in the Province of Granada, Andalusia, Spain. *Journal of Ethnopharmacology*, 139(2), pp.429–439.

Bennett, B.C. & Prance, G.T., 2000. Introduced Plants in the Indigenous Pharmacopoeia of Northern South America INTRODUCED PLANTS IN THE INDIGENOUS PHARMACOPOEIA OF NORTHERN SOUTH AMERICA. *Economic Botany*, 54(1994), pp.90–102.

Betz, C. et al., 2016. Cell behaviors and dynamics during angiogenesis. *Development*, 143(13), pp.2249–2260. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27381223.

Birch, K.A. et al., 1992. Calcium/calmodulin transduces thrombin-stimulated secretion: studies in intact and minimally permeabilized human umbilical vein endothelial cells. *The Journal of cell biology*, 118(6), pp.1501–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1522120.

Birch, K.A. et al., 1994. Prolonged peak elevations in cytoplasmic free calcium ions, derived from intracellular stores, correlate with the extent of thrombin-stimulated exocytosis in single human umbilical vein endothelial cells. *Journal of Cellular Physiology*, 160(3), pp.545–554. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7521337.

Buitrón, X. 1999. Ecuador: uso y comercio de plantas medicinales, situación actual y aspectos importantes para su conservación. Traffic International, Quito.

Burri, P.H., Hlushchuk, R. & Djonov, V., 2004. Intussusceptive angiogenesis: Its emergence, its characteristics, and its significance. *Developmental Dynamics*, 231(3), pp.474–488. Available at: http://doi.wiley.com/10.1002/dvdy.20184.

Byga, A. & Balslevb, H., 2004. *FACTORS AFFECTING LOCAL KNOWLEDGE OF PALMS IN NANGARITZA VALLE~ SOUTHEASTERN ECUADOR Journal of EthnO[iioliDgy 24(2): 255-278 Fall/Winter 2004*, Available at: https://ethnobiology.org/sites/default/files/pdfs/JoE/24-2/BygBalslev2004.pdf.

Cerón, C.E. & D. Gaybor. 1994. Plantas medicinales de los mercados de Guaranda. En: Memorias de las XVIII Jornadas Ecuatorianas de Biología. SEB; Universidad Técnica de Ambato, Ambato.

Cerón, C.E., C. Montalvo, J. Umenda & E. Umenda. 1994a. Etnobotánica y notas sobre la diversidad vegetal en la comunidad Cofán de Sinangüé, Sucumbíos, Ecuador, Vol. 2. Editcar, Quito.

Clark, P.R. et al., 2007. Increased ICAM-1 Expression Causes Endothelial Cell Leakiness, Cytoskeletal Reorganization and Junctional Alterations. *Journal of Investigative Dermatology*, 127(4), pp.762–774. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17195014.

Cragg, G.M. & Newman, D.J., 2001. Natural Product Drug Discovery in the Next Millennium. *Pharmaceutical Biology*, 39(sup1), pp.8–17. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21554167.

Cragg, G.M., Newman, D.J. & Snader, K.M., 1997. Natural Products in Drug Discovery and Development. *Journal of Natural Products*, 60(1), pp.52–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9014353.

D, V. der M., Swan, G.E. & Botha, C.J., 2001. *Journal of the South African Veterinary Association.*, [South African Veterinary Association.]. Available at: https://jsava.co.za/index.php/jsava/article/view/651/625.

Dothel, G. et al., 2013. Animal models of chemically induced intestinal inflammation: Predictivity and ethical issues. *Pharmacology & Therapeutics*, 139(1), pp.71–86. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/23563278.

Egan, K. & FitzGerald, G.A., 2006. Eicosanoids and the vascular endothelium. *Handbook of experimental pharmacology*, (176 Pt 1), pp.189–211. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16999220.

Estrella, E. 1995. Plantas medicinales Amazónicas: realidad y perspectivas. Tratado de Cooperación Amazónica, Lima.

Feng, D. et al., 1998. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *The Journal of experimental medicine*, 187(6), pp.903–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9500793.

Frangogiannis, N.G., 2006. Targeting the inflammatory response in healing myocardial infarcts. *Current medicinal chemistry*, 13(16), pp.1877–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16842199.

Gentry, A.H. 1990. Floristic similarities and differences between southern Central America and upper and central Amazonia. 141–157. In: A.H. Gentry (ed.), Four neotropical rainforests. Yale University Press, New Haven.

Goepfert, C. et al., 2000. CD39 modulates endothelial cell activation and apoptosis. *Molecular medicine (Cambridge, Mass.)*, 6(7), pp.591–603. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10997340.

Guerrero, F. 2005. Población indígena y afroecuatoriana en Ecuador: Diagnóstico sociodemográfico a partir del censo de 2001. Comisión Económica para América Latina y el Caribe (CEPAL), Santiago de Chile.

Heltianu, C., Simionescu, M. & Simionescu, N., 1982. Histamine receptors of the microvascular endothelium revealed in situ with a histamine-ferritin conjugate: characteristic high-affinity binding sites in venules. *The Journal of cell biology*, 93(2), pp.357–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7201474.

Hooijmans, C.R. & Ritskes-Hoitinga, M., 2013. Progress in Using Systematic Reviews of Animal Studies to Improve Translational Research. *PLoS Medicine*, 10(7), p.e1001482. Available at: https://dx.plos.org/10.1371/journal.pmed.1001482.

Hunt, B.J. & Jurd, K.M., 1998. Endothelial cell activation. A central pathophysiological process. *BMJ (Clinical research ed.)*, 316(7141), pp.1328–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9563977.

Jørgensen, P.M. & S. León-Yánez (eds.). 1999. Catalogue of the vascular plants of Ecuador. Missouri Botanical Garden Press, St. Louis.

Judd, W.S., Campbell, C.S. & Kellogg, E.A., 2002. *Plant systematics : a phylogenetic approach*,

Kennedy, D.O. & Wightman, E.L., 2011. Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Advances in nutrition (Bethesda, Md.)*, 2(1), pp.32–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22211188.

Keramaris, N.C. et al., 2008. Translational research: From benchside to bedside. *Injury*, 39(6), pp.643–650. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18508055.

Kilkenny, C. et al., 2010. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *Journal of Pharmacology and Pharmacotherapeutics*, 1(2), p.94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21350617.

Kilkenny, C. et al., 2009. Survey of the Quality of Experimental Design, Statistical Analysis and Reporting of Research Using Animals M. McLeod, ed. *PLoS ONE*, 4(11), p.e7824. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19956596.

Klein, D. et al., 2014. Nestin(+) Tissue-Resident Multipotent Stem Cells Contribute to Tumor Progression by Differentiating into Pericytes and Smooth Muscle Cells Resulting in Blood Vessel Remodeling. *Frontiers in Oncology*, 4, p.169. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25019063.

Kliebenstein, D.J., 2013. Making new molecules — evolution of structures for novel metabolites in plants. *Current Opinion in Plant Biology*, 16(1), pp.112–117. Available at: https://www.sciencedirect.com/science/article/pii/S1369526612001744?via%3Dihub.

Kohn, E.O. 1992b. La cultura médica de los runas de la región amazónica ecuatoriana. Colección Hombre y Ambiente No. 21. Abya-Yala, Quito.

Korshunov, V.A., Schwartz, S.M. & Berk, B.C., 2007. Vascular Remodeling. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 27(8), pp.1722–1728. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17541029.

Kothari, B. 1993. Ñucanchic panpa janpicuna/Plantas medicinales del campo. Abya-Yala, Quito.

Lans, C. et al., 2009. Medicinal plants used in British Columbia, Canada for reproductive health in pets. *Preventive Veterinary Medicine*, 90(3–4), pp.268–273. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19482367.

Ley, K. & Reutershan, J., 2006. Leucocyte-endothelial interactions in health and disease. *Handbook of experimental pharmacology*, (176 Pt 2), pp.97–133. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/16999226.

Lorant, D.E. et al., 1991. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *The Journal of cell biology*, 115(1), pp.223–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1717478.

Lückhoff, A. & Clapham, D.E., 1992. Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca2+-permeable channel. *Nature*, 355(6358), pp.356–358. Available at: http://www.nature.com/articles/355356a0.

Marchesi, V.T., 1961. The site of leucocyte emigration during inflammation. *Quarterly journal of experimental physiology and cognate medical sciences*, 46, pp.115–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13766496.

Martin, M., Mathias, E. & McCorkle, C.M., 2001. Ethnoveterinary medicine: an annotated bibliography of community animal healthcare. *Ethnoveterinary medicine: an annotated bibliography of community animal healthcare.* Available at: https://www.cabdirect.org/cabdirect/abstract/20013095127.

Martin, M.U. & Wesche, H., 2002. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochimica et biophysica acta*, 1592(3), pp.265–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12421671.

McCorkle, C.M., 1995. Back to the future: Lessons from ethnoveterinary RD&E for studying and applying local knowledge. *Agriculture and Human Values*, 12(2), pp.52–80. Available at: http://link.springer.com/10.1007/BF02217297.

McGonigle, P. & Ruggeri, B., 2014. Animal models of human disease: Challenges in enabling translation. *Biochemical Pharmacology*, 87(1), pp.162–171. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23954708.

Mentzer, S.J. & Konerding, M.A., 2014. Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. *Angiogenesis*, 17(3), pp.499–509. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24668225.

Mulvany, M., 1999. Vascular remodelling of resistance vessels: can we define this? *Cardiovascular Research*, 41(1), pp.9–13. Available at: https://academic.oup.com/cardiovascres/article-lookup/doi/10.1016/S0008-6363(98)00289-2.

Niu, J. et al., 2003. G Protein βγ Subunits Stimulate p114RhoGEF, a Guanine Nucleotide Exchange Factor for RhoA and Rac1. *Circulation Research*, 93(9), pp.848–856. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14512443.

Ortega, F. 1988. Hierbas medicinales de Quito urbano. Ministerio de Salud Pública; Instituto Nacional de Investigaciones Nutricionales y Médico Sociales, Quito.

Petrache, I. et al., 2003. The Role of the Microtubules in Tumor Necrosis Factor-α–Induced Endothelial Cell Permeability. *American Journal of Respiratory Cell and Molecular Biology*, 28(5), pp.574–581. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1270701.

Pober, J.S. et al., 1987. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *Journal of immunology (Baltimore, Md. : 1950)*, 138(10), pp.3319–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3494766.

Pober, J.S., 1988. Warner-Lambert/Parke-Davis award lecture. Cytokine-mediated activation of vascular endothelium. Physiology and pathology. *The American journal of pathology*, 133(3), pp.426–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2462353.

Pober, J.S. & Cotran, R.S., 1990. The role of endothelial cells in inflammation. *Transplantation*, 50(4), pp.537–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2219269.

Pober, J.S. & Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology*, 7(10), pp.803–815.

Prescott, S.M., Zimmerman, G.A. & McIntyre, T.M., 1984. Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when stimulated with thrombin. *Proceedings of the National Academy of Sciences of the United States of America*, 81(11), pp.3534–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6587368.

Ramakrishna, A. & Ravishankar, G.A., 2011. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, 6(11), pp.1720–1731.

Renna, N.F., de las Heras, N. & Miatello, R.M., 2013. Pathophysiology of Vascular Remodeling in Hypertension. *International Journal of Hypertension*, 2013, pp.1–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23970958.

Rodríguez, G. 1995. La faz oculta de la medicina Andina. Colección Amaru. Abya-Yala, Quito.

Roumenina, L.T. et al., 2016. Endothelial cells: source, barrier, and target of defensive mediators. *Immunological Reviews*, 274(1), pp.307–329.

Sandercock, P. & Roberts, I., 2002. Systematic reviews of animal experiments. *The Lancet*, 360(9333), p.586. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12241927.

Sarker, S.D., Latif, Z. & Gray, A.I., 2006. Natural Product Isolation. In *Natural Products Isolation*. Totowa, NJ: Humana Press, pp. 1–25. Available at: http://link.springer.com/10.1385/1-59259-955-9:1.

Schenkel, A.R. et al., 2002. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nature Immunology*, 3(2), pp.143–150. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11812991.

Schillhorn van Veen, T.W., 1997. Sense or nonsense? Traditional methods of animal parasitic disease control. *Veterinary Parasitology*, 71(2–3), pp.177–194. Available at: https://www.sciencedirect.com/science/article/pii/S0304401797000319.

Schultes, R.E. & R. Hofmann. 2000. Plantas de los dioses. Fondo de Cultura Económica, México D.F.

Severino L, Russo R, Autore G, Marzocco S, De Tommasi N. (2008) Use of phytotherapics in dogs and cats. Pharmacologyonline, 2, 12-21.

Teixeira, M.M., Williams, T.J. & Hellewell, P.G., 1993. Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin. *British journal of pharmacology*, 110(4), pp.1515–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8306095.

Triggle, C.R. et al., 2012. The endothelium: influencing vascular smooth muscle in many ways. *Canadian Journal of Physiology and Pharmacology*, 90(6), pp.713–738. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22625870.

Ulloa Ulloa, C. & D. Neill. 2005. Cinco años de adiciones a la flora del Ecuador 1999-2004. Editorial UTPL, Loja.

Ulloa Ulloa, C. & P.M. Jørgensen. 1995. Árboles y arbustos de los Andes del Ecuador. Abya-Yala, Quito.

Ungvari, Z. et al., 2018. Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. *Nature Reviews Cardiology*, 15(9), pp.555–565. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29795441.

Valencia, R., N. Pitman, S. León-Yánez & P.M. Jørgensen (eds.). 2000. Libro rojo de las plantas endémicas del Ecuador 2000. Herbario QCA, Pontificia Universidad Católica del Ecuador, Quito.

Varea, M.T. 1922. Botánica Médica Nacional. Tip. Vicente León, Latacunga.

Wanzala W, Zessin K H, Kyule N M, Baumann M P O, Mathias E and Hassanali A 2005: Ethnoveterinary medicine: a critical review of its evolution, perception, understanding and the way forward. Livestock Research for Rural Development. Volume 17, Article #119. http://www.lrrd.org/lrrd17/11/wanz17119.htm

Willms-Kretschmer, K., Flax, M.H. & Cotran, R.S., 1967. The fine structure of the vascular response in hapten-specific delayed hypersensitivity and contact dermatitis. *Laboratory investigation; a journal of technical methods and pathology*, 17(3), pp.334–49. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/6051014.

Yoo, S.Y. & Kwon, S.M., 2013. Angiogenesis and its therapeutic opportunities. *Mediators of inflammation*, 2013, p.127170. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23983401.

Zavoico, G.B. et al., 1989. IL-1 and related cytokines enhance thrombin-stimulated PGI2 production in cultured endothelial cells without affecting thrombin-stimulated von Willebrand factor secretion or platelet-activating factor biosynthesis. *Journal of immunology (Baltimore, Md.: 1950)*, 142(11), pp.3993–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2497185.

Zhang, J. et al., 2010. Biomarkers of Endothelial Cell Activation Serve as Potential Surrogate Markers for Drug-induced Vascular Injury. *Toxicologic Pathology*, 38(6), pp.856–871. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20716788.

Zhang, R.-Y. et al., 2013. The angiogenic factors and their soluble receptors in sepsis: friend, foe, or both? *Critical Care*, 17(4), p.R178. Available at: http://ccforum.biomedcentral.com/articles/10.1186/cc12857.