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ANIMAL CELL CULTURES AS *IN VITRO* MODELS FOR THE STUDY OF PHYTOEXTRACTS BIOLOGICAL ACTIVITY

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A Francesco,

"Doce ergo me suavitatem inspirando caritatem,
doce me disciplinam donando patientiam,
doce me scientiam illuminando intellegentiam."

Sant'Agostino
(Enarrationes in Psalmos, 118, 17, 4)

Summary

Abstract

Plants, herbs and ethnobotanicals have been used since ancient times and are still used throughout the world for health promotion and treatment of diseases, not only in human medicine but also in veterinary medicine. In recent years, the increasing interest in the use of herbal medicines involves critical issues, concerning the quality and safety of natural products in relation to their health claims. Consequently, the research to obtain scientific evidence on phytochemical characterization, molecular basis of biological activity and effectiveness of traditional remedies is increasingly needed.

The main purpose of this research project was the evaluation of the biological activity of selected anti-inflammatory and anticancer phytoextracts in *in vitro* models based on cell cultures, in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes on the base of the "3Rs" principles.

The first one was an inflammatory model based on primary culture of porcine aortic endothelial cells (pAECs) stimulated by LPS. This *in vitro* model was employed to evaluate the biological activity of different *Boswellia serrata* extracts and a *Cucumis sativus* extract. The obtained results confirmed the anti-inflammatory activity of both extracts on endothelial cells, suggesting a potential pharmaceutical application for cardiovascular health. However, *B. serrata* extracts showed also cytotoxicity or proliferative stimulation, depending on the dose and the formulation. Denying the common belief that herbal products are safe because they are natural, attention should be paid when these herbal supplements are used in human and animal phytotherapy, alone or as adjuvant of conventional therapies.

The second model was the canine osteosarcoma cell line (D-17) employed to evaluate the cytotoxicity and anti-proliferative effects of a hydroalcoholic extract of *Artemisia annua*, compared to its main active compound artemisinin, used as a reference. Dogs are unique animal models of cancer in that they generally experience spontaneous disease and share significant clinical and biological similarities with humans. The preliminary results confirmed the cytotoxicity of *A. annua* also in this canine tumour cell line. Hydroalcoholic extract showed much more potent cytotoxic effect than pure artemisinin, inducing a significant increase of G_2/M phase of cell cycle. A complete characterization of the phytoextract will be necessary to accurately quantify artemisinin and better

understand the possible plant compounds that act synergistically for the final cytotoxic effect.

In conclusion, the *in vitro* models based on animal cell cultures have shown to be a significant preclinical tool, contributing to the application of the "Replacement" principle. Finally, this study confirmed the importance of evidence-based research in phytotherapy for a quality and safe use of herbal medicine, not only in human but also in veterinary medicine.

Introduction

Herbal medicine

Traditional medicine is "the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness" (WHO, 2018). In traditional medicine, the attention is oriented on the whole condition of the individual, rather than on the particular disease, and the use of herbs is the most important part of all traditional medicines (Schmidt et al., 2008; Wachtel-Galor and Benzie, 2011).

Traditional Chinese medicine (TCM) has an ancient history of more than 3000 years and is the most important example of traditional medicines (Xutian et al., 2009). The oldest known herbal text in the world is *The Devine Farmer's Classic of Herbalism*, compiled about 2000 years ago in China and many herbal pharmacopoeias and monographs on individual herbs were developed through these accumulated knowledges.

During the past 100 years, the development and production of chemically synthesized drugs have revolutionized health care in most parts of the world. However, particularly in developing countries, many populations still rely on traditional practitioners and herbal medicines for their primary care. In China, traditional medicine accounts for around 40% of all health care delivered and more than 90% of general hospitals have units for traditional medicine. In Africa up to 90% and in India up to 70% of the population depend on traditional medicine for their primary care (WHO, 2005). However, during the last two decades, the use of traditional medicine is not limited to developing countries, but the public interest in natural remedies has increased considerably even in industrialized countries, with expanding use of botanicals. In the United States, around 38% of adults and 12% of children used some form of traditional medicine in 2007 (Barnes et al., 2008; Wachtel-Galor and Benzie, 2011).

Principally, herbal medicines are used for health promotion and therapy of chronic conditions. However, the use of traditional remedies increases when conventional medicine is ineffective in the treatment of diseases, such as in advanced cancer and in new infectious diseases. Moreover, traditional medicines are widely perceived as natural

and safe, and, for this reason, not toxic. This is not necessarily true, especially when herbs are taken together with prescription drugs, medicines over-the-counter, or in mixtures with other herbs (Canter and Ernst, 2004; Cohen and Ernst, 2010; Wachtel-Galor and Benzie, 2011).

Traditional and Complementary Medicine (T&CM) is increasing and expanding, particularly with the opportunity to buy natural products on line in Internet. The T&CM field now plays an important role in the economic development of many countries. At the same time, with prevailing current global financial constraints, use of T&CM for self-health care, health promotion and diseases prevention may actually reduce healthcare costs (WHO, 2013). The commercial value of the ethnobotanical market is constantly expanding and should not be underestimated. For example, in 1995, in Germany the total turnover of non-prescription-bound herbal medicines in pharmacies was equal to almost 30% of the total turnover of non-prescription-bound medicines, and in the United States, the annual retail sales of herbal products was estimated to be US$ 5.1 billion (Wachtel-Galor and Benzie, 2011).

Currently, herbal products are employed for the treatment of chronic and acute conditions and various disorders such as cardiovascular diseases, asthma, lung diseases, depression, inflammation, and to boost the immune system. In 2003, in China, traditional herbal medicines played a strategic role to contain and treat severe acute respiratory syndrome (SARS) and in Africa, a traditional herbal medicine, the Africa flower, has been used for decades to treat wasting symptoms associated with HIV (Tilburt and Kaptchuk, 2008). In Europe, Germany and France are countries leading in the use of herbal medicines and in most developed countries, one can find essential oils, herbal extracts or herbal teas being sold in pharmacies with conventional drugs (Wachtel-Galor and Benzie, 2011).

Plants are rich in a variety of compounds. Many of these are secondary metabolites including aromatic substances, phenols or their oxygen-substituted derivatives such as tannins (Hartmann, 2007). Most of these compounds have antioxidant properties. Natural products and plant constituents can be used directly as therapeutic agents, but ethnobotanicals are important for pharmacological research and drug development because they can be used as starting materials for the synthesis of drugs or as models for

pharmacologically active compounds (Li and Vederas, 2009). About 200 years ago, morphine, the first pharmacologically active pure compound, was obtained from opium extracted from seeds pods of the poppy *Papaver somniferum*. This discovery showed that drugs can be purified from plants and administered in precise dosages (Hartmann, 2007). The discovery of penicillin enhanced this method and the active compounds obtained from the plants continued to increase (Li and Vederas, 2009). With this trend, products derived from plants and natural sources (such as fungi and marine microorganisms) or analogues inspired by them have contributed greatly to the commercial drug production. Examples include the cardiac stimulant digoxin obtained from foxglove (*Digitalis purpurea*); antibiotics (e.g., penicillin, erythromycin); salicylic acid, a precursor of aspirin, derived from willow bark (*Salix spp*.); reserpine, an antipsychotic and antihypertensive drug obtained from *Rauwolfia spp.*; and antimalarials such as quinine derived from *Cinchona* bark and lipid-lowering agents (e.g., lovastatin) obtained from a fungus (Li and Vederas, 2009; Schmidt et al., 2008). Also, more than 60% of cancer therapeutics on the market or in testing are derived or inspired by natural products. For treatment of cancer, over 70% of drugs approved worldwide are based on natural products or mimetics, many of which were improved with combinatorial chemistry. Cancer therapeutics from plants include paclitaxel, isolated from the Pacific yew tree; combretastatin, derived from the South African bush willow; camptothecin, derived from *Camptotheca acuminata* is used to obtain irinotecan and topotecan. It is also estimated that about 25% of the drugs prescribed worldwide are derived from plants and 121 active compounds are in use (Sahoo et al., 2010). Between 2005 and 2007, 13 drugs obtained from natural products were approved in the United States. More than 100 natural product-based drugs were in clinical studies (Li and Vederas, 2009), and of the total 252 drugs in the World Health Organization's (WHO) essential medicine list, 11% are exclusively of plant origin (Sahoo et al., 2010; Wachtel-Galor and Benzie, 2011).

Regulations, quality, safety, and scientific evidence

WHO has recognized the important contribution of traditional medicine to provide essential care and developed "The WHO Traditional Medicine Strategy 2014 –2023" (WHO, 2013). In 1989, the U.S. Congress established the Office of Alternative Medicine within the National Institutes of Health to encourage scientific research in the field of

traditional medicine (https://nccih.nih.gov/) and in the same year, the European Scientific Cooperative on Phytotherapy (ESCOP) was founded with the aim of advancing the scientific status and harmonization of phytomedicines at the European level (http://escop.com/).

The increasing interest and great expansion of traditional medicines worldwide, bring with them important challenges. There are international diversity and national policies regarding the regulation of the production and use of herbal products, consequently there is need to improve quality, safety, and scientific evidences of botanicals in relation to health claims (Sahoo et al., 2010; WHO, 2005).

In Europe, the Directive 2001/83/EC released in 2001 by the European Parliament and by the Council of Europe provides the Community code relating to medicinal products for human use. This Directive shall apply to industrially produced medicinal products for human use intended to be placed on the market in Member States, including herbal medicines (Directive 2001/83/EC, 2001). However, in 2004 the European Parliament and Council amended Directive 2001/83/EC by enacting Directive 2004/24/EC, providing the guidelines for the use of traditional herbal medicinal products (Directive 2004/24/EC, 2004). In this directive a simplified registration procedure “traditional use registration” is established for those herbal medicinal products, for which a “bibliographical or expert evidence to the effect that the medicinal product in question, or a corresponding product has been in medicinal use throughout a period of at least 30 years preceding the date of the application, including at least 15 years within the Community” (Directive 2004/24/EC, 2004). After demonstrating the necessary number of years of traditional safe use, the herbal products can enter in the European pharmaceutical regime with the simplify procedure called "registration of traditional use". Directive 2004/24/EC also established the Committee on Herbal Medicinal Products (HMPC) at the European Medicines Agency (EMA). The HMPC is responsible for compiling and assessing scientific data on herbal substances, preparations and combinations, to support the harmonisation of the European market. Moreover, the HMPC establishes European monographs covering the therapeutic uses and safe conditions of well-established and/or traditional use for herbal substances and preparations (HMPC, 2004).

During the years, herbal medicine has been commonly used for enhancement of the quality of life, health promotion and for treatment and prevention of diseases. The holistic approach to health care makes herbal medicine very attractive to consumers, but it also makes scientific evaluation very difficult because so many factors must be considered. Herbal products are in widespread use and although they are believed as safe, they are often used in mixture and are obtained from plant sources with their own variability in species, growing conditions, and biologically active constituents. Herbal extracts may be adulterated, contaminated and may contain toxic compounds. The quality control of herbal medicines has a direct impact on their safety and efficacy but there is a lack of evidence-based research to assess quality, safety and effectiveness of many commercial products (Ernst et al., 2005; Wachtel-Galor and Benzie, 2011). As a consequence, these data are available on the molecular composition and quality of many herbal products not only due to lack of government requirements or international policies but also due to lack of adequate or accepted analytical techniques for characterizing and evaluating traditional medicines. Though the wide popularity of herbal supplements and botanicals, some herbal products on the market are likely to be of low quality and efficacy, and do not contain the effective claimed active substances, even if the same herb has been shown to have a biological activity in controlled studies using high-quality phytoextract (Wachtel-Galor and Benzie, 2011).

The most important advantage of herbal medicines over conventional drugs with a well characterized molecular profile is the presence of multiple active compounds that together can provide a synergic effect that may not be achievable by any single compound, but it is the result of the complete phytocomplex. Ideally, the composition of the botanical extract must be standardized and free of any potential hazards, and plants should be grown under controlled conditions, because the environment can significantly affect the phytochemical profile and the efficacy of the botanical product. In practice, the situation is very different. Herbal extracts contain hundreds of compounds, many of which are unknown and chemical fingerprinting is in its early stages and is lacking for most herbs. This makes standardization of botanicals difficult, although few of them can be produced with a standardized amount of a key component or class of components. However, even when such key compounds have been identified and a standard content is agreed or suggested, there is no guarantee that individual commercial herbal products actually

contain these essential components and in the claimed concentration (Wachtel-Galor and Benzie, 2011).

Research into quality, safety, biological activity and clinical efficacy of the different herbal medicines of common usage is needed. Newly emerging scientific approaches, genomic testing and chemical fingerprinting techniques using hyphenated testing platforms are now available for definitive authentication and quality control of herbal products (Patel et al., 2010; Wachtel-Galor and Benzie, 2011).

The values of Evidence-based Medicine and Evidence-based Research (Kelly et al., 2015; Sackett et al., 1996; Timmermans and Mauck, 2005) should also be applied to herbal medicine. Research is also needed to obtain science-based information on dosage, contraindications and effectiveness of most natural compounds. Herbal products can be safely used in human and animal phytotherapy only applying scientific principles.

Veterinary herbal medicine

Since ancient times, with a traditional history that can be traced back over millennia, herbal medicines were used also in animal care. In the rural areas worldwide, the veterinary medicine deals with knowledge, methods, practices, skills and beliefs about caring for their livestock. In some remote areas, people have great undocumented traditional knowledge about animal diseases, herbal treatments, formulations, etc., but most of these traditional treatments were limited to restricted populations (Rastogi et al., 2015). In 1998, McCorkle defined Ethnoveterinary Medicine (EVM) as: “The holistic, interdisciplinary study of local knowledge and its associated skills, practices, beliefs, practitioners, and social structures pertaining to the healthcare and healthful husbandry of food, work, and other income-producing animals, always with an eye to practical development applications within livestock production and livelihood systems, and with the ultimate goal of increasing human well-being via increased benefits from stockraising” (McCorkle and Martin, 1998). However, the most studied element of EVM is veterinary ethnopharmacopoeia, especially the use of botanicals. These knowledge and skills are hundreds or even thousands of years old. Classic cases include Ayurveda in India (Rastogi et al., 2015) and herbal medicine in China (Lin and Panzer, 1994; Xie and Eckermann-Ross, 2012). Ethnoveterinary is practised in several parts of the world, in

Africa (Assefa and Bahiru, 2018; Gabalebatse et al., 2013; McGaw and Eloff, 2008; Ndhlovu and Masika, 2013), in Asia (Khattak et al., 2015; Rastogi et al., 2015; Suroowan et al., 2017), in America (Lans, 2016) and also in Europe (Mayer et al., 2014; Piluzza et al., 2015).

In recent years, the increasing attention over the spread of antibiotic resistance, has raised the interest in phytotherapy, with the aim to reduce the use of antibiotics in livestock. Veterinary herbal medicinal products are not authorised centrally in the Europe, but by the national governments. Nevertheless, it is permitted to sell feed additives containing plant material in different formulations, with claims concerning optimisation of nutrition, support and protection of the physiological conditions, unless they do not contain a claim declaring that they will prevent, treat or cure a disease. Due to their easy access, botanical products are primarily sold as feed additives, and they may be used as presumed remedies (Blanco-Penedo et al., 2018).

Scientific information regarding veterinary phytotherapy is rare. Research is needed to validate efficacy also in veterinary herbal medicine (Mayer et al., 2014). Applying analytical methods, such as metabolomics, could help to better identify the chemical composition, and ensure standardisation and safety of the herbal products. Recent development of high-throughput and "omics" technologies might accelerate studies of the mechanisms underlying the biological activity and, therefore, support the effective use of these compounds (Blanco-Penedo et al., 2018).

An adaptation of the European legislation would potentially make it possible to register traditional veterinary herbal medicinal products through a simplified registration procedure or support the registration as health-supporting feed supplements (zootechnical feed additives). Some experts suggested to harmonize veterinary and human regulations, adapting the requirements for safety assessments to the specific circumstances surrounding botanicals that have been used for a long time and that are included in European monographs for human use (Blanco-Penedo et al., 2018).

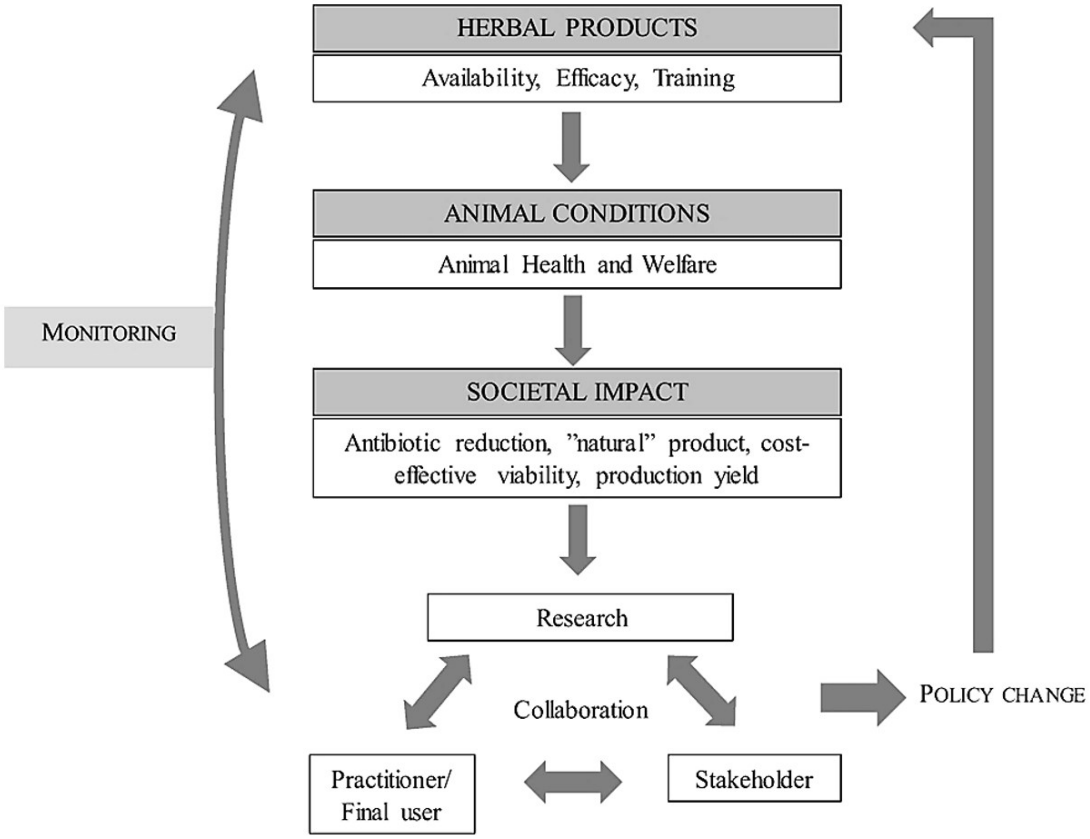

Figure 1. *Disciplines and impact flows related with the main challenges in phytotherapy (Blanco-Penedo et al., 2018).*

Only coordinated and sustained efforts by all players in animal health care as well as developers of regulatory framework will allow possibilities to release the potential therapeutic ability of veterinary phytotherapy.

In vitro models as alternatives to animal testing

The Principles of the "3Rs"

The publication of *"The Principles of Humane Experimental Technique"* by W.M.S. Russell and R.L. Burch in 1959 (Russell and Burch, 1959) marks the birth of the principle of the "3Rs".

The authors proposed the principles of **Replacement**, **Reduction** and **Refinement** (the "3Rs") as the key strategies of a systematic framework aimed at achieving the goal of human experimental techniques. Russell and Burch saw replacement as the ultimate goal for laboratory animal based research, education and testing, with the other two, reduction and refinement, being more readily achievable in the short term (European Commission Environment DG, 2016).

Replacement

The authors proposed to distinguish relative and absolute replacement. In relative replacement, animals are still required, though in actual experiment they are exposed, probably or certainly, to any distress at all. In absolute replacement, animals are not required at all at any stage of the experiments. Consequently, good absolute replacement may be regarded as the final ideal of replacement. But when relative replacement is combined with great reduction, as in the use of tissue culture, it may be very welcome indeed, and such developments are among the most important in the whole progress of research.

The first option is the case of non-recovery experiments on living and intact but completely anesthetized animals. In relation to contingent inhumanity, it is important that the anaesthesia is general and sufficiently deep, and its time-course properly synchronized with the treatment. The second one is the case of experiments in which animals are still required, but only to furnish preparations after being painlessly killed. This already constitutes a further advance. The remaining types of relative replacement involve work on the isolated cells, tissues, or organs of vertebrates.

Tissue culture represents a bridge between relative and absolute replacement, in which vertebrate animals are not required at all. Turning to absolute replacement, four main subdivisions may be distinguished: the use (outside the vertebrate body) of metazoan endoparasites, higher plants, microorganisms (protozoa, bacteria, molds, etcs.), and non-living physical and chemical systems (Russell and Burch, 1959).

Reduction

The concept of reduction extends to any approach that will result in fewer animals being used to achieve the same objective, including maximising the information obtained per animal, reducing the number of animals used in the original procedure and/or limiting or avoiding the subsequent use of additional animals.

By performing more than one procedure on the same animals it is possible to reduce the number of animals used, where this does not decrease the scientific objective or result in poor animal welfare. However, the benefit of reusing animals should always be balanced against any adverse effects on their welfare, considering the lifetime experience of the individual animal. As a result of this potential important conflict, the reuse of animals should be considered on a case-by-case basis (European Commission Environment DG, 2016).

Refinement

Today, the term refinement applies to all aspects of animal use, from the time the experimental animal is born until its death and signifies the modification of any procedure or husbandry and care practice to minimise pain, suffering, distress or lasting harm and enhance its welfare.

When an animal experiences pain, suffering or distress, there are often accompanying physiological, immunological and behavioural changes which may increase the variability of scientific results. Therefore, refinement is also likely to improve data quality and contribute to Reduction (European Commission Environment DG, 2016).

The “3Rs” in European legislative framework

The protection of animal rights and in particular those used for scientific purposes has long been a subject of interest for the Council of Europe. In 1986, the first milestone was achieved, when the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes was open for signature. This Convention is designed to reduce both the number of experiments and the number of animals used for scientific purposes and it encourages research without animal use, except where there is no validate alternative and it promotes research into alternative methods. The Council of Europe Convention paved the way for the EU’s Directive 86/609/EEC, adopted in 1986, as the provisions in it are based on the Convention (European Directorate for the Quality of Medicines - EDQM).

However, the present Directive 2010/63/EU on the protection of animals used for scientific purposes, for the first time in EU legislation spells out the principle of the “3Rs” and makes it a firm legal requirement. The principles of Replacement, Reduction and Refinement must be considered systematically at all times when animals are used for scientific purposes in the EU. Under the Directive, the term "scientific purposes" covers all uses of animals for the purposes of basic, translational and applied research, regulatory testing and production as well as for the purposes of education and training.

Furthermore, the Directive ensures that its application goes beyond that of the original, narrower interpretation of the “3Rs” only in the context of choice of methods. The Directive enlarges the Refinement also to cover all animal breeding and care, that is, to ensure refinement during housing, breeding and care even if animals are not undergoing a scientific procedure (Directive 2010/63/EU, 2010).

What are alternative approaches?

The term ‘alternatives’ in this context includes all assays, tests, methods, techniques, tools, strategies and approaches etc. that contribute to the practical implementation of the “3Rs”. That is:

- to obtain the required information without the use of live animals;
- to reduce the numbers of animals whilst obtaining the same level and quality of information;
- to refine the use of live animals so as to cause less pain, distress or suffering, or improve the welfare of the animals.

Alternative methods provide opportunities to extend the principles of "3Rs", but, at the same time, they aim to develop better and more predictive scientific tools to protect human and animal health and the environment (European Commission Environment DG, 2016).

The European Commission's involvement in activities targeted to the validation of alternative approaches to animal testing started in 1991, with the launch of ECVAM (the European Centre for the Validation of Alternative Methods), hosted by the Joint Research Centre located in Ispra, Italy. The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) has been formally established in 2011, due to the increasing need for new methods to be developed and proposed for validation in the European Union.

EURL ECVAM has a long tradition in the validation of methods which reduce, refine or replace the use of animals for safety testing and efficacy/potency testing of chemicals, biologicals and vaccines. Research laboratories are able to submit to EURL ECVAM for scientific validation the alternative methods to animal testing that they have developed. EURL ECVAM also promotes the development and dissemination of alternative methods and approaches, their application in industry and their acceptance by regulators (EURL ECVAM).

In July 2013, the European Commission announced the creation of European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) in response to the provision of Directive 2010/63/EU on the protection of animals used for scientific purposes which requests that EU Member States assist the European Commission in the validation of alternative methods. Currently there are a total of 37 members of EU-NETVAL, selected against pre-defined eligibility criteria (including the European Commission's own *in vitro* GLP test facility operated by EURL ECVAM, which coordinates the network) and endorsed by the National Contact Points.

EU-NETVAL's mission is to provide support for EURL ECVAM validation studies that serve to assess the reliability and relevance of alternative methods that have a potential to replace, reduce, or refine the use of animals for scientific purposes.

In addition to the wide range of techniques and capabilities, the network offers an opportunity to share knowledge and to collaborate for the promotion of the development and use of alternative approaches. The knowledge and expertise contained within EU-NETVAL is extremely valuable for translating scientific innovation in the "3Rs" into practical applications in the academic, industrial, and regulatory domains (EU-NETVAL).

Aim of the study

Plants, herbs and ethnobotanicals have been used since ancient times and are still used throughout the world for health promotion and treatment of diseases, not only in human medicine but also in veterinary medicine. In recent years, the increasing interest in the use of herbal medicine involves important challenges. The first is the international diversity of policies concerning the regulation of production and use of herbs, their quality and safety in relation to health claims. Consequently, the second is the need of research to obtain scientific evidence on the molecular basis of biological activity of traditional remedies.

The current European regulation on the protection of animals used for scientific purposes (Directive 2010/63/EU), for the first time in EU legislation spells out the principle of the "3Rs" and promotes the development of alternative methods.

The main purpose of this research project is the evaluation of the biological activity of selected anti-inflammatory and anticancer natural phytoextracts in *in vitro* cellular models.

Considering swine an important animal model for translational medicine, an inflammatory model developed in primary culture of porcine aortic endothelial cells (pAECs) induced by LPS was chosen. The cytotoxicity and the anti-inflammatory activity of two traditional Ayurvedic remedies, *Boswellia serrata* and *Cucumis sativus* extracts was evaluated using this *in vitro* model.

Naturally occurring tumours in dogs have many clinical and biological similarities to human cancers and make the dog an important spontaneous animal model in comparative medicine. Therefore, the canine osteosarcoma cell line (D-17) was chosen to evaluate the cytotoxicity and anti-proliferative effects of a hydroalcoholic extract obtained from *Artemisia annua* L., used for centuries in Chinese Traditional Medicine as antimalarial and recently proposed as antitumoral drug.

Experimental part 1

Translational research: swine as animal model

The European Society for Translational Medicine (EUSTM) defines Translational Medicine as an "interdisciplinary branch of the biomedical field supported by three main pillars*:* bench side*,* bedside and community. The goal of Translational Medicine is to combine disciplines, resources*,* expertise*,* and techniques within these pillars to promote enhancements in prevention*,* diagnosis*,* and therapies*"* (Cohrs et al., 2015).

The use of animal models in preclinical research is pivotal to bridge the translational gap to the clinic field (Denayer et al., 2014). The species of origin of an *in vitro* model is also of great importance and may increase the translational impact of a study. Ideally, the best choice would be of human origin; however, this has its own limitations due to sample availability, in addition most available samples coming from diseased tissue with altered phenotypes. To address this, swine could be a suitable alternative. (Olayanju et al., 2018). The pig has several distinct advantages that have made this species a useful translational animal model for human research. In particular, there are important anatomic, physiological, metabolic and genetic similarities to human beings. As a consequence, pig is deservedly one of the most important animal model for preclinical studies (Gonzalez et al., 2015; Groenen et al., 2012; Perleberg et al., 2018; Prather, 2013; Stricker-Krongrad et al., 2016).

In 2012, the Swine Genome Sequencing Consortium sequenced the genome of *Sus scrofa* and is continually characterizing and updating it (Groenen et al., 2012). The achievement of the complete swine genome sequence project has greatly accelerated the process of gene targeting and identification of conserved genes, confirming the relevance of pig as a preclinical translational model (Perleberg et al., 2018; Prather et al., 2013; Walters et al., 2017). The swine genome has 18 chromosome pairs and 2 sex chromosomes of approximately 2.7 Gb, about 7% smaller than the human genome but the overall similarity of the transcribed swine genome is approximately 85%. In comparison, canine and mouse genomes are 14% smaller than the human one. Naturally occurring mutations associated with human diseases have been identified in homologous regions of porcine genome showing phenotypes comparable to those of humans (Schomberg et al., 2016).

Despite the important need of husbandry, many advantageous practical factors, such as breeding characteristics, make pigs an important animal model for human research. Pigs mature relatively quickly for a large species (6-7 months), have a short gestation period (~114 days) and produce large litters (~10 piglets per litter), depending on the breed. Centuries of pig domestication have established suitable housing conditions, including specific pathogen-free conditions, which require only minor adaptation for research (Meurens et al., 2012; Perleberg et al., 2018). Furthermore, pigs have advantages over other animal models in that ethical and societal concern presumably is lower for utilization of a farm animal as a research model in comparison with companion animals and nonhuman primates (Prather et al., 2013; Schomberg et al., 2016).

In particular, the use of miniature pigs in research has been continuously expanding for over a decade. Beyond genetic, anatomic, and physiologic similarities of the conventional swine breeds to humans, miniature pigs also have the additional advantages of size and slower growth curves like humans. At full maturity, conventional breeds can reach 249 - 306 kg and pose important needs in husbandry and handling in the biomedical research setting. In contrast, at equivalent maturity, miniature swine weigh 68 - 91 kg, the weight of an average human male. Their size also allows for the use of medical equipment and procedures that are used in human clinical settings, allowing researchers to employ state-of-the-art medical innovations in research, while giving direct translatability of experimental knowledge gained to humans (Schomberg et al., 2016; Stricker-Krongrad et al., 2016).

The intestinal microbial ecosystem plays an important role in maintaining health. Pigs are human-sized omnivorous with nutritional requirements comparable to humans and share similar metabolic and intestinal physiological processes. This likely contributes to their comparable mucosal barrier physiology and enteric microbiota, as well as susceptibility to selected enteric pathogens, in fact pigs also exhibit similar intestinal syndromes to humans. Similarities in the intestinal microbial ecology between pigs and humans have made the pig an important non primate animal model for research on dietary modulation of intestinal microbiota as a therapeutic or preventive tool (Gonzalez et al., 2015; Heinritz et al., 2013).

Moreover, pigs are the species of choice as a model for highly prevalent human diseases: diabetes, metabolic syndrome, obesity and cardiovascular diseases (Bassols et al., 2014; Swindle et al., 2012). In particular, the surgical community has recognized swine to be an excellent anatomical and physiological model for the human cardiovascular system since decades. The pig heart, coronary vessels, vasculature, and blood flow are very similar to those of humans, and overall, pigs are better suited to study cardiovascular diseases than rodent and canine models. Atherosclerosis and myocardial infarction can be induced in swine by providing diets with nutrient compositions that are known to increase the risk for cardiovascular disorders in humans (Prather et al., 2013; Swindle et al., 2012).

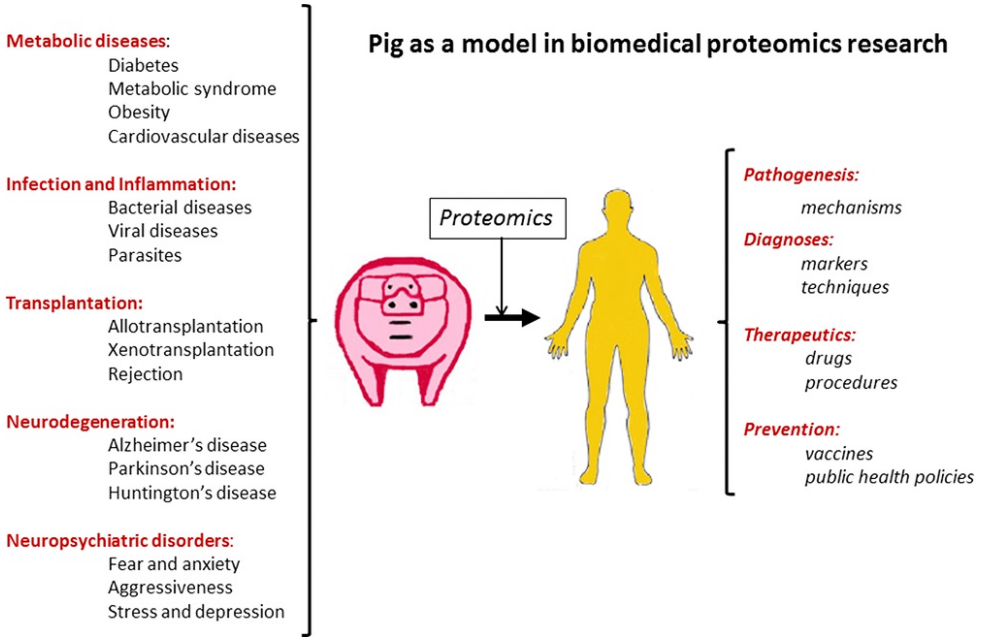

Figure 2. *Pig as a model in biomedical proteomics research (Bassols et al., 2014).*

Although it is an excellent model in its natural state, pig is even more attractive for the biomedical community due to the possibility to obtain genetic engineered animals for specific human diseases. The application of genetic modification technology to pigs has greatly increased their value to biomedicine. Advancements in "gene editing" (e.g., CRISPR/Cas9 system) now allow researchers to build upon the human-like genome template and replicate genetic conditions responsible for human diseases and improve histocompatibility of swine organs for xenotransplantation. The most promising and clinically relevant genetically modified porcine models of human diseases for

translational biomedical research include cardiovascular diseases, cancers, diabetes mellitus, Alzheimer's disease, Huntington's disease, cystic fibrosis, retinitis pigmentosa, spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (Fig. 3) (Perleberg et al., 2018; Prather et al., 2013; Walters et al., 2017).

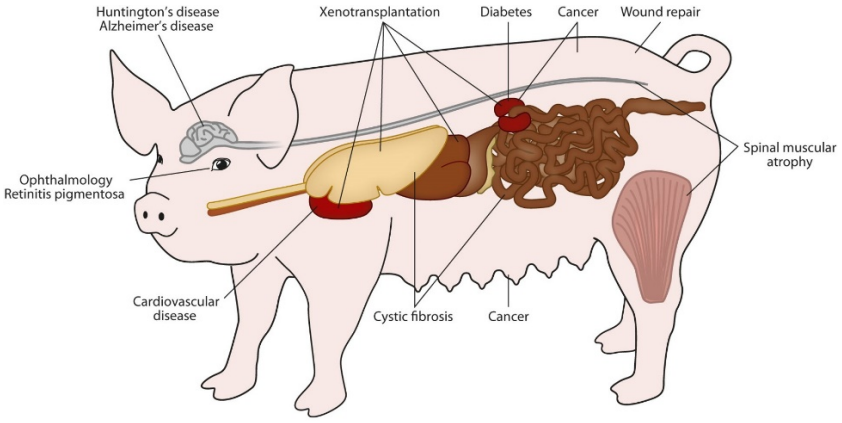

Figure 3. *Organ systems for which genetically engineered pigs have been created (Prather et al., 2013).*

In the last decade, swine has been considered the most suitable animal model in the development of stem cell-based therapy, regenerative medicine and transplantation. Porcine Mesenchymal Stem Cells (pMSCs) were isolated from several sources (bone marrow, adipose tissue, peripheral blood, aorta, coronary vessels, etc.) and share differentiation potential, cell surface markers and gene expression with human MSCs. Despite immense proliferation and differentiation potential of pMSCs, their low immunogenicity, immunosuppressive and immunomodulatory properties make them a promising candidate for cellular therapy (Bharti et al., 2016; Casado et al., 2012; Zaniboni et al., 2015, 2014). Recently, it has been demonstrated that, in 56% of cases, the pig is the large animal of choice for regenerative medicine in the areas of cardiovascular, orthopaedic and wound-healing research. Preclinical trials for toxicology and regenerative medicine products have traditionally relied on rodent models, but the pig was more in recent years (Walters et al., 2017).

With an increasing need for swine models, it is essential that the genomic tools, models and services be readily available to the scientific community. In 2003, the US National Institutes of Health (NIH) at the University of Missouri funded the National Swine

Resource and Research Center (NSRRC). It is a facility with the aim to provide high-quality biomedical swine models to the scientific community (Walters et al., 2017).

Porcine Aortic Endothelial Cells (pAECs)

The endothelium is a cellular monolayer that lines the lumen of blood vessels and plays an important physiological role in vascular homeostasis. Moreover, the endothelium is responsible for different functions including the modulation of vascular tone, the vascular permeability, the maintenance of blood fluidity, the regulation of inflammation and immune response, the prevention of thrombosis and overall the maintenance of vessel integrity. Endothelium impairment is a complex pathophysiological event that includes both the increased activation of endothelial cells and the onset of endothelial dysfunction (Chistiakov et al., 2015; Incalza et al., 2018).

The endothelium is more than inert blood vessel lining tissue, in fact endothelial cells are active players in the formation of new blood vessels both in health and diseases by an active and regulated phenomenon of angiogenesis (Eelen et al., 2018).

The vascular endothelium is the first functional organs that develops during embryogenesis and matures into a closed cardiovascular system to conduct vasculature blood flow through an intricate network of large- to medium-size vessels extending into micrometer-size capillaries. During early embryo development, a primitive vascular plexus is formed in a process termed vasculogenesis. This primitive plexus subsequently undergoes substantial remodelling by vessel intussusception whereby a pre-existing capillary splits in two adjacent vessels or by sprouting angiogenesis whereby a new capillary sprouts of a pre-existing vessel. Sprouting angiogenesisis the more frequently studied and better-understood form of vessel formation, and typically entails a series of highly orchestrated processes driven by identified pro-angiogenic stimuli (Fig. 4) (Eelen et al., 2018).

On the other hand, pathological excessive angiogenesis causes many diseases including cancer, pulmonary arterial hypertension and many eye diseases characterized by ocular neovascularization (Eelen et al., 2018).

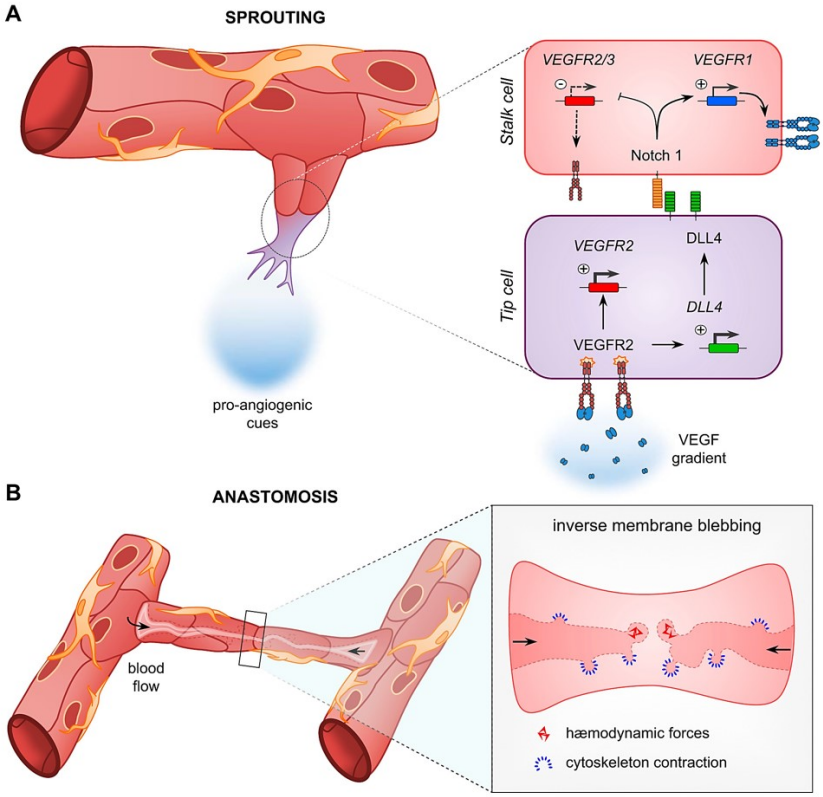

Figure 4. *General concepts in angiogenesis: tip versus stalk specification and anastomosis. A: in sprouting angiogenesis, a pro-angiogenic growth factor gradient induces tip and stalk cell formation in a pre-existing vessel to form a new sprout. B: newly formed sprouts from neighboring vessels meet and fuse through a process termed anastomosis (Eelen et al., 2018).*

Furthermore, endothelial cells have a key position in the beginning, progression, control and resolution of the vascular dysfunction (Cahill and Redmond, 2016; Chistiakov et al., 2015; Endemann and Schiffrin, 2004; Jamwal and Sharma, 2018).

Endothelial activation is the first step observed and is defined as a pro-inflammatory, pro-coagulant and pro-thrombotic state of the endothelial cells, characterized by the expression of cell-surface adhesion molecules required for the recruitment and attachment of inflammatory cells (Liao, 2013). Endothelial activation is induced by cytokines secreted by tissues and organs under endogenous and exogenous pro-inflammatory stimuli, such as lipopolysaccharide (LPS). 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 status is

not counterbalanced by the synthesis of protective molecules, the endothelial activation converts into the endothelial dysfunction and then in the vascular disease (Incalza et al., 2018; Leligdowicz et al., 2018; Sprague and Khalil, 2009).

The endothelial dysfunction (ED) is thus defined as an imbalance in the production of the vasodilator and vasoconstrictor factors, which predisposes the vasculature toward a pro-thrombotic and pro-atherogenic phenotype, characterized by vasoconstrinction, leukocyte adhesion, platelet activation, mitogenesis, pro-oxidation, impaired coagulation, vascular inflammation, atherosclerosis and thrombosis. Endothelial dysfunction has been linked to many diseases, including atherosclerosis, diabetes mellitus, coronary artery diseases, hypertension and hypercholesterolemia (Incalza et al., 2018).

Primary cell cultures are increasingly used as a significant tool in cellular and molecular biology. Since primary cells are non-transformed and non-immortalized, they faithfully reproduce *in vivo* phenotype and reach more physiologically significant results. In particular, primary cultures of endothelial cells are important for the studies of vascular dysfunction in cardiovascular diseases.

At the BMC Laboratory (Cellular and Molecular Biochemistry and Biotechnology) in the Department of Veterinary Medical Sciences of the University of Bologna, a method for the isolation of endothelial cells from porcine thoracic aorta was developed (Bernardini et al., 2005).

Briefly, thoracic aortic traits were collected from adult pigs at a local slaughterhouse in accordance with the "Replacement" of principle of the "3Rs" (Russell and Burch, 1959) inserted in the European Directive 2010/63 / EU on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010). After collection, aortas were transferred to the laboratory within 1 hour on ice. Then aortas were washed in sterile saline solution added with ampicillin (1 mg/ml) (Sigma Alderich, St Louis, MO, USA) and amphotericin B (1%) (Gibco-Life technologies Carlsbad CA, USA), then they were washed in sterile Dulbecco Phosphate Buffer Solution (DPBS, Gibco-Life technologies) added with antibiotics/antimycotics solution (10%) (Gibco-Life technologies). After ligation of all arterial side branches (Fig. 5A), aortas were cannulated with modified syringe cones and silicone tubes to set up a closed system (Fig. 5B). The vessels were

gently flushed with DPBS to remove residual blood (Fig. 5C), then they were filled with DPBS added with 0.2% collagenase (Sigma Alderich) and clamped at both ends. After 20 minutes of incubation at 37 °C, the collagenase solution was collected, and the vessels were washed with DPBS with 10% fetal bovine serum (FBS) (Gibco-Life technologies). The solutions were centrifuged at 800 x *g* for 10 minutes. The cellular pellet was resuspended in 1 mL human endothelial basal growth medium (Gibco-Life technologies) supplemented with 5% FBS and 1% antibiotics-antimycotics (Gibco-Invitrogen). Cell number and viability (85–90%) were determined using a Burker chamber under a phase-contrast microscope. Approximately 3 X 10^5 cells were placed in T-25 primary tissue culture flasks (T25-Falcon, Becton-Dickinson, Franklin Lakes, NJ, USA) in a 5% CO_2 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 checked by immunocytochemistry for endothelial cell markers: CD31, VIII factor and VE-Caderine. Then cells were expanded till 20th passages.

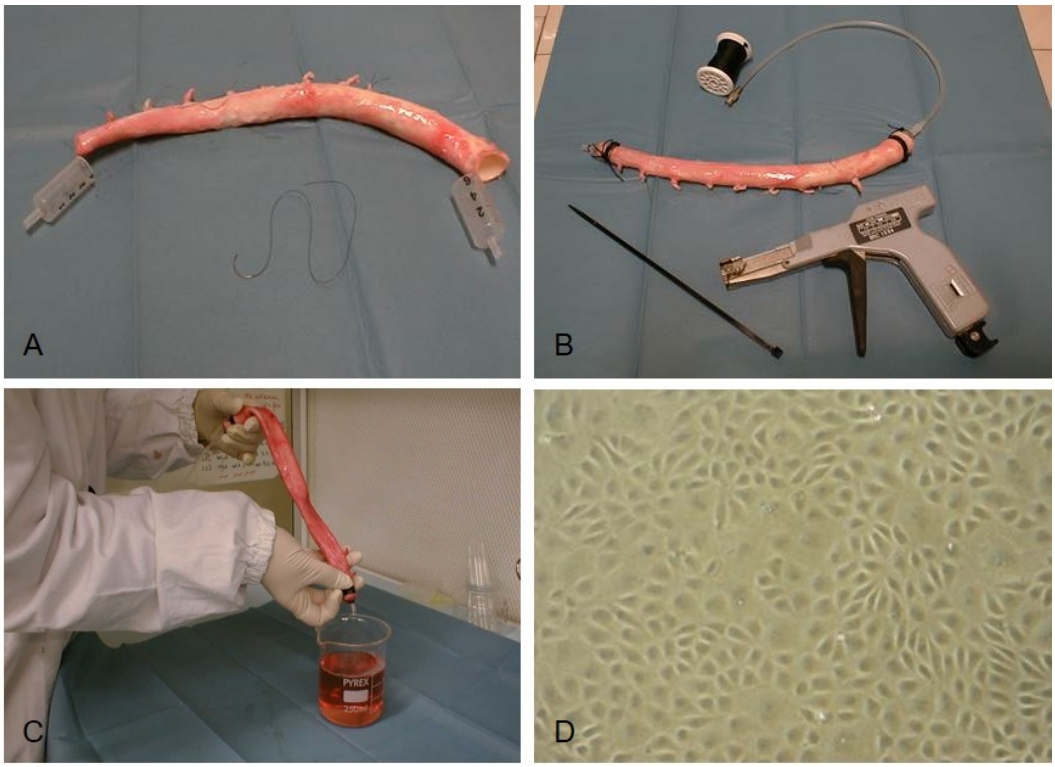

Figure 5. *Isolation of endothelial cells from porcine thoracic aorta. (A) ligation of all arterial side branches; (B) set up a closed system; (C) flushing with DPBS to remove residual blood and verify the closing of the system; (D) porcine Aortic Endothelial Cells (pAECs).*

The complete procedure is available at this link http://www.portaledidatticovet.org/vet02/78-fisiologia/335-colture-cellulari.html

Kindly acknowledgement to Professor Monica Forni for availability the video.

In full accordance with the principle of "Replacement" (Directive 2010/63/EU, 2010; Russell and Burch, 1959), 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.

pAECs were used to study the vascular response to different physical, chemical and biological stimuli as *in vitro* model. In particular, the effects of the magnetic field (Bernardini et al., 2007), the toxic effects induced by the pollutant Tributyltin (TBT) (Botelho et al., 2015) and the alteration induced by components of the outer membrane of the spirochete *Treponema denticola* (OMT) (Bernardini et al., 2010a) were evaluated on pAECs. Moreover, pAECs were employed to develop an *in vitro* inflammatory model induced by lipopolysaccharide (LPS) (Bernardini et al., 2012, 2010b, 2005), preceding the endotoxic shock *in vivo* model and confirming swine as a relevant animal model for translational medicine (Forni et al., 2005; Zannoni et al., 2012, 2010).

In this thesis, the activity of traditional remedies, used since ancient times in Ayurvedic medicine, was evaluated on *in vitro* inflammatory model induced by LPS. In particular, the anti-inflammatory activity of *Boswellia serrata* extracts (Bertocchi et al., 2018) and *Cucumis sativus* extract (Bernardini et al., 2018) was evaluated on pAECs.

Boswellia serrata

Boswellia is a relatively small genus belonging to the family of *Burseraceae,* with 28 distinct species (The Plant List, 2013) distributed in Africa, in the Arabian Peninsula and in India. The best known are *B. serrata* Roxb. ex Colebr., native of India, *B. frereana* Birdw. and *B. papyrifera* (Caill. ex Delile) Hochst., from East Africa (Somalia and Ethiopia) and *B. sacra* Flueck, from the Arabian peninsula (Oman, Yemen and Southern Saudi Arabia) (Brendler et al., 2018; Iram et al., 2017; Shah et al., 2009).

The taxonomic classification of *Boswellia serrata* Roxb. ex Colebr. is the following

- Kingdom: *Plantae*
  - Subkingdom: *Tracheobionta*
    - Superdivision: *Spermatophyta*
      - Division: *Magnoliophyta*
        - Class: *Magnoliopsida*
          - Order: *Sapindales*
            - Family: *Burseraceae*
              - Genus: *Boswellia*
                - Species: *Boswellia serrata* Roxb. ex Colebr.

B. serrata grows to a height of up to 15 m. It is deciduous and has a spreading crown with drooping branches (Fig. 6). It has a short bole (up to 5 m, sometimes longer) with an average girth of 1.2 - 1.8 m. The greyish-green to ashy or reddish bark is very thin, with the outer layer peeling off in thin, papery flakes. Leaves are variable in size, 20 - 45 cm, alternate and imparipinnate, predominantly at the end of branches. White flowers occur in axillary racemes, 10 - 20 cm long. The fruits are trigonous, three-valved drupes, with compressed and pendulous seeds (Brendler et al., 2018).

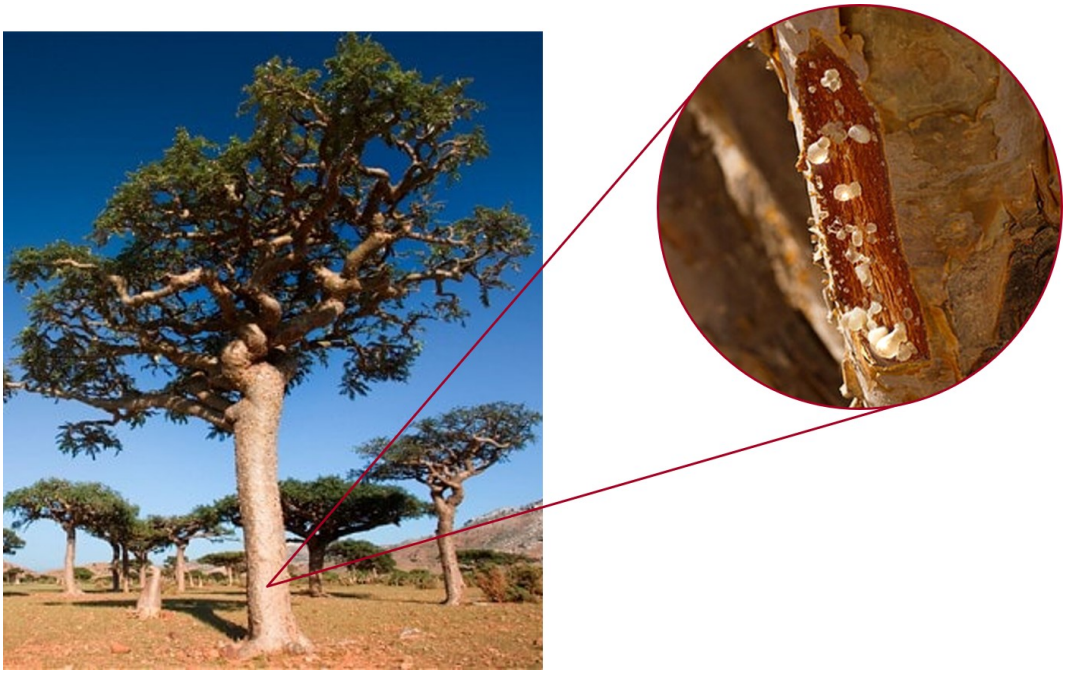

Figure 6. *Indian frankincense, Boswellia serrata tree and a particular of exudate derived by incision of the bark of B. serrata tree.*

The dried exudate from the bark of *B. serrata* tree is an oleogum resin which is commonly known as Indian Frankincense, Indian olibanum, Incense or Salai guggal (Fig. 6). The dried gum appears in transparent, yellow brown fragrant tears around 5 cm long and 2 cm thick (Fig. 7). The tears are brittle and translucent with a waxy surface. The resin burns easily and produces a characteristic, resinous balsamic odour and taste (Brendler et al., 2018).

Figure 7. *Frankincense olibanum resin (photo taken by Peter Presslein).*

Incense was known to all the ancient civilisations and used in rituals and prayers to the gods. Frank “pure” incense and myrrh were the finest and most scarce substances used in religious ceremonies. Because of their rarity and great cost, the gifts of the Magi were a sign of wealth and sacrifice. Beyond that, there is medical evidence that gold, frankincense and myrrh were important for wound healing, used by many cultures and societies for thousands of years. Babylonians, Hindus, Buddhists, Shintoists, Greeks and Romans used incense in their ritualistic ceremonies. The oldest written document, which mentions frankincense as a drug is the papyrus Ebers. Remedies containing preparations from frankincense were used by Hippocrates, Celsus, Galenus and Dioskurides. The use of the oleogum resin of *B. serrata* (salai guggal) is described in Ayurvedic text books (Charaka Samhita, 1st - 2nd century AD and in Astangahrdaya Samhita, 7th century AD). Medical preparations containing the bark or the oleogum resin were used to treat a variety of diseases. These included diseases of the respiratory tract, diarrhoea, constipation, flatulence, central nervous diseases and others. Olibanum was still a remedy in the beginning of the 20th century in Europe. Thus, olibanum is mentioned in the supplement to the 6th edition of the German Pharmacopoeia, which appeared in 1926 (Ammon, 2006).

The oleogum resin of different species of *Boswellia* contains more than 200 phytochemicals. These compounds include 5-9% essential oil, 65-85% alcohol-soluble resin, and the remaining 21-22% is water-soluble gum (polysaccharidic fraction and polymeric substances).The content and composition of oleogum resin may vary from species to species depending upon age, quality of resin, geographical distribution (Al-Yasiry and Kiczorowska, 2016; Ammon, 2006; Iram et al., 2017). The resin contains specific and characteristic pentacyclic and tetracyclic triterpenes. Among the pentacyclic triterpenes, boswellic acids are unique to *Boswellia* genus and responsible for many of the pharmacological effects (Fig. 8). Other compounds are tetracyclic triterpenic acids among which tirucallic acids were also shown to be biologically active (Ammon, 2006; Iram et al., 2017).

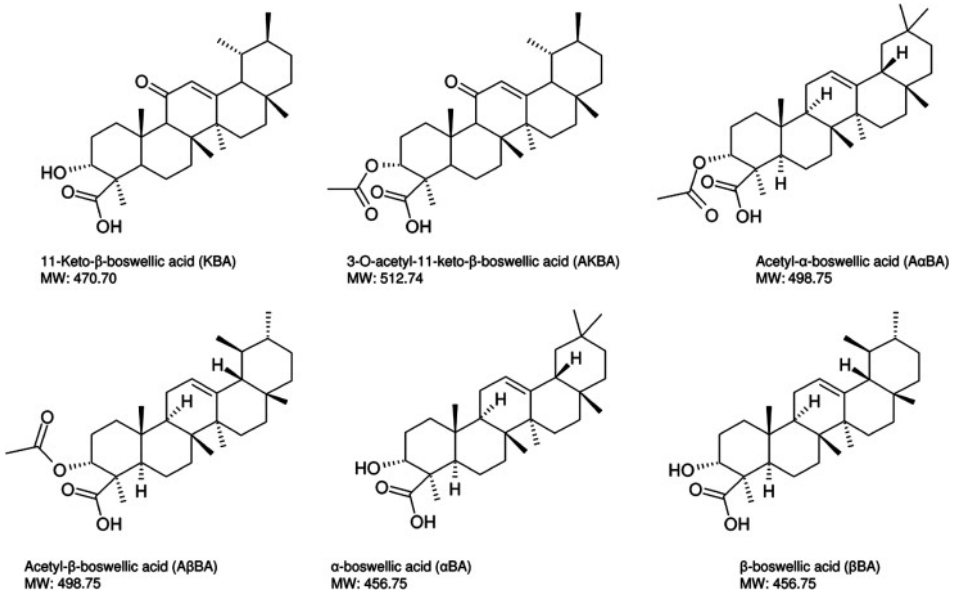

Figure 8. *Structures of major Boswellic acids (Gerbeth et al., 2013).*

The pharmacological effects of *B. serrata* have been mainly attributed to boswellic acids, especially 11-keto-b-boswellic acid (KBA) and acetyl-11-keto-b-boswellic acid (AKBA), which were proposed as selective 5-lipoxygenase (5-LO) inhibitors. However, other important targets of boswellic acids include topoisomerases, angiogenesis, and cytochrome p450 enzymes. Recently, other components of the phytocomplex, such as β-boswellic acid (βBA), have been suggested as anti-inflammatory molecules, acting through inhibition of serine protease cathepsin G (catG) and microsomal prostaglandin E synthase (mPGES) (Abdel-Tawab et al., 2011; Ammon, 2010; Iram et al., 2017).

In the first paper six different *B. serrata* dry extracts and one hydroenzimatic extract were analysed. The first aim of this study was to perform a comparative analysis of AKBA content and *in vitro* antioxidant power of different dry and aqueous extracts of *B. serrata* gum resin as a tool for the evaluation of the quality of the extracts. Furthermore, their ability to modulate the immune system regulatory properties was investigated in *ex vivo* human Peripheral Blood Mononuclear Cells (hPBMCs) model, in collaboration with researcher of Universities of Camerino and Perugia.

In the second paper the chemical characterization was further investigated focusing on HPLC quantification of KBA and βBA. Then, the primary culture of porcine aortic endothelial cells (pAECs) was chosen as an ideal *in vitro* model to study the anti-inflammatory and angiogenic properties of two different formulations of *B. serrata* extracts in comparison with pure AKBA, KBA, and βBA.

First paper

Antioxidant and *Ex Vivo* Immune System Regulatory Properties of *Boswellia serrata* Extracts

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Research Article

Antioxidant and *Ex Vivo* Immune System Regulatory Properties of *Boswellia serrata* Extracts

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Boswellia serrata (BS) is an important traditional medicinal plant that currently represents an interesting topic for pharmaceutical research since it possesses several pharmacological properties (e.g., anti-inflammatory, antimicrobial, and antitumour). The safety and versatility of this dietary supplement should allow for its use in numerous pathological conditions; however the quality of the extracts needs to be standardized to increase the clinical success rate resulting from its use. In the present study, different commercially available *B. serrata* extracts were employed to compare their AKBA content and in vitro antioxidant power. Furthermore, their ability to modulate the immune system regulatory properties was investigated. Our results showed that the AKBA content varied from 3.83 ± 0.10 to 0.03 ± 0.004%, with one sample in which it was not detectable. The highest antioxidant power and phenolic content were shown by the same extract, which also exhibited the highest AKBA concentration. Finally, the BS extracts showed the ability to influence the regulatory and effector T-cell compartments. Our results suggest that frankincense should be further investigated for its promising potentiality to modulate not only inflammation/oxidative stress but also immune dysregulation, but attention should be paid to the composition of the commercial extracts.

1. Introduction

The gum resin of *Boswellia serrata* (BS), a traditional treatment of Ayurvedic medicine in India also identified as Indian frankincense, Salai Guggal, or Indian olibanum, has been used for centuries as a remedy for many health problems [1].

Indeed, the anti-inflammatory, antiarthrogenic, and analgesic activities of its dried resinous gum (guggulu), derived from tapping the *Boswellia* tree, have been recognized since ancient times [2]. The inflammatory response represents the first-line defense of the body to tissue damage and/or to microbial invasion and it determines the recruitment of immune cells and some plasma proteins [3]. The final goal of inflammation is healing, elimination of the external or internal inflammation *noxae,* and the restoration of homeostasis.

This immune response should be self-limiting but the persistence of the stimulus in predisposed subjects leads to chronification of the process and eventually to irreversible tissue injury. Indeed, persisting low-grade inflammation plays a key role in the pathogenesis of many chronic diseases [4] and most of these diseases are also associated with increased production of reactive oxygen species (ROS), which results in oxidative stress [5]. Therefore, inflammation is tightly linked with oxidative stress [6] by an interdependent relationship and both participate in the pathogenesis of many chronic diseases [4].

During recent decades, many authors have investigated the mechanisms of action of BS extracts related to the inflammatory process. Studies in animal models showed that the ingestion of a defatted alcoholic extract of *Boswellia* decreased polymorphonuclear leukocyte infiltration and migration as well as primary antibody synthesis [7, 8] and led to almost total inhibition of the classical complement pathway [9]. In vitro studies revealed that the boswellic acids, a group

of pentacyclic triterpenoid compounds, and their acetylated derivatives inhibit the biosynthesis of leukotrienes, the proinflammatory 5-lipoxygenase products which cause increased permeability [10], in a dose dependent manner [11]. In addition, Cuaz-Pérolin et al. [12] observed that 3-acetyl-11-keto-beta-boswellic acid (AKBA) was a natural inhibitor of the transcription factor NFkB, whose presence is a prerequisite for the formation/action of cytokines/chemokines involved in inflammatory reactions.

Therefore, these natural compounds can dampen the inflammatory response, but also simultaneously reduce oxidative stress, as observed by Umar et al. [13].

In recent years, extracts from the gum resin of BS have been shown to target both the humoral and adaptive immune responses [14] eventually interfering with the inflammatory cascade [15].

However, to the best of our knowledge, no studies have yet investigated whether the BS extracts can exert any effects on specific T-cell subsets whose balance is crucial for the maintenance of immune homeostasis: the regulatory T-cells (Tregs) and the proinflammatory Th1/Th17 cells. In the present study, different commercially available *B. serrata* extracts were employed as follows: (i) to compare their composition and in vitro antioxidant power; (ii) to test their ability to modulate Treg/Th1/Th17 cells *ex vivo*.

2. Materials and Methods

2.1. Chemicals and Plant Material. All chemicals used were of analytical reagent grade from Sigma-Aldrich (St. Louis, MO, USA). Six (A, B, C, D, E, and F, resp.) of the seven BS oleogum resins utilized in the present study were commercially available and were certified for a content of boswellic acids of 65%. The pale yellow or white amorphous powders were insoluble in water but soluble in methanol and dimethyl sulfoxide (DMSO). The seventh (G) BS extract was an aqueous extract obtained by a process of bioliquefaction based on enzyme biocatalysis [16] and was kindly offered by its producer (Phenbiox, Calderara di Reno, Bologna, Italy).

2.2. TLC Analysis. BS extract separation was performed on 20 × 20 cm silica gel plates with a fluorescent indicator at 254 nm (Sigma-Aldrich, St. Louis, MO, USA). Pentane and diethyl ether (2 : 1) containing 1% (v/v) of acetic acid were used as a mobile phase. Twenty mg of BS extracts was dissolved in 300 μL of ethanol, sonicated for 5 minutes, and centrifuged. Clear supernatant (10 μL) was carefully layered at 1.5 cm from the bottom of the plate giving an elution distance of 9 cm. After the separation, plates were observed at 254 nm and developed with anisaldehyde (5 mL) in glacial acetic acid (50 mL) and H_2SO_4 (1 mL). The TLC analysis of BS G extract was not performed due to its particular formulation.

2.3. HPLC-DAD Analysis. A HPLC system (Beckman Coulter, Brea, CA, USA), comprising a 116 pump, a 507 automatic autosampler, an UV-Diode Array 168 detector, and integration software 32 Karat, was used for the analysis of seven BS extracts. Samples were prepared by dissolving extracts in methanol. Briefly, the separation was performed using a reverse phase column Luna C18 5 μm 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) with a guard column PR C-18 5 μm 15 × 4.6 mm (Phenomenex, Torrance, CA, USA). Chromatographic separation was achieved in isocratic conditions at room temperature. The mobile phase was a mixture of phosphoric acid (H_3PO_4 10 mM in water) and acetonitrile (19 : 81 v/v). The flow rate was 1 mL/min, and the injection volume was 50 μL. The analyses were made at two different wavelengths (210 and 260 nm) and UV spectra were recorded in the range of 190–300 nm. A standard stock solution was prepared by dissolving 5 mg of AKBA analytical standard (Sigma-Aldrich, 5 mg, batch number BCBN2928V, CAS number 416619) in methanol (5 mL). The calibration curve was obtained by analyzing nine serial dilutions (50 ppm, 25 ppm, 15 ppm, 10 ppm, 7.5 ppm, 5 ppm, 2.5 ppm, 1 ppm, and 0.5 ppm) of the stock solution and by plotting the peak area measured at 260 nm against AKBA concentrations. The following equation of the curve was obtained:

$$y = 79739x - 5414, \quad R^2 = 0.999. \tag{1}$$

The AKBA peaks were identified on the basis of the retention time on the chromatogram at 260 nm. All measurements were performed in triplicate and data were reported as mean ± SD.

2.4. Quantification of Total Phenolic Content (Folin-Ciocalteu Method). The total content of polyphenolics was determined by a colorimetric method as described by Singleton and Rossi [17] and adapted to a 96-well plate format. Briefly, the seven BS extracts were redissolved in 1 mL of methanol and 100 μL/well of each extract was dispensed into a flat bottom 96-well tissue culture plate (Becton Dickinson, Lincoln Park, NJ); then 150 μL Folin-Ciocalteu reagent (1 mL Folin-Denis' reagent in 4 mL H_2O) was added. The plate was incubated for 10 min at 37°C. Next, 50 μL of a saturated Na_2CO_3 solution in H_2O was added to each well and the plate was incubated for a further 10 min. The absorbance was measured at 765 nm. A standard calibration curve was plotted using gallic acid (0–300 mg/L). The results were expressed as g of gallic acid equivalents (GAE) per g of dry weight of BS extract. The results were expressed as the average of three measurements.

2.5. Antioxidant Activity. Free radical scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) on a microplate analytical assay according to the procedures described by Srinivasan et al. [18], while the total radical scavenging capacity of the same products was measured by the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay as modified by Re et al. [19], for application to a 96-well microplate assay.

Finally, the determination of antioxidant activity by FRAP assay was carried out according to the procedure described by Müller et al. [20], monitoring the reduction of $Fe3^+$-tripyridyl triazine (TPTZ) to blue-colored $Fe2^+$-TPTZ.

Trolox was used as standard in all assays and the ability of BS extracts to scavenge the different radicals was expressed as tocopherol-equivalent antioxidant capacity (mmol TE/g of product) and, for DPPH and ABTS assays, also as IC_{50}, the latter defined as the concentration of the tested material required to cause a 50% decrease in initial DPPH/ABTS concentration. All measurements were performed in triplicate and reported as mean ± standard deviation (SD).

2.6. Immune Responses

2.6.1. Cell Proliferation Assay. Human peripheral blood mononuclear cells (PBMCs) of seven healthy donors (HD; 5 male and 2 female, age mean ± standard deviation: 47 ± 12.1 years) were isolated from fresh heparinized venous blood (10 mL/HD) by gradient separation. The study was approved by the local ethics committee (CEAS Umbria) and written informed consent was obtained from participants in accordance with the Declaration of Helsinki. The final concentration of live cells was adjusted to 1×10^6/mL in complete medium (RPMI-1640 medium containing 10% heat-inactivated serum, L-glutamine (2 mM), Euroclone®, penicillin (100 U/mL), and streptomycin (100 μg/mL), Biochrom^{AG}, Berlin). PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) cell tracer (BioLegend, San Diego, CA), dispensed into flat bottom 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) (1 mL/well), and cultured for 5 days at 37°C in 5% CO_2. For proliferation stimuli were either 1 μg/mL of pokeweed mitogen (PWM; Sigma-Aldrich Co. Ltd., Saint Louis, Missouri) or 1.2 μg/mL of phytohemagglutinin (PHA; Biochrom^{AG}, Berlin), in the presence or absence of two BS extracts (0.1 μg/mL). BS extract A revealed the highest in vitro antioxidant power; BS extract G was obtained with a different extraction method compared to other BS compounds (by Phenbiox srl). So lymphocytes were exposed, in the culture medium, to an AKBA concentration of 3.8 ng/mL that resulted below the mean maximal concentration of 6 ng/mL detected in human plasma after an oral administration of a BS dry extract in fasted condition [21].

A negative control was represented by PBMC cultured without any mitogen/extract (C), so that the base proliferation could be estimated [22]. Therefore, nine different experimental theses for each blood sample were tested. Flow cytometry analyses were performed on a standard FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA) running the CellQuestPro™ software. The results of the lymphocyte proliferation assay were expressed as a percentage (%). Furthermore, the lymphocyte proliferation index (LPI) was calculated with the following formula:

$$\text{LPI} = \frac{(\text{FP} - \text{BV})}{\text{BV}} * 100, \quad (2)$$

where the FP values are represented by the "final percentages" of cell proliferation (after 5 days in culture with BSs and with/without the mitogens), whereas the BV values are represented by the "basal values" obtained by cells either stimulated (with PHA and PWM) or not (CTR) with the mitogen but without BSs [23].

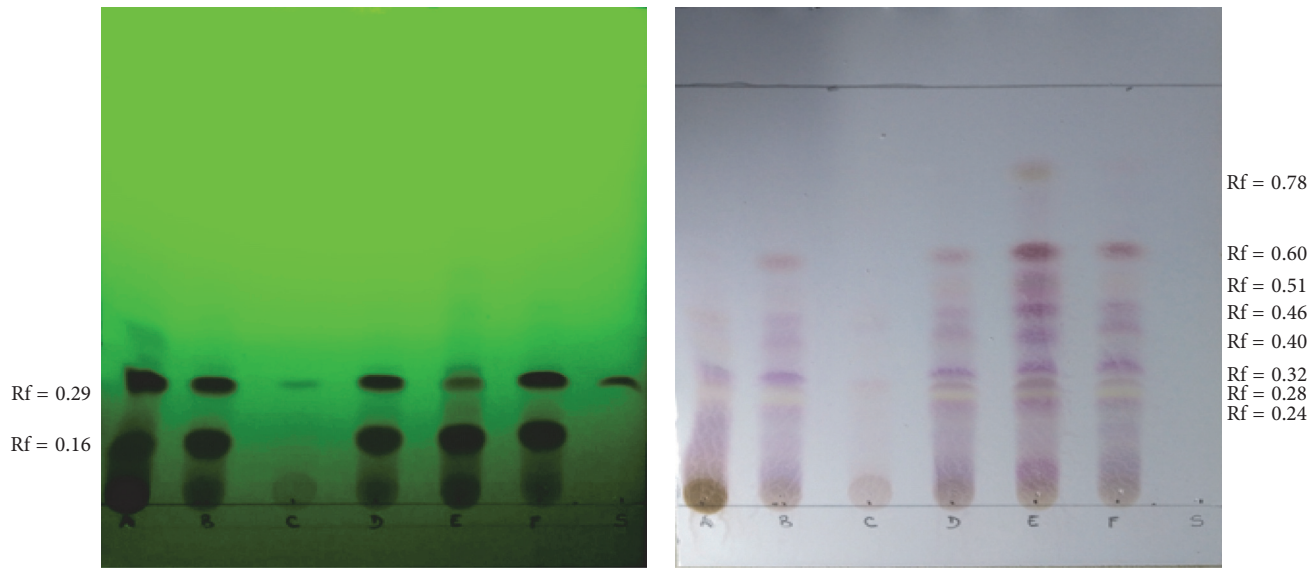
2.6.2. Phenotypic Characterization of Peripheral Blood Mononuclear Cells. PBMCs obtained from the seven volunteers were seeded further (1×10^6 cells/well) into additional flat bottom 24-well tissue culture plates and cultured, with or without PHA, for 5 days at 37°C in 5% CO_2. After culture, six-hour in vitro stimulation with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μg/mL ionomycin, and 1 μL/mL BD GolgiPlug™ (BD Biosciences) in complete medium was performed. For surface staining, fluorescein isothiocyanate (FITC), PeCy7, or APC labelled antihuman CD4, CD3, and CD25 and respective isotypes were used (BD Biosciences, San Jose, CA, USA, Immunotools). Then, cells were permeabilised with 0.1% saponin blocking buffer after 4% paraformaldehyde fixation to perform intracellular staining with Alexa Fluor 647 or Phycoerythrin (PE) antihuman IL-17 and INFγ, and their isotype controls were used (BD Biosciences). When required, cells were permeabilised with commercially available Forkhead box protein P3 stain buffer (BD Biosciences) for intracellular staining with PE-labelled mAb to human FoxP3 and respective isotype controls [24]. Debris was excluded by backgating to CD3 T-cells in forward scatter/side scatter (FSC/SSC) plots. Samples were analyzed using FACSCalibur flow cytometer (BD) and CellQuestPro software (BD).

2.7. Statistical Analysis. The results of immune responses are reported as mean ± standard error of the mean (SEM) from seven samples of different HD. The unpaired Student's t-test was used to compare biological data from controls with that from BS A or BS G treated samples, respectively (GraphPad Prism, 2007) [25]. p values < 0.05 for two-tailed test were considered statistically significant.

3. Results

3.1. TLC Chromatograms. The preliminary qualitative TLC screening of the BS extracts is shown in Figure 1. The UV analysis revealed two main spots visible in all samples, with the exception of extract C. The first spot had a Rf of 0.16, whereas the second spot had a Rf of 0.29. The use of the AKBA standard (lane S) allowed for the identification of this boswellic acid in the spot with Rf of 0.29. It is noticeable that extract C lacked the first spot and presented only traces of the spot corresponding to AKBA, while extract A presented the greatest spot referring to AKBA. Extracts A and E presented other components absorbing at 254 nm. Figure 1(b) demonstrates the pattern obtained after dyeing with anisaldehyde, heating, and color development. Similar profiles were obtained for extracts B, D, E, and F. Instead, extract E was characterized by a major number of spots; extract A lost the spots at the higher Rf; and extract C was found to have fewer components. The majority of samples shared the spots detected at Rfs of 0.24, 0.32, 0.40, 0.46, and 0.60.

3.2. HPLC-DAD Analysis. At 260 nm, the majority of extracts presented two major peaks: the first one, at Rt of 13.2 min, and



(a) (b)

Figure 1: UV detection at 254 nm (a) for UV-active boswellic acids. Chromatograms after dyeing with anisaldehyde (b). Rf values are reported for the most relevant spots. A–F: six different powder extracts of *Boswellia serrata* gum resin; S: 3-acetyl-11-keto-beta-boswellic-acid (AKBA) analytical standard (Rf = 0.29).

Table 1: AKBA quantification in *Boswellia serrata* extracts. Data are reported as mean ± SD (n = 3). Samples A–F = powder extracts, sample G = hydroenzymatic extract.

Sample	Concentration[§]	% in *B. serrata* extract[#]
A	38.30 ± 1.01	3.83 ± 0.10
B	17.18 ± 0.05	1.72 ± 0.005
C	3.08 ± 0.06	0.31 ± 0.01
D	24.35 ± 1.87	2.43 ± 0.19
E	nd[*]	nd
F	21.07 ± 0.16	2.11 ± 0.02
G	0.29 ± 0.04	0.03 ± 0.004

[§]AKBA concentration is expressed as mg/g of powder extract, with the exception of sample G (mg/mL of hydroenzymatic extract); [#]AKBA percentage is expressed as g/100 g of powder extract, with the exception of sample G (g/100 mL of hydroenzymatic extract); [*]nd = not detectable.

the second one, identified as AKBA by the use of an analytical standard, at Rt of 26 min. Other minor peaks were also present. AKBA concentrations for each sample, calculated on the basis of the peak area and the calibration curve, are shown in Table 1. Extract A presented significantly higher amounts of AKBA as compared with the other samples. Extract C presented only a small peak of AKBA and was lacking the first peak.

Other components in the BS extracts were visualized at 210 nm. The chromatograms of all the BS extracts analyzed at the different wavelengths to highlight the variability of the components present in the samples are reported in Figure 2.

3.3. Determination of Total Phenolic Content and Antioxidant Capacity of Boswellia serrata Extracts. All the BS extracts utilized in the present study exhibited a relatively low content in phenolics ranging from 7.68 ± 0.9 mg gallic acid equivalent (GAE)/g (extract A) to 0.11 ± 0.05 mg GAE/g (aqueous extract G) (Table 2). The chemical complexity of the extracts, often mixtures of many compounds with differences in functional groups, polarity, and chemical behavior, could lead to scattered results, depending on the antioxidant test employed. For these reasons, in the present study, the BS extracts were screened for their free radical scavenging and reducing properties through three test systems: (a) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, (b) monitoring of the reduction power of $Fe3^{+}$ (FRAP assay), and (c) evaluating the total radical scavenging capacity (ABTS assay). All the BS extracts analyzed showed relative radical scavenging activities in all the assays employed, revealing antioxidant powers lower (from 34 to nearly 580 times) than that of Trolox (positive control, Table 2).

However, between the BS extracts investigated, the BS extract A showed the highest scavenging reducing power and the highest polyphenolic content.

3.4. Immunomodulatory Activity. The in vitro lymphocyte proliferation (CFSE assay) was not influenced by the BS extracts if cells were cultured without any activator (data not shown) or if stimulated by PHA. However, when cells were activated by PWM, the addition of BS extracts induced a significantly higher lymphocyte response (Figures 3(a) and 3(b)). No significant differences were observed between the two types of BSs for the LPI (Figures 3(c) and 3(d)).

The in vitro regulatory or Th1/Th17 proinflammatory responses (Figures 4(d), 4(e), and 4(f)) were not significantly modulated by the addition of the BS extracts when PBMCs were triggered by PHA. Neither type of utilized extract (A versus G) elicited an altered response.

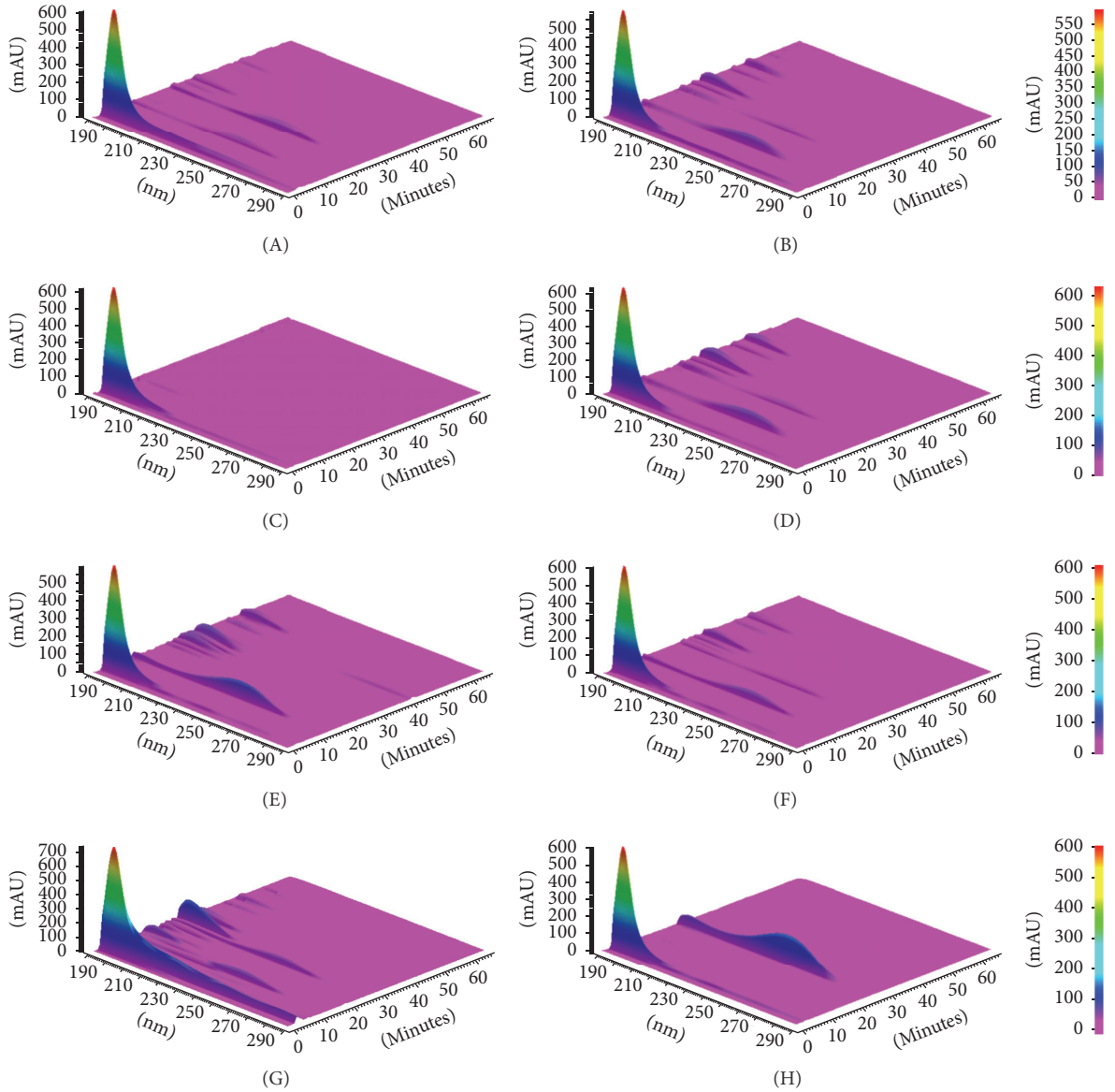


Figure 2: Chromatograms of *Boswellia serrata* extracts (BS) after HPLC-DAD analysis. BS (A)–(F) extracts were diluted 1 : 400 in methanol, whereas, BS (G) extract was diluted 1 : 20 in methanol. The chromatogram of the AKBA analytical standard is also reported (H). The absorbance (mAU) is reported on the *y*-axis, wavelength (nm) on *x*-axis, and the retention time (minutes) on the *z*-axis.

Table 2: In vitro radical scavenging activity and polyphenolic content of different *Boswellia* extracts.

Boswellia serrata extracts	Polyphenols mg GAE/g	DPPH		ABTS		FRAP
		TEAC[a] μmol TE/g	IC_{50}[b] μg/ml	TEAC μmol TE/g	IC_{50} μg/ml	TEAC μmol TE/g
A	7.68 ± 0.9	31.8 ± 0.7	340.2 ± 3.5	151.8 ± 10.6	79.26 ± 1.8	66.89 ± 3.5
B	1.43 ± 0.5	4.48 ± 0.08	2416 ± 12.5	37.54 ± 2.5	320.30 ± 4.5	ND
C	0.56 ± 0.2	3.83 ± 0.08	2823 ± 27.5	1.92 ± 0.1	6250 ± 17.5	ND
D	1.09 ± 0.4	6.29 ± 0.12	1720 ± 13.8	27.8 ± 1.9	431.62 ± 6.5	ND
E	1.09 ± 0.3	5.41 ± 0.11	1998 ± 17.5	18.7 ± 1.2	641.75 ± 9.5	ND
F	0.96 ± 0.3	5.20 ± 0.15	2080 ± 22.5	20.6 ± 21.4	581.94 ± 8.5	ND
G	0.11 ± 0.05	1.85 ± 0.03	5820 ± 32.5	1.76 ± 0.1	6800 ± 22.5	ND
Positive control						
Trolox			10.85 ± 0.2		3.01 ± 0.2	

[a]TEAC = Trolox equivalent (TE) antioxidant concentration. [b]IC_{50} = The concentration of compound that affords a 50% reduction in the assay; GAE = gallic acid equivalent. ND = not detectable.

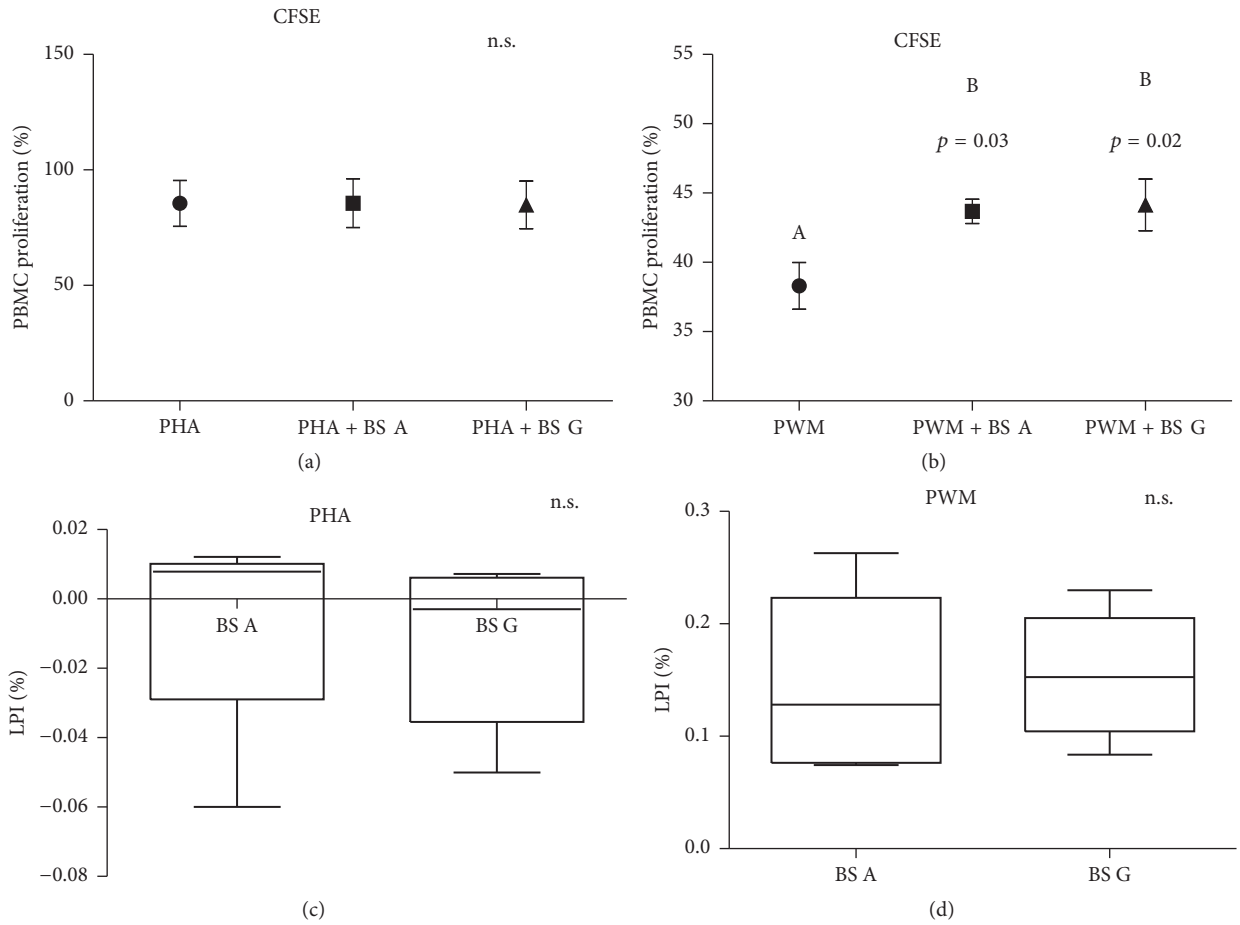


Figure 3: *Boswellia serrata* (BS) extract (A or G) effects on lymphocyte proliferation assay. Data are shown as mean ± SEM of seven independent experiments. PBMCs were cultured with phytohemagglutinin (PHA; graphics (a) and (c)) or pokeweed mitogens (PWM; graphics (b) and (d)) and stained with carboxyfluorescein diacetate succinimidyl ester cell tracer (CFSE). The lymphocyte proliferation index (LPI) was calculated as reported in the text. ^{A,B}Different letters for $p < 0.05$. n.s. = not significant.

However, when cells were not PHA pulsed, an increase of FOXP3$^+$ cells was observed in PBMCs cultured with the BS extracts. In particular, a tendency towards a higher number of regulatory cells was observed for extract A ($p = 0.079$), whereas extract G led to a significant increase of FOXP3$^+$ cells ($p = 0.045$; Figure 4(a)).

Furthermore, higher number of Th17$^+$ cells, although not significant, was again observed when PBMCs were cultured with the extract G (Figure 2(C)).

4. Discussion

The therapeutical efficacy of BS extracts has been extensively investigated in arthritis, asthma, diabetes mellitus, colitis, and cancer [26, 27] in light of their antioxidant and anti-inflammatory activities [28]. Indeed, all these diseases share a persistent dysregulation of redox status that contributes to the intensity and duration of the inflammatory response and therefore to the induction and perpetuation of chronic inflammation.

It is noteworthy that the phytochemical content of *B. serrata* oleogum resin is dependent on both the botanical origin and the geographical origin [29]. Usually it consists of 30–60% triterpenes (such as α- and β-boswellic acids, lupeolic acid), 5–10% essential oils, and 20–35% polysaccharides [30]. According to Singh et al. [31], in aqueous and ethanolic extracts of *B. serrata*, it is possible to recognize alkaloids, carbohydrates, phytosterols, terpenoids, phenolic compounds, flavonoids, and tannins. However, other authors found also glycosides, proteins, and saponins [32].

Furthermore, the wide variations of pharmacologically active molecules in commercial BS formulations could significantly affect the final product [33].

The first aim of this study was to perform a comparative analysis on the composition of different dry and aqueous extracts of *B. serrata* gum resin as a tool for the evaluation of the quality of the extracts.

Combination of TLC and HPLC analyses can be considered as a multidimensional analytical approach combining fast qualitative screening with an accurate and precise quantification of specific compounds. We decided to quantify AKBA because the boswellic acid, characteristic and unique to *Boswellia* genus, is considered the most effective, at least in *in vitro* studies. When analyzed at 260 nm, four of the

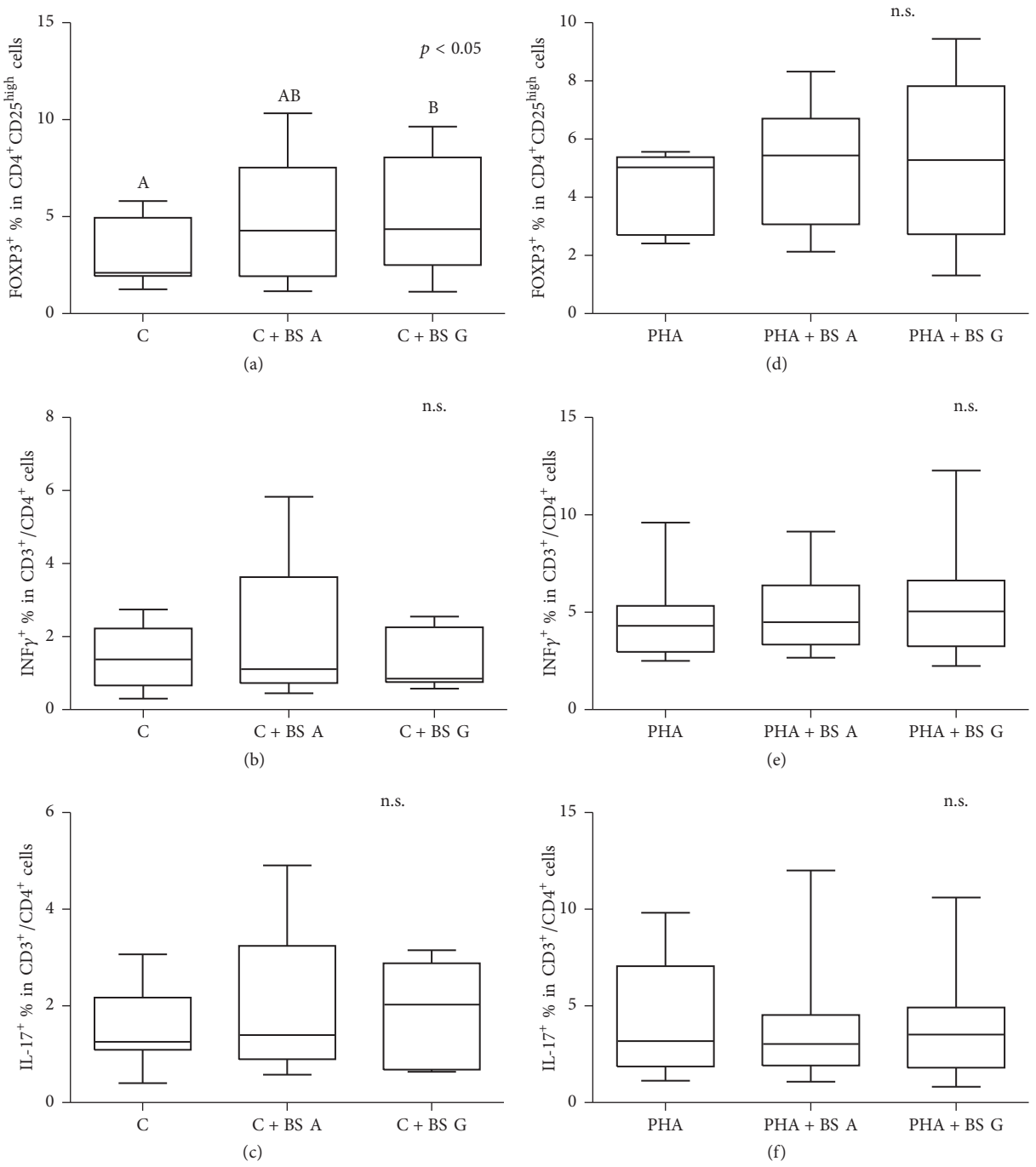


Figure 4: *Boswellia serrata* (BS) extract (A or G) effects on Treg ($CD4^+CD25^+FOXP3^+$ cells), Th1 lymphocyte ($INF\gamma^+$ cells), and Th17 cell ($IL\text{-}17^+$) responses. Data are shown as mean ± SEM of seven independent experiments. PBMCs were cultured in absence (controls, C; graphics (a), (b), and (c)) or presence of mitogen (phytohemagglutinin, PHA; graphics (d), (e), and (f)) and with/without BS extracts. ^{A,B}Different letters for $p < 0.05$. n.s. = not significant.

seven samples presented comparable profiles, characterized by the presence of two main peaks the second of which corresponds to AKBA. The other components of BS extracts, lacking the keto moiety, were visualized only at a less specific wavelength (210 nm) as already reported [34]. The AKBA concentrations detected in samples A, B, D, and F are similar to those reported by other authors [30, 35], while those found in extracts C and G were 10 or 100 times lower, respectively. It can be hypothesized that extract C belongs to *Boswellia* species other than *B. serrata*, due to a wrong botanical identification by local producers. Indeed, it has already been reported that the elution profile of *Boswellia frereana* gum resin lacks KBA and AKBA peaks [34], while *Boswellia sacra* gum resin contains much lower amounts of KBA and less AKBA than *B. serrata* [34, 36]. Concerning extract G, this aqueous extract showed a lower content of AKBA, but it

was enriched by other components of the phytocomplex, as demonstrated by the additional peaks obtained in the elution profile at 210 nm. The presence of these compounds is probably related to the particular and innovative extraction method [16].

The composition of BS extract E is challenging, due to the discrepant results in TLC and HPLC analysis. The spot at Rf of 0.26, a putative AKBA component, was not confirmed by a corresponding peak in the HPLC chromatogram at 260 nm. Other components of the phytocomplexes containing a keto moiety should have contributed to this spot and further analyses are needed to identify these molecules.

The antioxidative potential and radical scavenging activity of aqueous and ethanolic extracts of *B. serrata* are significantly correlated to their total phenolic and flavonoid content [31].

According to Kohoude et al. [37], the amount of phenolics in *Boswellia* genus (315 g/kg) is comparable to reference extracts rich in phenolic compounds. Despite what is reported in literature, the BS samples investigated here were all characterized by either relatively low antioxidant properties or total phenolic content. Indeed, the latter ranged from 0.11 ± 0.05 to 7.68 ± 0.9 mg GAE/g versus values of 28.46–12.73 mg GAE/g obtained by other authors in aqueous and ethanolic extracts, respectively [31].

According to the extraction procedure, the antioxidant activity increased with the polarity of the solvent. Other authors reported that the essential oil of *Boswellia dalzielii* was characterized by low antioxidant activity [36] and that this was due to the extraction method adopted (e.g., low polarity of the solvent) that determined the absence of phenolics, especially flavonoids.

In the present study, the extraction methods adopted by manufacturers or even the preservative systems used could be responsible for the low level of total phenolic and flavonoids compounds, which are contained mainly in the volatile essential oil component of the oleogum resin.

Furthermore, although theoretically the aqueous extract (extract G) should have characteristics closest to the natural product being obtained by an enzymatic hydrolysis that maintains the intact phytocomplexes [16], we observed that it was the one with the lowest antioxidant properties and total phenolic contents.

However, these results are at least partially in line with those of other authors reporting that the wild habitat samples, with a completely different profile as compared to the market samples, were those lacking antioxidant activity [28].

Nevertheless, the BS extracts of the present study were able to significantly modulate some immune responses investigated independently of the in vitro antioxidant activities. As reported in Figure 3(b), when cells were stimulated by PWM, a mitogen that stimulates B lymphocytes in the presence of T-cells, the PBMC proliferation was significantly increased ($p < 0.05$) by the addition of the BS extracts (0.1 μg/mL for both extracts A and G) and the LPI did not change between the two BS extracts (Figure 3(d)).

Conversely, when cells were activated by PHA, neither the PBMC proliferation (mainly, T-cells$^+$) nor the LPI were affected by the BS extracts (Figures 3(a) and 3(c)).

It has been previously reported that the BS could produce opposing effects on immune responses in vivo or in vitro. Potentially low concentrations of BSs increase stimulated proliferation of lymphocytes whereas higher concentrations are even inhibitory [14].

Sharma et al. [38] reported that a mixture of various boswellic acids in the range of 1.95–125 μg/mL inhibited mice splenocytes stimulated with lipopolysaccharides (LPS), PHA, alloantigen, and concanavalin A (ConA), in a concentration-dependent manner. Indeed, a significant inhibition of splenocytes to mitogens and alloantigens was observed starting from concentrations greater than 3.90 μg/mL.

On the other hand, Gayathri et al. [15] observed that 30 μg/mL of a crude methanolic BS extract is able to inhibit almost 80% of human lymphocyte proliferation. These data are in striking contrast with the observations of other authors [39] who tested the effect of 1 mg/mL of BS total alcoholic extract, gum, or volatile oil on human lymphocyte proliferation and observed no inhibition of cells stimulated with either PHA or Con A.

Besides the different lymphocyte proliferation assays applied, the BS concentrations used in the present study were 10 to 1000 times lower than those adopted in the cited articles and, at these doses, we obtained an effect on the lymphocyte proliferation only when cells were stimulated by PWM (B cells$^+$).

However, in mice treated with orally administered boswellic acids, the secondary antibody titres were appreciably enhanced at the lowest tested doses (25 mg/kg body weight versus 100–200 mg/kg) [38].

The dose of BS extracts we adopted was probably low enough to induce an effect on B cells$^+$ (activated by PWM), but too low to induce an inhibition of T lymphocyte proliferation, as reported by other authors [14, 39].

In the maintenance of T-cell balance a pivotal role is attributed to T-helper cells and regulatory T-cells [40, 41]. T-helper cells are defined as Th1-, Th2-, or Th17-cells and are characterized by differential expression of certain cytokines [42]. Th1-cells have the capacity to express the key cytokine interferon-γ (IFN-γ), whereas Th17-cells, a more recently described T-helper cell subset, evolutionally and functionally divergent from Th1 and Th2 cell subsets, are characterized by their ability to produce interleukin-17A (IL-17A) [43].

Regulatory T-cells (Tregs) suppress effector T-cells and, in humans, can be characterized by a $CD4^+CD25^{high}FoxP3^+$ phenotype [41].

In our study, we observed that when PBMCs from healthy controls were not activated by PHA mitogen, the presence of BS extract G in the culture medium determined a significant increase of Tregs (Figure 4(a)). Furthermore, the increased number of Treg cells in BS G treated samples was accompanied by a higher number, although not significant, of Th17$^+$ cells. Conversely, the BS extracts did not influence the number of Th1$^+$ cells (INFγ^+). When PBMCs were pulsed by PHA, no additional effect could be seen following the BS extract addition.

The recent evidence of a developmental plasticity between Treg and Th17 cells prompts the investigation of intermediate phenotypes that result from their reciprocal conversion

according to the surrounding microenvironment [44]. The presented results show a possible role for BS extracts in such a fine balance between these two cell subsets.

Furthermore, it is important to note that, at least in mice, Th17 lymphocytes can also function as B-cell helpers [45], mediating B-cell differentiation and antibody class switch recombination. The results of our lymphocyte proliferation assay showed that the BS extracts exerted a significant stimulatory effect on B+ cell proliferation, possibly mediated by an enhanced number of $Th17^+$ cells.

Many authors have demonstrated that *B. serrata* extracts turn out to be effective in the treatment of diseases such as inflammatory bowel disease and osteoarthritis in which inflammation and/or oxidative stress exert an important pathogenic role [2, 13, 30, 46].

However, BS extracts also exerted beneficial effects in some autoimmune diseases, such as rheumatoid arthritis [1], where chronic inflammation and an aberrant autoimmune response are hallmarks of the disease [47].

This *ex vivo* study provides evidence that *B. serrata* extracts, besides their reported capacity in dampening the inflammatory response together with counteracting the oxidative stress, were able to influence the regulatory and effector T-cell compartments.

In order to draw conclusions, it will be necessary to deepen the experiment on a wider case study. However, these preliminary results suggest that frankincense should be further investigated for its promising ability to interfere, possibly also through such regulatory mechanism, on immune dysregulation typical of various immune disorders, but attention should be paid to the quality of the commercial extracts which can show wide variations in their chemical composition.

Conflicts of Interest

Authors declare that there are no conflicts of interest regarding the publication of this paper.

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Second paper

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

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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-β-boswellic acid (KBA) and 3-O-acetyl-11-keto-β-boswellic acid (AKBA) were proposed to act as inhibitors of 5-lipoxygenase (5-LO) [11, 12]. Recently, other components of the phytocomplex, such as β-boswellic acid (βBA), 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 bioliquefaction 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 βBA [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 βBA. 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 βBA, 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 βBA (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, α-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 βBA concentrations and were prepared by dissolving extracts in methanol. KBA and βBA 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 βBA concentrations. The following equations of the curves were obtained:

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

The KBA and βBA 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 (3×10^5 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 μg 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 μg 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 μg/ml of AKBA.

Pure analytical grade BAs (KBA, AKBA, and βBA) 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 βBA, and *high*, corresponding to 380 ng/ml AKBA, 300 ng/ml KBA, and 800 ng/ml βBA. 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 (6×10^3 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 (6×10^3 cells/cm^2) were exposed to lipopolysaccharide (LPS) (25 μg/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 (4×10^4 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 μg dry extract/ml and 10 μg dry extract/ml) and extract G (2.4 μg resin/ml and 240 μg resin/ml) and pure BAs at low (3 ng/ml KBA, 3.8 ng/ml AKBA, and 8 ng/ml βBA) and high (300 ng/ml KBA, 380 ng/ml AKBA, and 800 ng/ml βBA) 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 βBA Quantification by HPLC-DAD Analysis. Representative chromatograms of KBA, AKBA, and βBA 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 βBA by the use of the analytical standard. KBA, AKBA, and βBA 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 βBA 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 μg 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 βBA 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 βBA 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

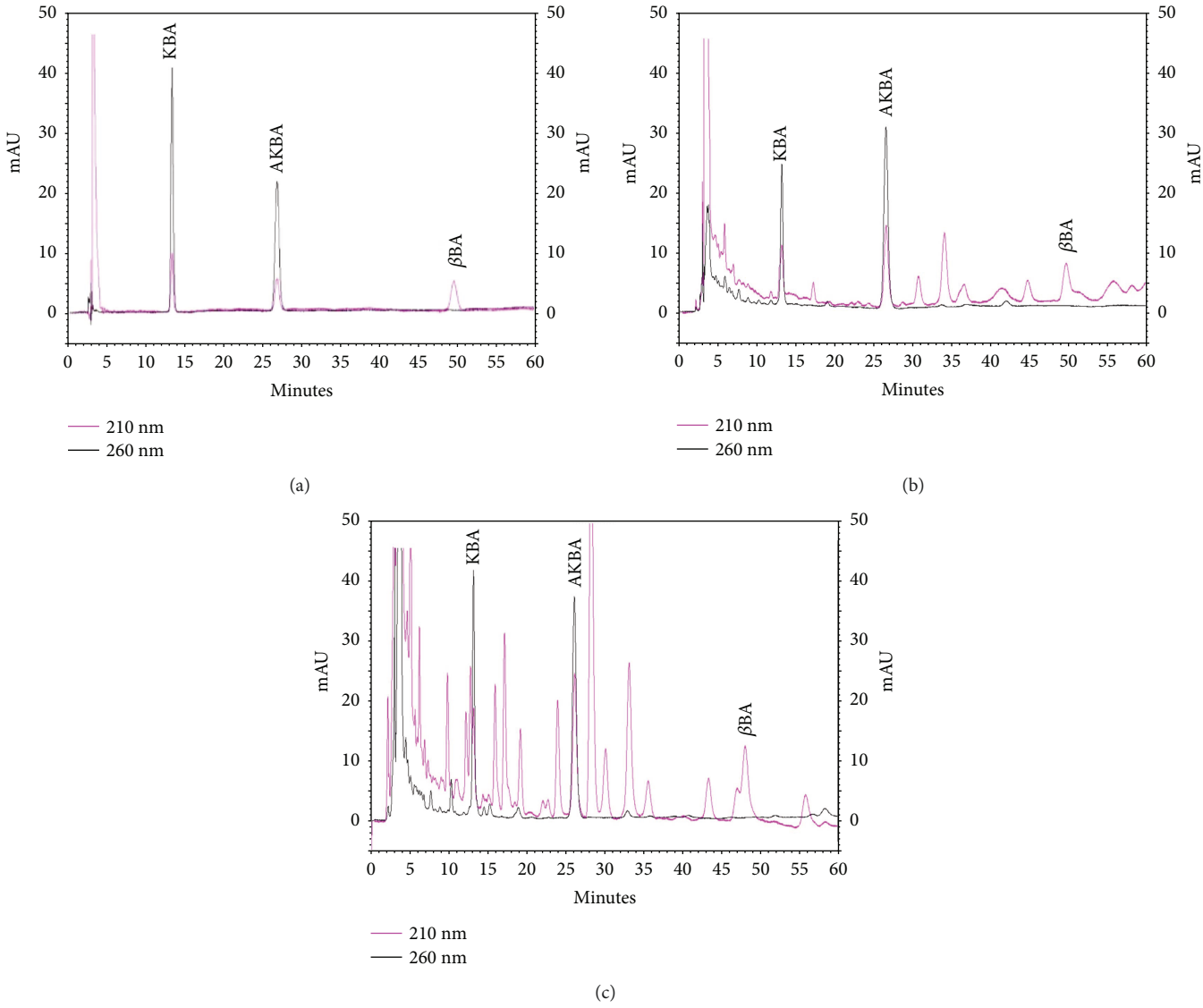

Figure 1: Representative chromatograms of pure analytical grade BAs (KBA, AKBA, and βBA) (25 ppm each) (a), extract A (b), and extract G (c) at 210 (pink chromatogram) and 260 nm (black chromatogram).

Table 1: KBA, βBA, 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[§]	βBA	AKBA[§]
A	$15.86 \pm 0.56^{**}$	$33.53 \pm 7.23^{*}$	$38.30 \pm 1.01^{**}$
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 TNFα-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

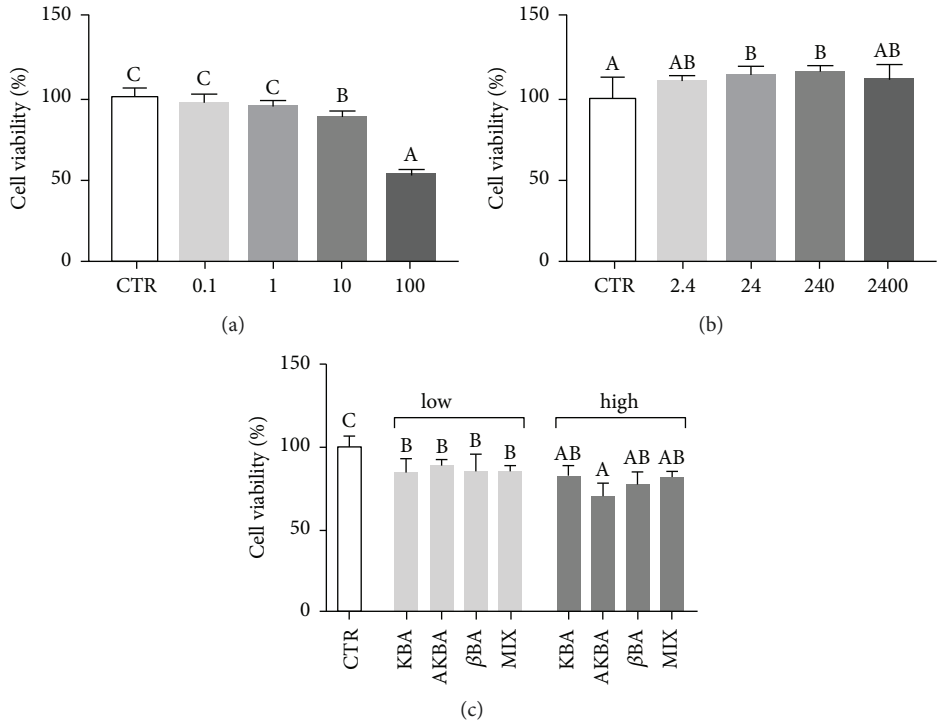

Figure 2: Effect of increasing doses of *B. serrata* extract A (0.1, 1, 10, and 100 μg dry extract/ml) (a), extract G (2.4, 24, 240, and 2400 μg resin/ml) (b), and pure BAs (*low*, corresponding to 3.8 ng/ml AKBA, 3 ng/ml KBA, and 8 ng/ml βBA, and *high*, corresponding to 380 ng/ml AKBA, 300 ng/ml KBA, and 800 ng/ml βBA) (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).

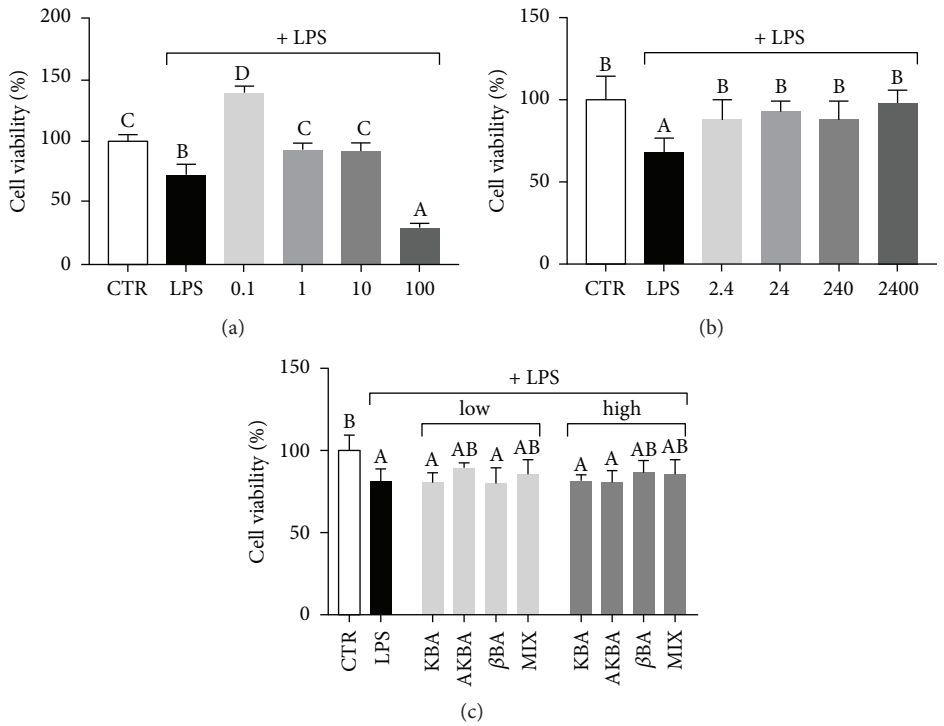

Figure 3: Effect of increasing doses of *B. serrata* extract A (0.1, 1, 10, and 100 μg dry extract/ml) (a), extract G (2.4, 24, 240, and 2400 μg resin/ml) (b), and pure BAs (*low*, corresponding to 3.8 ng/ml AKBA, 3 ng/ml KBA, and 8 ng/ml βBA, and *high*, corresponding to 380 ng/ml AKBA, 300 ng/ml KBA, and 800 ng/ml βBA) (c) on pAEC viability, in the presence of LPS (25 μg/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).

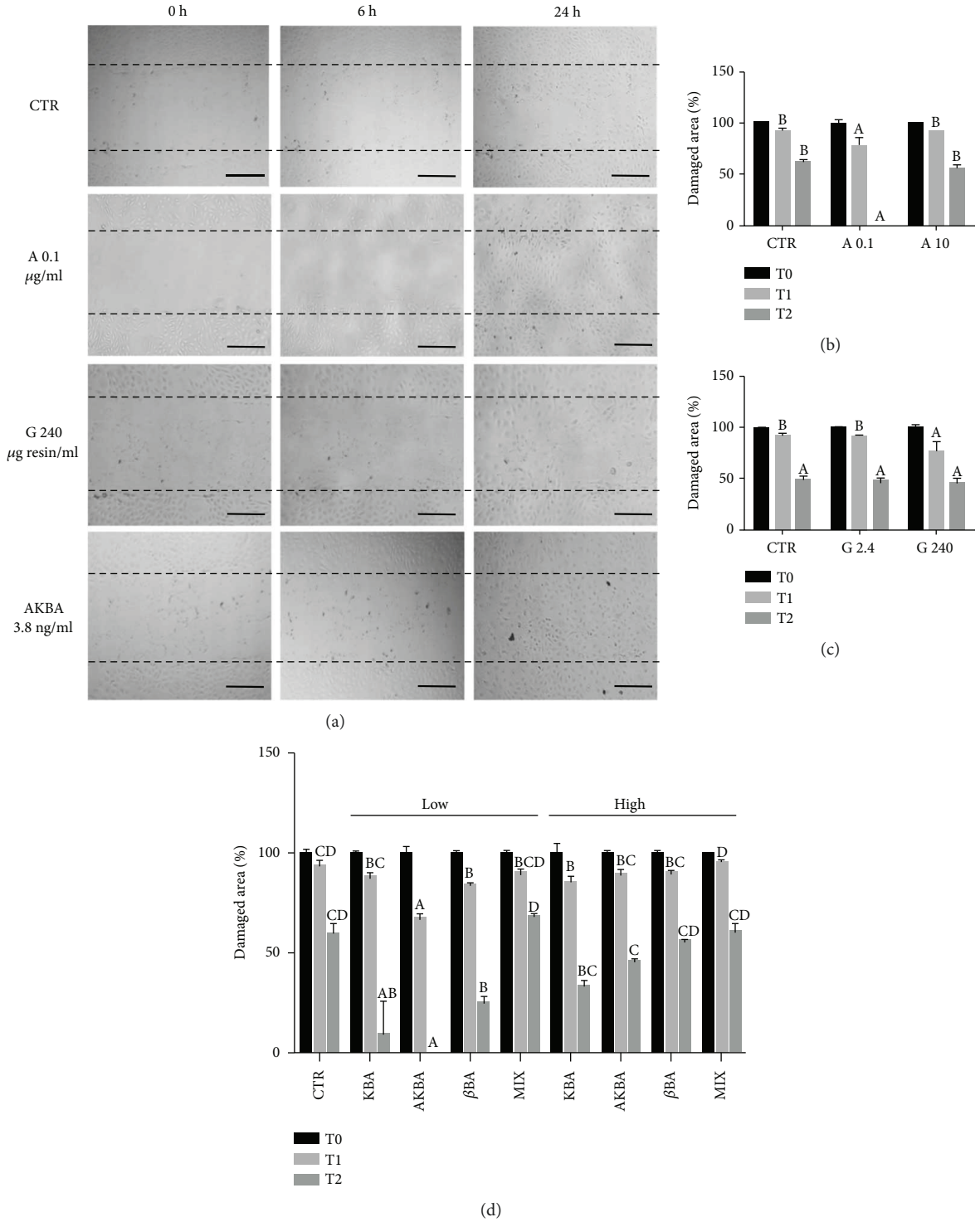

Figure 4: Effect of *B. serrata* extracts on pAEC migration capacity. Cells were scratch wounded and then treated with extracts A and G and pure BAs. Photographs were recorded at 0 h (T0), 6 h (T1), and 24 h (T2) after scratching. (a) Representative microscopic phase-contrast pictures showing the size of the scratch wound in different treatment groups compared with control. Scale bar, 200 μm. The extent of the damaged area (%) is reported for treatment with extract A (0.1 and 10 μg dry extract/ml) (b), extract G (2.4 and 240 μg resin/ml) (c), and pure BAs (*low*, corresponding to 3 ng/ml KBA, 3.8 ng/ml AKBA, and 8 ng/ml βBA, and *high*, corresponding to 300 ng/ml KBA, 380 ng/ml AKBA, and 800 ng/ml βBA) (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, βBA, 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 βBA, 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 β-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

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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)*

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Cucumis sativus

Cucumber (*Cucumis sativus* L.) belongs to the *Cucurbitaceae* family. It is commercially cultivated worldwide as a seasonal vegetable crop. It is native to India, found wild in the Himalayas from Kumaun to Sikkimand and cultivated throughout the country (Mukherjee et al., 2013).

The taxonomic classification of *Cucumis sativus* L. is the following

- Kingdom: *Plantae*
  - Subkingdom: *Tracheobionta*
    - Superdivision: *Spermatophyta*
      - Division: *Magnoliophyta*
        - Class*: Magnoliopsida*
          - Order: *Violales*
            - Family: *Cucurbitaceae*
              - Genus: *Cucumis*
                - Species: *Cucumis sativus* L.

Cucumber is an annually growing creeping vine. The leaves are hispidly hairy tralling or climbing type. Leaves are simple alternate, deeply cordate 3–5 lobed in both surfaces with a hairy margin denticulate. Flowers are yellow in colour; male flowers are clustered, bearing anthers with cohering, connective crushed or elevated above the cells whereas females are solitary thick covered with very bulbous based hairs. Fruits are compressed, elongated, ellipsoid, dorsiventrally convex and laterally ridged with variable size. Seeds are cream or white, testa hard and smooth. Cucumber has enclosed dicotyledonous seeds and it develops from a flower, and therefore it is classified as a fruit (Fig. 9) (Mukherjee et al., 2013).

Figure 9. *Cucumis sativus; Family: Cucurbitaceae (Original book source: Prof. Dr. Otto Wilhelm Thomé "Flora von Deutschland, Österreich und der Schweiz" 1885, Gera, Germany Permission granted to use under GFDL by Kurt Stueber Source).*

The medicinal properties of the cucumber have been described since ancient times. Different parts of the plant, *e.g.* leaf, fruit and seed have been explored for their therapeutic benefits. *C. sativus* fruits and seeds (Fig. 10) have important therapeutic value in the Indian Ayurvedic medicine. They are widely used for various skin problems including swelling under the eyes and sunburn. It is believed that they promote refreshing, cooling, healing, soothing, emollient and anti-itching effect to irritated skin. Several pharmacological activities including antioxidant, anti-inflammatory, antiwrinkle, antimicrobial, antidiabetic, and hypolipidemic potentials have been reported. Anti-

hyaluronidase and anti-elastase activities have been proved for its cosmetic potentials. (Mukherjee et al., 2013; Muruganantham et al., 2016; Park et al., 2015; Trejo-Moreno et al., 2018).

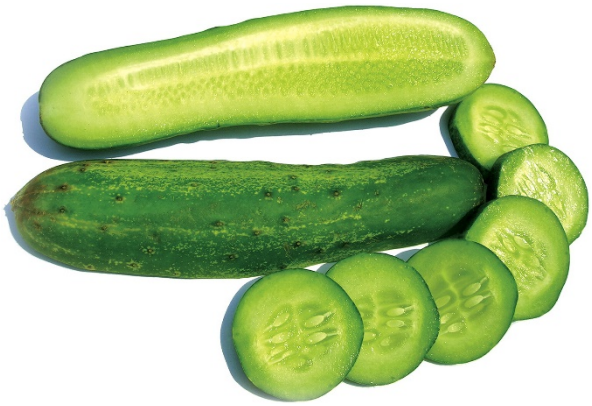

Figure 10. *C. sativus fruits and seeds (Mukherjee et al., 2013).*

Leaves, fruits and seeds of *C. sativus* contain numbers of phytoconstituents but the presence of cucurbitacins (triterpenoid substances-well-known for their bitterness and toxicity) is characteristic of the family *Cucurbitaceae*. Structurally, cucurbitacins have a tetracyclic cucurbitane nucleus skeleton, namely, 9b-methyl-19-nor lanosta-5-ene (Fig. 11), which is arbitrarily divided into twelve categories (Chen et al., 2005). Despite their toxic nature, cucurbitacins have been proved to possess pharmacological effectiveness against inflammation, cancer, artherosclerosis and diabetes (Kaushik et al., 2015).

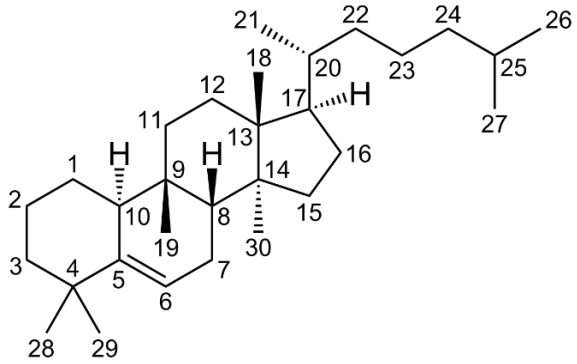

Figure 11. *Basic structure of cucurbitanes (9b-methyl-19-nor lanosta-5-ene) (Chen et al., 2005).*

In addition to cucurbitacins, several bioactive compounds have been isolated from cucumber including cucumegastigmanes I and II, cucumerin A and B, vitexin, orientin, isoscoparin, 2″-*O*-(6‴-(E)-p-coumaroyl) glucoside, apigenin 7-*O*-(6″-*O*-p-coumaroylglucoside) etc. Despite the wide use of cucumber in agricultural field, comparatively very few studies have been published about its chemical profile and its therapeutic potential (Mukherjee et al., 2013).

In the third paper, to provide new scientific evidence supporting the use of Cucumber in traditional medicine, the main purpose was to investigate whether a water/ethanol extract of *Cucumis sativus* L. fruit can attenuate the inflammation induced by lipopolysaccharide (LPS) in an *in vitro* model pAECs.

Third paper

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

Bernardini et al. BMC Complementary and Alternative Medicine (2018) 18:194
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RESEARCH ARTICLE Open Access

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 (3×10^5 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 3×10^3 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 4×10^4 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 4×10^4 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 (8×10^4 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).

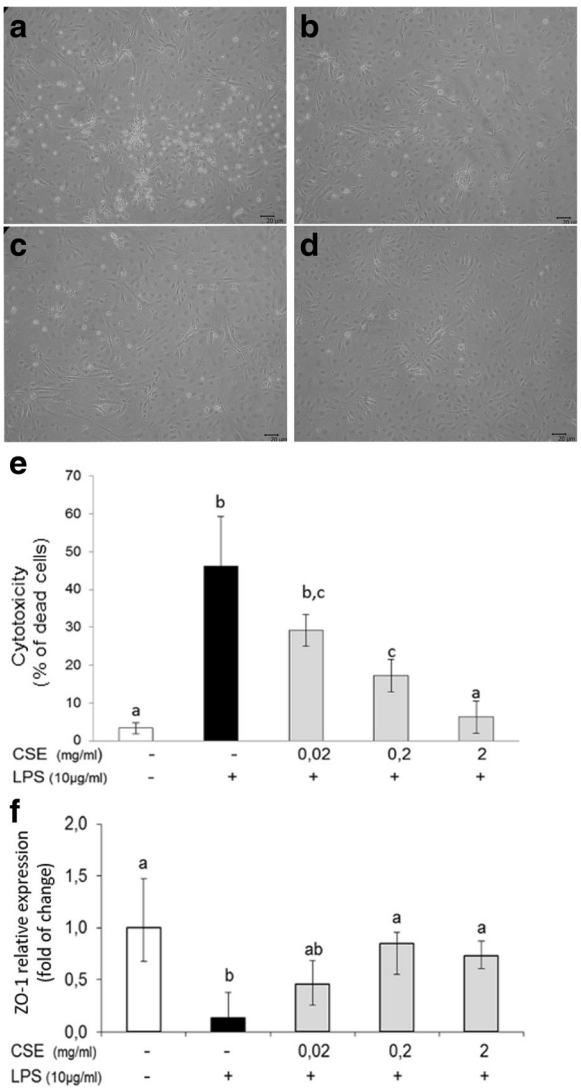

Fig. 1 Effect of *Cucumis sativus* L. extract (CSE) on LPS-induced pAECs toxicity. **a** Representative images of pAECs morphology under LPS (10 μg/ml) stimulation (**b, c, d)** and in the presence of increasing dose of CSE (0.02; 0.2; 2 mg/ml). **e** Protective effect of CSE on LPS-induced cytotoxicity; 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

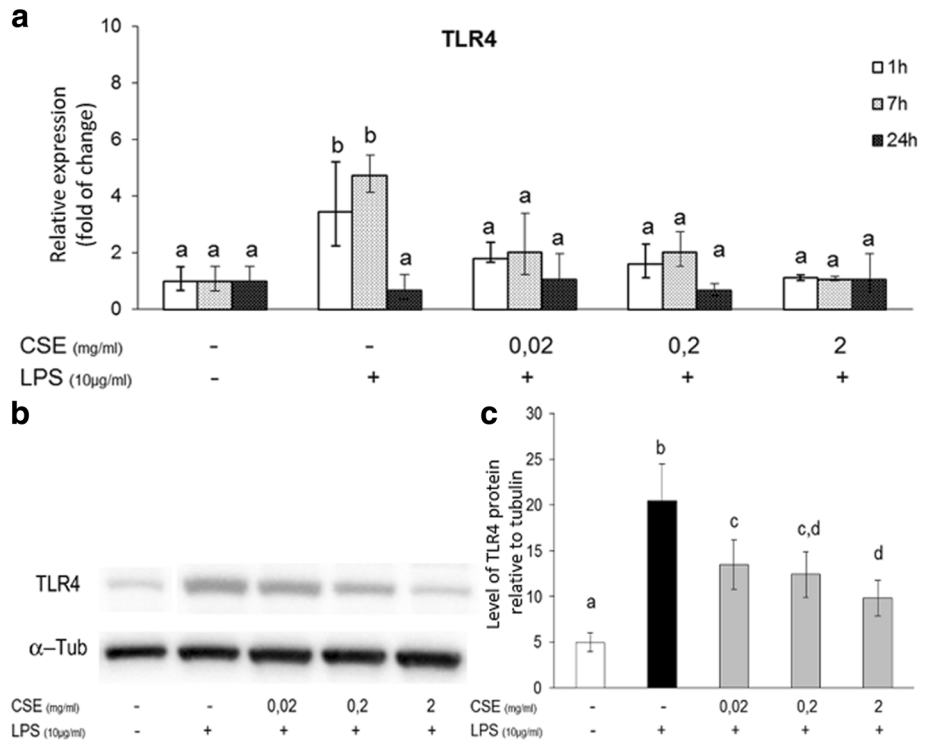

Fig. 2 Effect of *Cucumis sativus* L. extract (CSE) on LPS induced TLR4 expression. **a** Expression of TLR4 mRNA in pAECs treated with LPS (10 μg/ml) for different time (1, 7, 24 h) in the presence or absence of increasing doses of CSE (0.02; 0.2; 2 mg/ml). mRNA expression of TLR4 is determined by quantitative 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

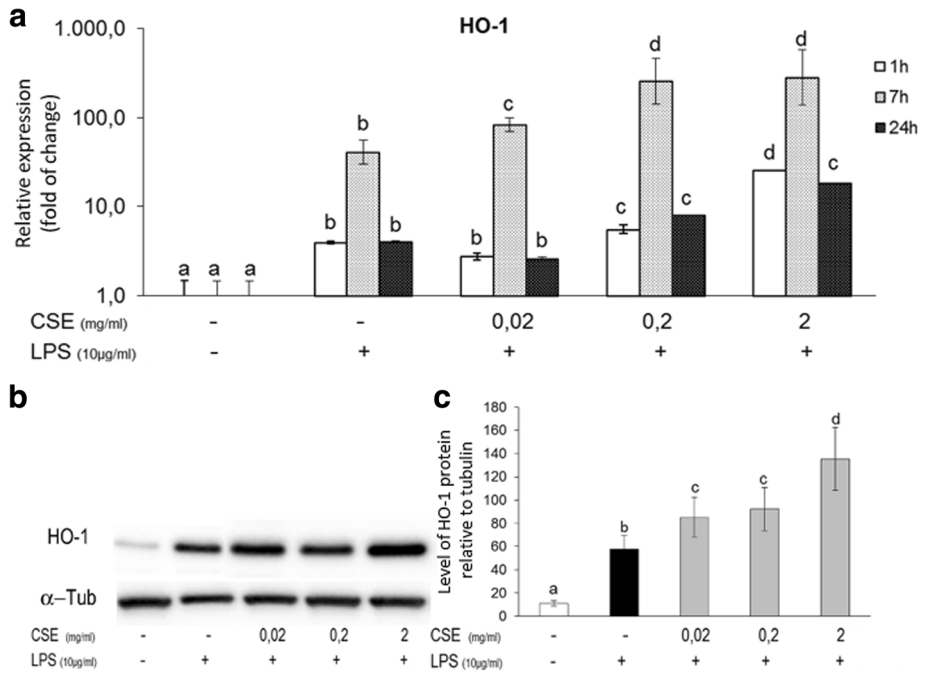

Fig. 3 Effect of *Cucumis sativus* L. extract (CSE) on LPS induced HO-1 expression. **a** Expression of HO-1 mRNA in pAECs treated with LPS (10 μg/ml) in the presence or absence of increasing doses of *Cucumis sativus* L. extract (0.02, 0.2, 2 mg/ml). mRNA expression of HO-1 as determined by quantitative 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**

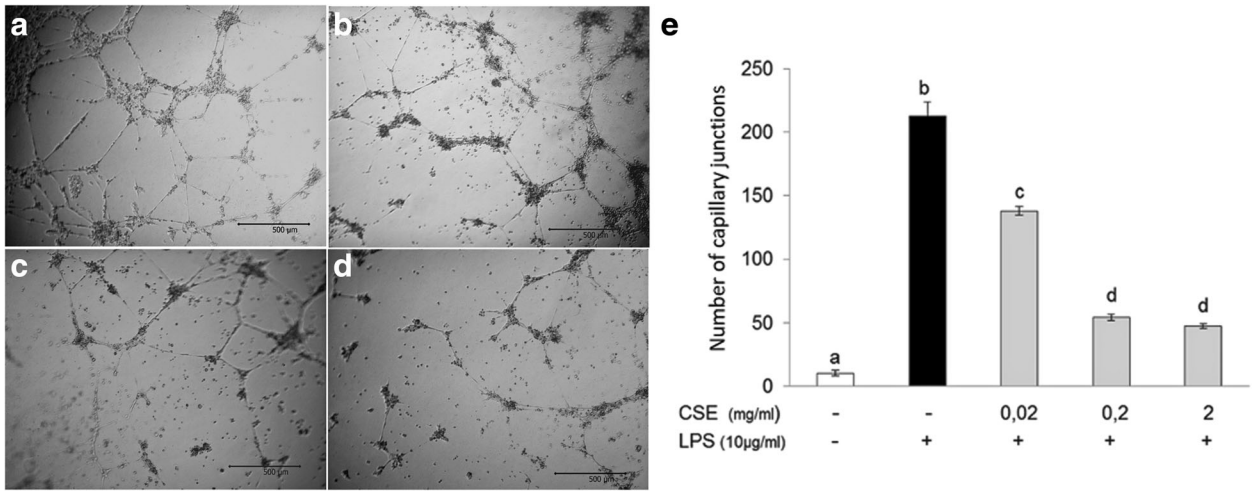

Fig. 4 Effect of *Cucumis sativus* L. extract on LPS-induced angiogenesis. pAECs were cultured on a extracellular matrix with LPS (10 μg/ml) in the absence (**a**) or presence of increasing doses (0.02; 0.2; 2 mg/ml) of *Cucumis sativus* L. extract (**b, c, d** respectively) for 18 h. LPS induced a capillary 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.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Experimental part 2

Comparative medicine: dog as spontaneous animal model

Man's best friend, *Canis familiaris*, plays an important role in the field of comparative medicine. The unique breeding history of the domestic dog offers an exceptional opportunity to explore the genetic basis of disease susceptibility, morphological variation and behavioural traits. The position of the dog within the mammalian evolutionary tree also makes it an important guide for comparative analysis of the human genome.

The history of the domestic dog traces back at least 15,000 years, and mitochondrial DNA (mtDNA) analyses have probably suggested dating far back to 100,000 years ago, until its original domestication in East Asia from the grey wolf, the dog's direct ancestor. Humans and dogs evolved through a mutually beneficial relationship, sharing living environment and food sources. In recent centuries, humans have selectively bred dogs that excel at herding, hunting and obedience. During domestication process, dog's behavioural patterns changed and have created breeds rich in behaviours that mimic human ones and support our needs. Dogs have also been bred for desired physical characteristics such as body size, skull shape, leg length, coat colour and texture, producing breeds with closely delineated morphological features. This evolutionary experiment has generated diverse domestic breed under strong selection, each dog breed represents an isolated breeding population and harbouring more morphological variability than those present within the remainder of the family *Canidae* (Lindblad-Toh et al., 2005; Parker et al., 2010).

As a consequence of these stringent breeding programmes and periodic population bottlenecks (for example, during the World Wars), many of the ~400 modern dog breeds also show a high prevalence of specific diseases, including cancers, blindness, heart disease, cataracts, muscular dystrophy, autoimmune disease, epilepsy, hip dysplasia and deafness. Many canine diseases are caused by mutations in the same genes as the corresponding diseases in humans. Moreover, most of dog's diseases are also frequently seen in the human population, with similar clinical manifestations. The high prevalence of specific diseases within certain breeds suggests that a limited number of loci underlies each disease, making their genetic dissection potentially more tractable in dogs than in humans (Lindblad-Toh et al., 2005).

Except for human, dog is the most highly studied animal in medical practice, with often available detailed family history and pathology data. Through the efforts of the American Kennel Club (AKC) and similar organizations worldwide, extensive genealogies and genetic data are easily accessible for most purebred dogs (Lindblad-Toh et al., 2005).

The dog genome is more similar to human genome than mouse genome, therefore, more of the human genomic sequence can be aligned to the genome of the dog than that of the mouse. Dogs have approximately the same number of genes as humans, most of them being close orthologues. Several canine diseases are caused by mutations in the same genes as the corresponding diseases in humans (Hytönen and Lohi, 2016). The sequencing of the canine genome and subsequent development of powerful gene discovery tools has remarkably facilitated gene discoveries in disease, morphology and behaviour. Currently, over 300 variants that underlie canine Mendelian disorders are recorded in the public database Online Mendelian Inheritance in Animals (OMIA) (OMIA, 2011) and novel mutations are continually discovered (Donner et al., 2016; Lindblad-Toh et al., 2005; Parker et al., 2010).

Moreover, pet dogs share also the environmental living and are exposed to the same environmental risk factors of their owners and are thus not only affected by genetic traits but also by "life style". Many of spontaneously occurring canine pathological disorders are analogous to human diseases such as diabetes, cancers, epilepsies, eye diseases and autoimmune diseases, and several rare monogenic diseases (Donner et al., 2016; Hytönen and Lohi, 2016).

Consequently, dog becomes an important comparative model for the development of new therapies for both species, but also for the better understanding of the molecular pathophysiology of human and dog diseases (Fig. 11) (Hytönen and Lohi, 2016).

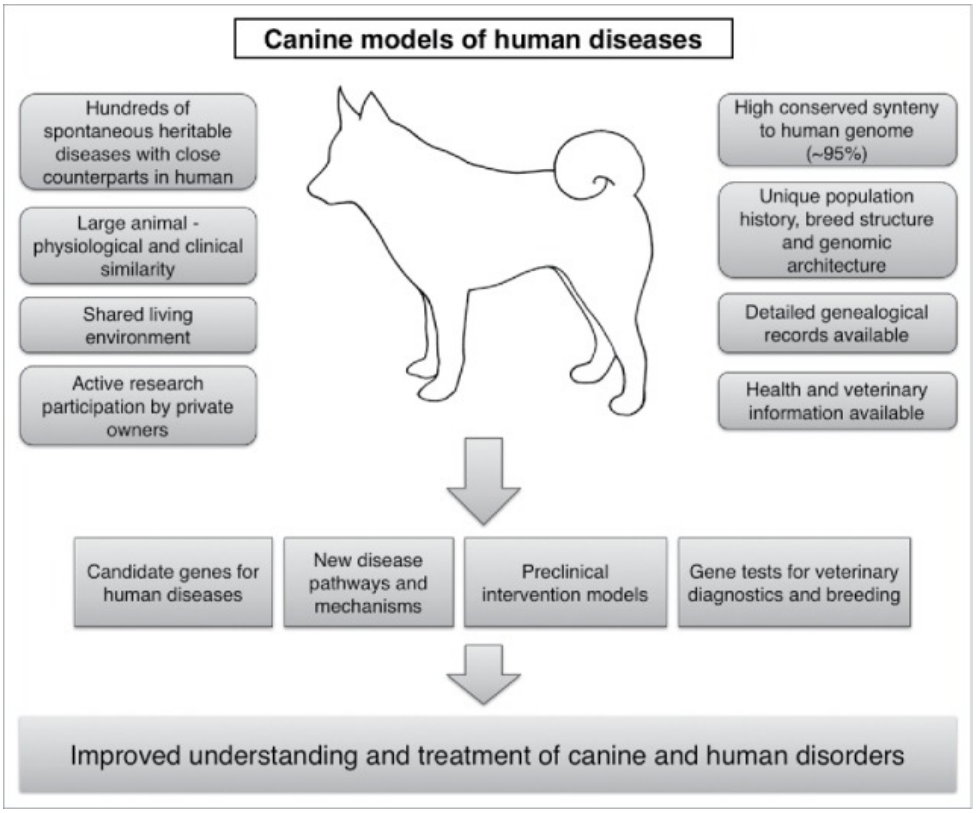


Figure 11. *Various clinical, genetic, physiological and environmental characteristics in dogs make them excellent comparative models for facilitating the understanding and treatment of human disorders (Hytönen and Lohi, 2016).*

In human oncology research, murine cancer models such as xenografts and transgenic mouse models have been extremely useful in the study of the complexity of human cancer biology. However, sometimes the tumour development and biological responses observed in murine models are not predictive of what happens in human tumours. Therefore, there is an increasing need for a more appropriate, spontaneous animal model that reflects the biologic complexity of human cancer. In this regard, companion animals (pet dogs and cats) seem to have many desired characteristics that fill the gap between *in vitro* and *in vivo* studies. Spontaneous tumours in companion animals, particulars in dogs, are a unique and underused resource as models for human cancer biology and for translational cancer therapeutics. Naturally occurring tumours in dogs have clinical presentation, histology, disease progression, and response to treatment similar to human cancers that are difficult to reproduce in other model systems (Pinho et al., 2012). Cancers in pet dogs often mirrors the biology and heterogeneity of human disease, including complex

interactions between the immune system and tumour cells, significant heterogeneity, development of chemotherapy resistance, and metastasis resulting in patient death (Gardner et al., 2016). The increasing interest of cancer researchers in this type of animal models and the growing number of publications in veterinary oncology makes comparative oncology of primary importance in translational medicine, allowing for the rapid and valid flow of data for clinical application in humans (Pinho et al., 2012). Recently advances in the development of genome techniques and commercially available high-throughput methodologies, like genotyping microarray, specific for dogs have increased the ability to understand mechanism and characterize canine cancers. Importantly, specific molecular pathways of human tumour are frequently observed in canine cancers, offering the opportunity to target those mechanisms in dogs and develop accurate preclinical assessment of novel therapeutics. The similarities between cancer in dogs and humans underscore the value of comparative and translational research for the benefit of both species. Clinical trials evaluating novel therapeutics in dogs affected by cancer provide dogs with state-of-the-art therapy at little or no cost and generate critical new data with direct applicability to subsequent human clinical studies. Consequently, international collaborative initiatives, as well as biomedical industry entities, now leverage client-owned dogs with spontaneous tumours to address central questions regarding the biologic activity, adverse event profile, appropriate dose/regimen, and pharmacodynamic/pharmacokinetic endpoints for novel cancer treatments with the ultimate goal of accelerating their application to human cancers (Gardner et al., 2016).

Dogs naturally develop cancers that share many characteristics with human malignancies, in particular osteosarcoma (Fenger et al., 2014), lymphoma (Marconato et al., 2013), leukaemias (Figueiredo et al., 2012; Gardner et al., 2016), mammary cancer (Liu et al., 2014; Pinho et al., 2012) and melanoma (Gillard et al., 2014; Simpson et al., 2014).

Osteosarcoma

Osteosarcoma (OSA) is the most common primary bone tumour in both people and dogs, but it is significantly more prevalent in dogs, with a reported incidence of 13.9/100,000 in dogs in contrast to 1.02/100,000 in humans. OSA commonly occurs in older dogs (median age 7 years); however, a bimodal distribution is present with a small peak in young dogs (average age 1 year). This is in contrast to human OSA, which is more frequent in adolescence (10- to 14-year-old age group) (Fenger et al., 2014). Amputation and adjuvant chemotherapy increase median survival times to 8-12 months from the 3-4 months generally achieved with amputation alone; however, 90% of dogs are euthanized within 2 years of diagnosis (Withrow et al., 2012). Moreover, the prognosis for OSA is caution in children; the overall 5-year survival rate is 67% in the nonmetastatic disease and 10-30% if metastases are found at initial diagnosis (Fenger et al., 2014). Genes and signalling-pathway alterations to disease pathogenesis are highly conserved in both the human and canine OSA, confirming the relevance of canine OSA as spontaneous animal model for OSA in paediatric research. Comparative genomic analyses have demonstrated abnormalities similar in human and dog OSA, this discover has identified novel molecular drivers that may be interesting targets for therapeutic intervention. Dysregulation of specific candidate genes implicated in the etiopathogenesis of OSA are found in both species, including mutations in the tumour suppressor genes p53, RB1, and PTEN and alterations of the oncogenes MYC and MET (Fenger et al., 2014). In conclusion, together these data suggest that the integration of dogs with OSA into comparative and translational cancer research has the potential to identify shared and novel molecular targets for therapeutic intervention, lending additional insight into the biology of OSA and ultimately advancing the care of children affected by this dangerous disease (Gardner et al., 2016).

D-17 canine osteosarcoma cell line

Canine Osteosarcoma cells line (D-17) was purchased from the "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna - Sez. Brescia". These cells were obtained from a metastasis to the lung of an osteosarcoma in a 11-year old female poodle dog (Riggs et al., 1974).

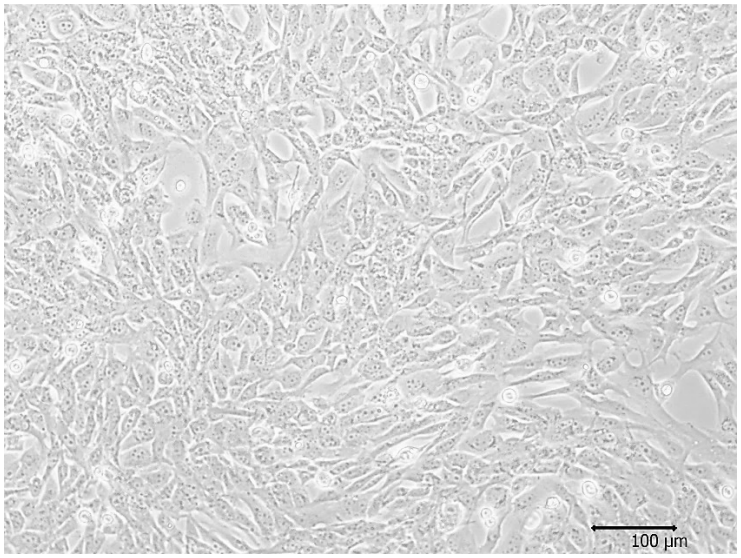


Figure 12. *D-17 canine osteosarcoma cell line.*

As reported in literature, D-17 canine osteosarcoma cells were a consolidate *in vitro* model for the studies on dog osteosarcoma (Gebhard et al., 2018, 2016; Lopez et al., 2018) and in comparative medicine (Bergeron et al., 2013; Shahi et al., 2015). Moreover, D-17 were used as an *in vitro* model for toxicology and pharmacology studies, to evaluate the efficacy of drugs and natural compounds (Helmerick et al., 2014; Seo et al., 2012; Zhao et al., 2018).

In this thesis, the cytotoxicity and anti-proliferative effects of a hydroalcoholic extract obtained from *Artemisia annua* L., used for centuries in Chinese Traditional Medicine and recently proposed as antimalaria and antitumoral drug, were evaluated on canine osteosarcoma cell line (D-17) (Fig. 12).

Artemisia annua

Artemisia annua L. is a member of the *Asteraceae* family of plants (formerly *Compositae*). Since ancient time, *A. annua* has been used in Traditional Chinese Medicine (TCM) for the treatment of several diseases. It is native to China but is widely distributed in the temperate, cool temperate and subtropical zones of the world. The Chinese name of the plant is Qinghao, which means green herb, but it is also known as sweet wormwood, Chinese wormwood sweet, annual wormwood, annual sagewort, annual mugwort, and sweet sagewort (Brown, 2010).

The taxonomic classification of *Artemisia annua* L. is the following

- Kingdom: *Plantae*
  - Subkingdom: *Tracheobionta*
    - Superdivision: *Spermatophyta*
      - Division: *Magnoliophyta*
        - Class*: Magnoliopsida*
          - Order: *Asterales*
            - Family: *Asteraceae*
              - Genus: *Artemisia*
                - Species: *Artemisia annua L.*

A. annua is an annual herb, that can naturally grow to 30-100 cm high. The stem is erect, ribbed, brownish or violet-brown. Leaves are alternate, alveolate, dark green, or brownish green. Odour is characteristic and aromatic, while the taste is bitter. It is characterized by large panicles of small globulous capitulums (2-3mm diameter), with whitish involucres, and by pinnatisect leaves which disappear after the blooming period, characterised by small (1-2 mm) pale yellow flowers having a pleasant odour (Fig. 13) (WHO, 2006).

Figure 13. *Artemisia annua L. (Original sources: Vuyck, L. (1906) Flora Batava. 22. Band. Vincent Loosjes, Haarlem. Tafel 1697 and Einjähriger Beifuß "Artemisia annua" photographed by Kristian Peters).*

In the western medicine, the notoriety of *A. annua* is linked to the antimalarial action against the parasite *Plasmodium falciparum*. In 1972, from *A. annua,* Youyou Tu, a Chinese pharmaceutical chemist, isolated artemisinin following low temperature extraction. Nowadays, artemisinin, its derivatives and Artemisinin Combination Therapy (ACT) belong to the established standard treatments of malaria worldwide. For this important discover, Youyou Tu was awarded the Nobel Prize in Physiology or Medicine in 2015.

Over 600 phytochemicals have been identified as constituents of *A. annua* L (Brown, 2010). The chemical composition of *A. annua* consists of volatile and non-volatile constituents. The volatile components are mainly attributable to essential oils with the

percentage of the latter being 0.2-0.25%. The main non-volatile ingredients include sesquiterpenoids, flavonoids and coumarins, together with proteins (such as β-galactosidase, β-glucosidase) and steroids (e.g. β-sitosterol and stigmasterol) (Brown, 2010; WHO, 2006).

This chemical composition is common among *Artemisia* genus, that counts more than 200 species, but the major and unique constituent of *A. annua* is the sequiterpene artemisinin (Brown, 2010).

Artemisinin is a sesquiterpene trioxane lactone, which contains an endoperoxide bridge essential for its biological activity (Fig. 14).

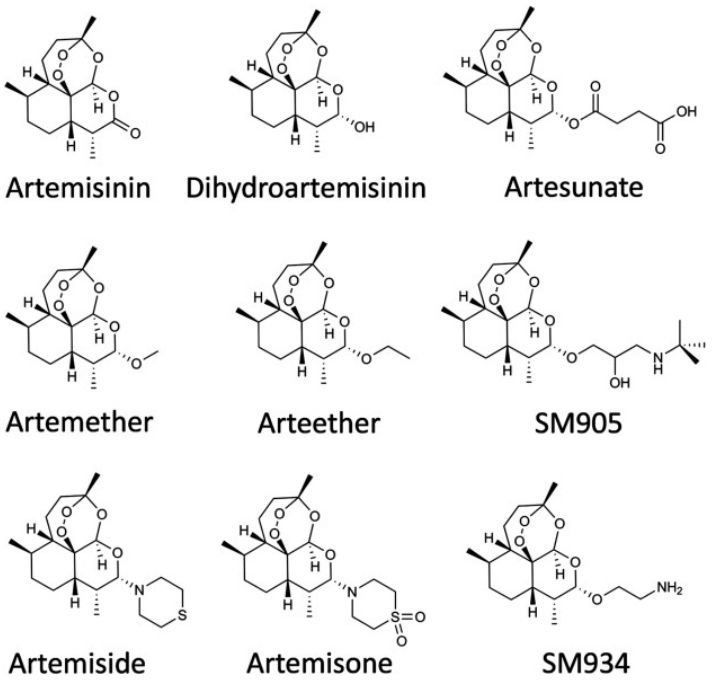


Figure 14. *Chemical structures of artemisinin and its derivatives (Ho et al., 2014).*

Artemisinin can be extracted from dried leaves, but its content is highly variable, ranging anywhere between 0.01% and 0.8% of the dry weight, depending on variety, environment and growth conditions, and can even be as high as 1.4% in some cultivated strains (Brown, 2010; Golenser et al., 2006; Van Der Kooy and Sullivan, 2013). The low amount of artemisinin extracted from the plant can represent a serious limitation for the commercialization of the drug. Moreover, artemisinin has low hydro and liposolubility,

and has a limited bioavailability. Different approaches have been used to increase content of artemisinin; metabolic and genetic engineering strategies have been developed not only in cell/tissue plant cultures but also in transgenic plants (Badshah et al., 2018; Brown, 2010; Golenser et al., 2006; Sun et al., 2017). In the last 30 years, to resolve these problems, several semisynthetic artemisinin derivatives (artemisinins) were developed (Fig. 14).

Although developed as antimalarials, artemisinin and its semisynthetic derivatives have been studied for many other therapeutic properties (Fig. 15).

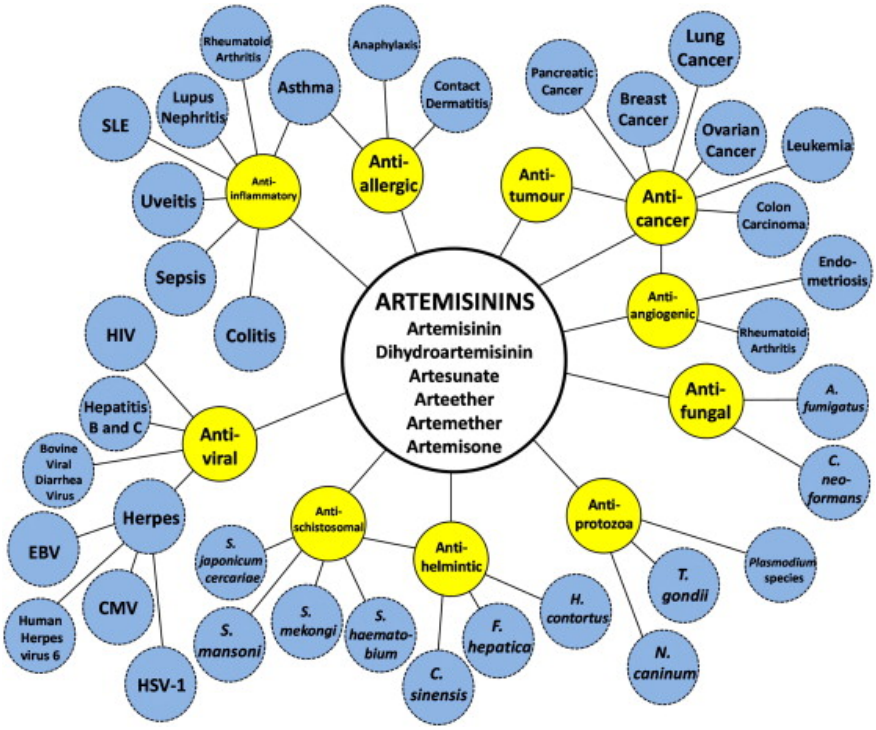


Figure 15. *Bubble map outlining various biological activities of artemisinins and their potential clinical applications in different diseases (Ho et al., 2014).*

Artemisinin and its derivatives have been reported to possess strong inhibitory effects against viruses (e.g. *Human cytomegalovirus*), protozoa (e.g. *Toxoplasma gondii*), helminths (e.g. *Schistosoma* species and *Fasciola hepatica*) and fungi (e.g. *Cryptococcus neoformans*). Moreover, artemisinins have demonstrated anti-inflammatory effects and have been evaluated in autoimmune diseases, allergic disorders and septic inflammation. The anti-inflammatory effects of artemisinins have been attributed to the inhibition of

Toll-like receptors, Syk tyrosine kinase, phospholipase Cγ, PI3K/Akt, MAPK, STAT-1/3/5, NF-κB, Sp1 and Nrf2/ARE signaling pathways (Ho et al., 2014).

Since the late 1990s, anticancer properties of artemisinins have been well known and there has been rapid multiplication of *in vitro*/*in vivo* studies, case reports and clinical trials on the antitumor properties of artemisinins (Bhaw-Luximon and Jhurry, 2017).

The endoperoxide moiety is the key of the bioactivity of artemisinin-type drugs. Its cleavage leads to reactive oxygen species (ROS) formation and induces oxidative stress. Furthermore, in the presence of ferrous iron or reduced heme, artemisinins can convert itself into cytotoxic carbon-centred radical, a highly potent alkylating agent, to induce direct oxidative damage to cancer cells (Bhaw-Luximon and Jhurry, 2017; Efferth, 2017).

Oxidative stress and DNA damage block cell cycle progression and induce various cell death modes: apoptosis, necrosis, necroptosis, autophagy and ferroptosis, confirming the crucial role of iron for artemisins anticancer activity (Efferth, 2017).

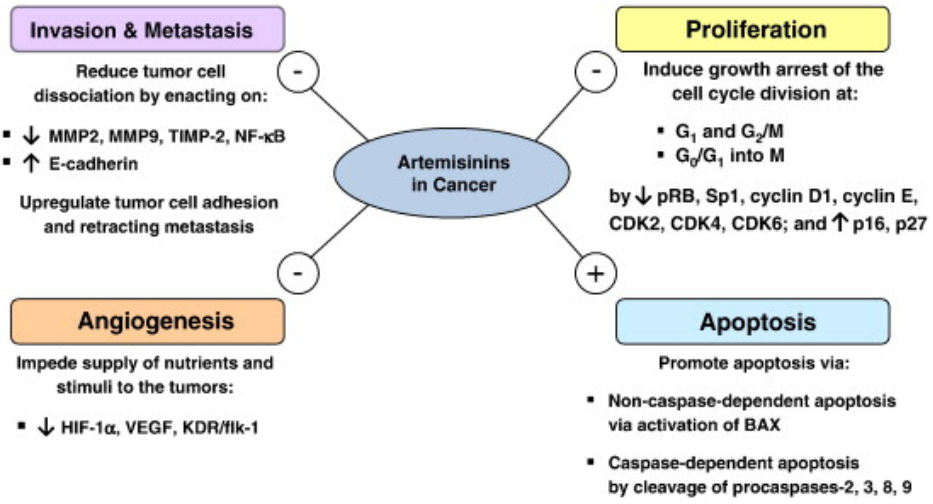


Figure 16. *Overview of the anti-cancer mechanisms of action for artemisinins (Ho et al., 2014).*

Like several natural products, another important advantage of artemisinin and its derivatives is that they act in a multi-specific manner against tumours. Indeed, artemisinins reduce cell proliferation, inhibit angiogenesis and tissue invasion of the

tumour, as well as cancer metastasis (Fig. 16). In particular, artemisinis act to tumour-related signal transduction pathways (e.g. Wnt/ β -catenin pathway, AMPK pathway), signal transducers (NF-κB, MYC/MAX, AP-1, CREBP, mTOR etc) and proteolytic enzymes, such as MMPs. Complex interactions through different pathways may enhance the anticancer effect of artemisinins (Bhaw-Luximon and Jhurry, 2017; Efferth, 2017; Ho et al., 2014).

In the fourth preliminary paper, the cytotoxicity and anti-proliferative effects of a hydroalcoholic extract obtained from *Artemisia annua* L., were evaluated *in vitro* on canine osteosarcoma cell line (D-17), considering the importance of canine osteosarcoma in veterinary oncology and the relevance of dog as spontaneous comparative model.

Preliminary paper

Preliminary study to evaluate the cytotoxic effects of *Artemisia annua* L. and artemisinin on D-17 canine osteosarcoma cell line

Preliminary study to evaluate the cytotoxic effects of Artemisia annua L. and artemisinin on D-17 canine osteosarcoma cell line

Abstract

Artemisia annua L. has been used for centuries in Chinese Traditional Medicine. Although developed as antimalarials, its active compound artemisinin and its semi-synthetic derivatives have been also investigated for their anticancer property, with interesting and promising results. Artemisinin is a sesquiterpene trioxane lactone, which contains an endoperoxide bridge essential for its activity. Reduced heme or ferrous iron are believed to be responsible for the activation of the endoperoxide bond, leading to the formation of ROS and carbon centred radicals, that act as alkylating agents. In veterinary medicine, canine osteosarcoma represents a highly aggressive tumour that often leads to therapeutic failure due to lung metastasis and development of chemo-resistance, highlighting the urgent need of novel therapies. Naturally occurring tumours in dogs have many clinical and biological similarities to the human counterpart and make the dog an important spontaneous animal model in comparative medicine. The aims of this research were to evaluate the cytotoxicity and the anti-proliferative effect of pure artemisinin and a commercial hydroalcoholic extract obtained from *A. annua* on canine osteosarcoma cell line (D-17). The last aim was to optimize an analytical protocol for the determination of intracellular iron in D-17 cells. Both artemisinin and hydroalcoholic extract induced cytotoxic effect in a dose-dependent manner. Pure artemisinin caused an increase of cells in S phase, whereas the hydroalcoholic extract induced an evident increase in G_2/M phase. A significant decrease of iron concentration was measured in D-17 cells treated with pure artemisinin and hydroalcoholic extract compared to untreated cells. In conclusion, although preliminary, the data obtained in this study are indicative of a more potent cytotoxic activity of the hydroalcoholic extract than pure artemisinin, indicating a possible synergistic effect of the phytocomplex. Considering the similarities between human and canine osteosarcoma, progress in

deepening knowledge and improving therapeutic protocols will probably be relevant for both species, in a model of reciprocal translational medicine.

Introduction

Since ancient time, *Artemisia annua* L. (family *Compositae*) has been used as medicinal plant in the treatment of several diseases in Traditional Chinese Medicine (Brown, 2010). Nowadays, the reputation of *A. annua* is linked to its antimalarial activity. In 2015, Youyou Tu was awarded the Nobel Prize in Physiology or Medicine for isolating the active molecule artemisinin from *A. annua*. Currently, artemisinin, its derivatives and Artemisinin Combination Therapy (ACT) belong to the established standard treatments of malaria worldwide (Efferth, 2017). Over 600 phytochemicals have been identified as constituents of *A. annua,* but its phytochemistry is dominated by sesquiterpenoids, flavonoids and coumarins, together with proteins (such as β-galactosidase, β-glucosidase) and steroids (e.g. β-sitosterol and stigmasterol) (Brown, 2010). However, *A. annua* is distinguished from the other 200 species of the *Artemisia* genus by the exclusive presence of artemisinin, the active compound extracted from dried leaves (WHO monograph on good agricultural and collection practices (GACP) for *Artemisia annua* L.). Artemisinin is a sesquiterpene trioxane lactone, which contains an endoperoxide bridge essential for its biological activity. The low amount of artemisinin extracted from the plant, its low hydro and liposolubility and its limited biodisponibility can represent a serious limitation for the standardization and commercialization of the drug. In the last 30 years, several semisynthetic artemisinin derivatives were developed with different strategies, including genetic engineering (Badshah et al., 2018; Brown, 2010; Sun et al., 2017). Although developed as antimalarials, artemisinin and its semisynthetic derivatives, have been also investigated for many other therapeutic properties, such as antiviral, antimicrobial and anti-inflammatory activities (Ho et al., 2014). However, since the late 1990s, anticancer properties of artemisinins have been well known and there has been a rapid increase of *in vitro* and *in vivo* studies, case reports and clinical trials on the antitumor properties of artemisinins (Bhaw-Luximon and Jhurry, 2017). The endoperoxide moiety is the key of the bioactivity of artemisinin-type drugs. Its cleavage leads to reactive

oxygen species (ROS) formation and induces oxidative stress. Furthermore, in the presence of ferrous irons or reduced heme, artemisinin can convert itself into cytotoxic carbon-centred radical, a highly potent alkylating agent, to induce direct oxidative damage to cancer cells (Bhaw-Luximon and Jhurry, 2017; Efferth, 2017). Complex interactions through different pathways may enhance the anticancer effect of artemisinins. Indeed, artemisinins induce apoptosis and ferroptosis, reduce cell proliferation through cell cycle arrest, inhibit angiogenesis and tissue invasion of the tumour, as well as cancer metastasis (Bhaw-Luximon and Jhurry, 2017; Efferth, 2017; Ho et al., 2014).

In veterinary medicine, osteosarcoma (OSA) accounts for 2-5% of all canine malignancies (Farcas et al., 2014) and for 80-85% of all bone tumours (Chun and de Lorimier, 2003; Morello et al., 2011). OSA is characterized by locally aggressive and highly metastatic behaviour, that leads to early mortality (Morello et al., 2011; Szewczyk et al., 2015). Affected bone is destroyed by the tumour, that typically extends to surrounding soft tissues. Metastases spread mainly via haematogenous route and mostly to the lungs (Morello et al., 2011; Pollard and Kitchen, 2017; Szewczyk et al., 2015). Other metastatic sites are represented by other bones, visceral organs, brain, subcutaneous tissue and skin. Metastasis to regional lymph nodes are quite uncommon (4.4-9%) (Morello et al., 2011). Treatment for OSA involves surgery to remove primary tumours and on occasion distant metastasis. Surgery involves either amputation of the limb or limb salvage/sparing procedures. However, dogs treated with surgery alone have a short median survival time (Simpson et al., 2017). Surgery combined with neoadjuvant and/or adjuvant chemotherapy prolongs the survival of dogs with OSA and the protocols include doxorubicin, cisplatin, carboplatin and lobaplatin used alone or in combination (Morello et al., 2011; Simpson et al., 2017; Szewczyk et al., 2015).

Spontaneously occurring OSA in dogs have clinical presentation, biological behaviour, response to treatment and disease progression similar to human OSA, that are difficult to reproduce in other model systems (Fenger et al., 2014; Gardner et al., 2016; Pinho et al., 2012). OSA is the most common primary malignancy of bone both in dogs and humans. It is significantly more prevalent in dogs, with an

incidence rate 27 times higher in dogs than in people. OSA commonly occurs in older dogs (median age 7 years), while in humans is more common in adolescence (10- to 14-year-old age group) (Gardner et al., 2016; Simpson et al., 2017). The site of OSA development in children and dogs is strikingly similar, with a predilection for the weight-bearing region of long bones. Approximately 75% of canine OSA occurs in the appendicular skeleton, with the most common sites in the distal radius and proximal humerus. In human OSA, long bones are affected in up to 90% of cases, with the distal femur, proximal tibia, and the proximal humerus being the most common locations (Fenger et al., 2014; Simpson et al., 2017). Furthermore, mutations of specific genes involved in the etiopathogenesis of OSA are found in both species, including mutations in the tumour suppressor genes p53, RB1 and PTEN and alterations of the oncogenes MYC and MET (Gardner et al., 2016). However, therapeutic failures are recurrent in both dogs and humans and are mainly due to the development of multiple resistance and metastatic spread, making the development of new therapies essential.

Therefore, to provide new scientific evidence to support antitumor activity of *A. annua* L. in *in vitro* animal model, the main purposes of the present research were to evaluate the cytotoxicity and the effects on cell cycle of pure artemisinin and a commercial hydroalcoholic extract obtained from *A. annua* on canine osteosarcoma cell line (D-17). Considering the crucial role of iron on artemisinin activity, the last aim was to optimize an analytical protocol for the determination of intracellular iron in D-17 cells.

Materials and Methods

Chemicals and reagents

All reagents were obtained from Sigma Aldrich (St. Louis, MO, USA), if not otherwise specified, and were Ultrapure grade, included 98% pure artemisinin (CAS number: 63968-64-9). Minimum Essential Media (MEM), heat inactivated fetal bovine serum (FBS) and Dulbecco's phosphate buffered saline (DPBS) were purchased from Gibco-Life technologies (Carlsbad CA, USA). A commercial hydroalcoholic extract obtained from *A. annua* and composed by 65% ethanol, 20%

of aerial parts and water. This extract contains 2 mg artemisinin/ml (corresponding to 7 mM) as declared by the producer. All plastic supports were purchased from Falcon, Beckton-Dickinson (Franklin Lakes, NJ, USA).

Cell culture and treatment

Canine osteosarcoma cells line (D-17) was purchased from the "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – Sez. Brescia". Cells were cultured in Minimum Essential Media (MEM) added with 2 mM L-Glutamine and FBS (5%) in a 5% CO_2 atmosphere at 37°C. The first seeding after thawing was always performed in T-75 tissue culture flasks ($4x10^6$ cells/flask) and successive experiments were conducted in T-25 flasks (cell cycle analysis and iron quantification) and in 96-well assay plates (cytotoxicity). Artemisinin was dissolved in DMSO to obtain a 50 mM stock solution then diluted in culture medium to obtain the required concentrations. Hydroalcoholic extract of *A. annua* was directly diluted in culture medium to obtain the required concentrations. For each treatment, the same concentration of the specific vehicle was used as control, DMSO for artemisinin and a solution of 65% ethanol for hydroalcoholic extract.

Cytotoxicity

D-17 were seeded in a 96 wells plate (approximately 1.5×10^4 cells/well) and exposed for 24 h to increasing concentration of pure artemisinin (50, 100, 250, 500, 750, 1000, 2000 µM) or increasing doses of *A. annua* hydroalcoholic extract corresponding to artemisinin concentrations of 14, 35, 70, 140, 280, 420, 700 µM, on the base of the declared concentration of 2 mg artemisinin/ml in hydroalcoholic extract. Cytotoxicity was measured using tetrazolium salt (*In Vitro* Toxicology Assay Kit, MTT based). The formazan absorbance was measured at a wavelength of 570 nm, using Infinite® F50/-Robotic Absorbance microplate readers TECAN (Life Science). The background absorbance of multiwall plates at 690 nm was also measured and subtracted from the 570 nm measurements.

The concentrations of artemisinin required for 50% inhibition of cell viability (IC_{50}) were calculated by Prism Graph Pad software and the IC_{50} values were used for subsequent experiments.

Cell cycle analysis

For the analysis of cell cycle in flow cytometry, aliquots of $1x10^6$ cells in duplicate, for each treatment (IC_{50} standard artemisinin, IC_{50} *A. annua* hydroalcoholic extract) were washed from growth medium by centrifuging at 240 x g for ten minutes. Then, the resulting pellet was resuspended in 1 ml of a solution containing 0.1% sodium citrate, 0.1% Nonidet, 10 µg/ml of RNAse and 50 µg/ml of propidium iodide (final concentration $1x10^6$ cells/ml). The PI fluorescence is acquired by flow cytometer (Epics Beckman Coulter) equipped with an Argon Ion laser tuned at 488 nm. PI red fluorescence (600 nm) is analysed on a linear scale and data analyses is performed using the software program "ModFit" (USA).

Iron quantification

For the iron determination was used a Spectra AA-20 atomic absorption spectrometer (Varian) equipped with a GTA-96 graphite tube atomizer and the sample dispenser. The optimization of the analytical method was obtained as it is given by (Tüzen, 2003) with minor changes: the graphite tubes employed were coated GTA tubes (Agilent Technologies, Germany), the hollow cathode lamp current was 7 mA and measurements were performed at 248.3 nm resonance lines using a spectral slit width of 0.2 nm. During spectrophotometer readings, internal argon flow rate in the partition graphite tubes was maintained at 300 ml/min and was interrupted in the atomization phase. Ramp and hold times for the drying, pyrolysis, atomization and cleaning temperatures are reported in Table 1 and were optimized to obtain maximum absorbance without significant background absorption so no background correction was necessary. For the same reasons, sample volume was optimized to 20 µl.

Table 1. Furnace conditions for the determination of iron in D-17 cells.

Phases of programme	Temperature C°	Ramp time (seconds)	Hold time (seconds)
Drying 1	110	1	20
Drying 2	130	5	30
Pyrolisis	1100	15	10
Atomization	2200	0.6	5
Cleaning	2600	1	2

The calibration curve was obtained by diluting 1 mg/ml standard stock solution of iron (BDH Chemicals, Poole, England) with MilliQ water to obtain working standards containing 0, 20, 40 and 60 ng/ml of iron and by plotting the absorbance at 248.3 nm against iron concentrations. The equation of the curve was y = 0.0109x and the calculated regression coefficient (r) was 0.993.

For the quantification of iron, for each treatment (IC_{50} standard artemisinin, IC_{50} *A. annua* hydroalcoholic extract), D-17 cells were harvested, counted and centrifuged at 800 x g for 10 min. The pellet was washed twice with DPBS, then resuspended in a solution of 1 M HNO_3 overnight, at a final concentration of $1x10^6$ cells/ml. The samples were centrifuged at 1500 x g for 10 min to precipitate cellular debris. The detection limit (LOD), defined as the concentration corresponding to 3 times the standard deviation of 6 blanks, was 0.8 ng/ml. Iron concentration is reported as ng Fe/1 x 10^6 cells.

Statistical analysis

Cytotoxicity assay was performed on six replicates and IC_{50} concentrations were obtained by Prism Graph Pad software. Data of iron quantification were analysed with a one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* comparison Test. Data of cell cycle were analysed with two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons. Differences of at least $p < 0.05$ were considered significant. Statistical analysis was carried out using Prism Graph Pad software.

Results

Effect of *Artemisia annua* hydroalcoholic extract and artemisinin on cell viability

The effect of artemisinin pure standard and *A. annua* hydroalcoholic extract was evaluated on D-17 cells by MTT assay. Both artemisinin and hydroalcoholic extract induced a decrease of cell viability at all concentrations and exerted cytotoxic effect in a dose-dependent manner. Both provoked an increased number of round and detached cells after 24 h, more evident in the presence of hydroalcoholic extract (Fig. 1D and E). Data obtained from MTT analyses were elaborated to assess the concentration of artemisinin required for 50% inhibition of cell viability (IC_{50}): the values corresponded to 548 µM for the standard and 65 µM for the hydroalcoholic extract (Fig. 1 A and B). Artemisinin contained in hydroalcoholic extract was found to exert cytotoxic activity with a dose one order of magnitude lower than pure artemisinin.

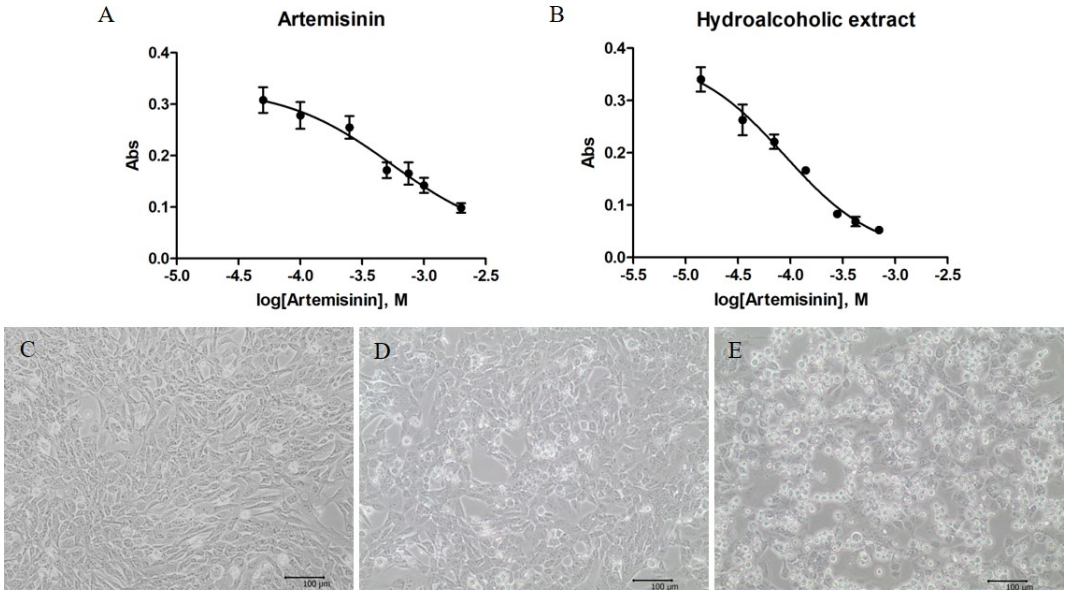


Figure 1. Effect of artemisinin and *A. annua* hydroalcoholic extract on D-17 cells. Dose-response curve: D-17 cell viability upon treatment with different concentrations of (A) pure artemisinin and (B) *A. annua* hydroalcoholic extract. Representative images of D-17 cell morphology: (C) untreated cells; (D) in the presence of artemisinin (IC_{50}=548 µM) and (E) hydroalcoholic extract (IC_{50}=65 µM).

Effect of *Artemisia annua* hydroalcoholic extract and artemisinin on cell cycle

The effect of *A. annua* hydroalcoholic extract and artemisinin on D-17 cell cycle was evaluated by flow cytometry. Untreated cells presented a typical cytogram of a diploid cell population (Fig. 2A and D). As shown in Figure 2B, C and D, significant changes in D-17 cell cycle were highlighted in the presence of both pure artemisinin and hydroalcoholic extract. A significant decrease ($p<0.0001$) of cells in G_0/G_1 phase was observed after both treatments. Pure artemisinin caused a significant increase ($p<0.01$) of cells in S phase, while hydroalcoholic extract induced an evident significant ($p<0.0001$) increase of cells in G_2/M phase.

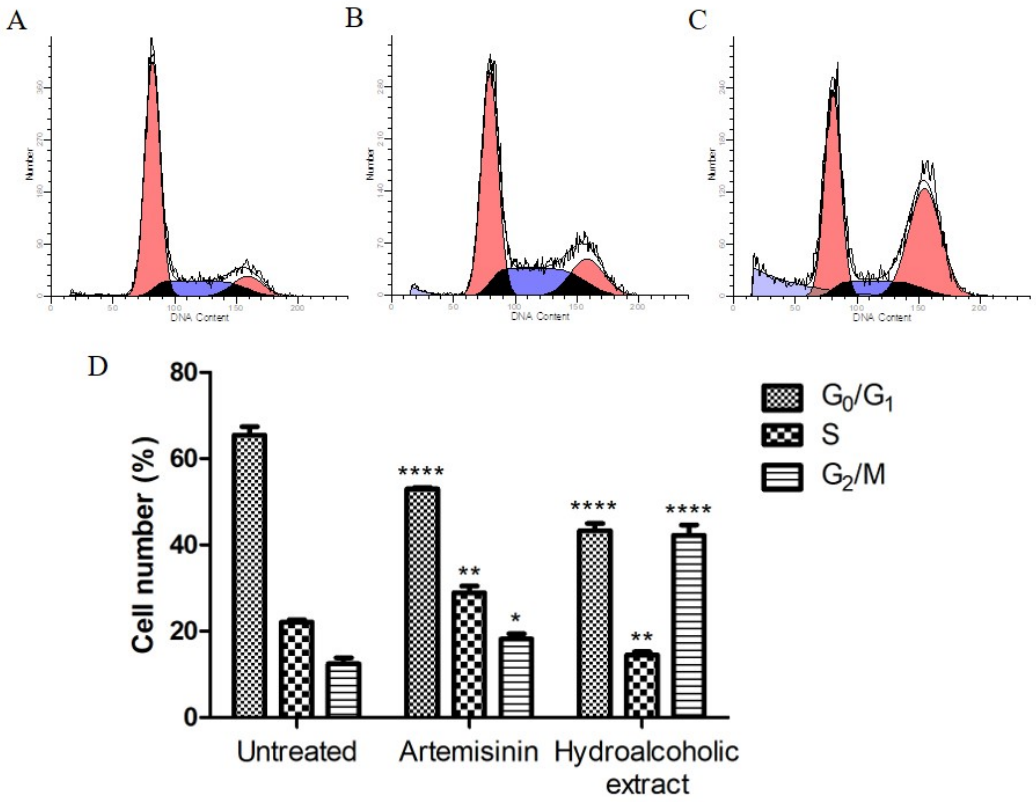
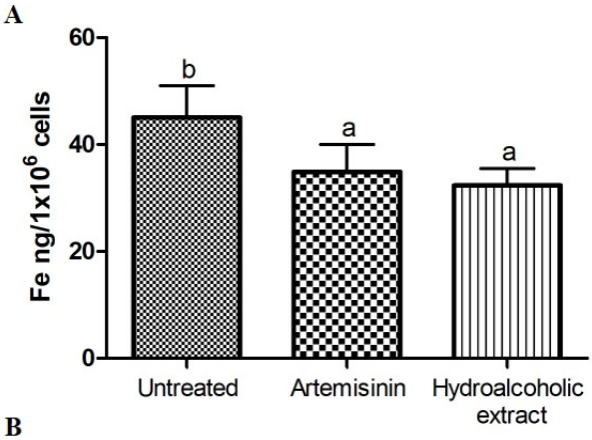


Figure 2. Cell cycle analysis of D-17 cells after 24 hours of treatment: (A) untreated cells; in presence of (B) 548 µM artemisinin and (C) hydroalcoholic extract corresponding to 65 µM artemisinin; (D) Cell cycle distribution. Data are reported as mean ± SD (n = 2). Significant differences *vs* untreated cells are indicated by *($p<0.05$), by **($p<0.01$) and ****($p<0.0001$) (two-way-ANOVA followed by the Bonferroni multiple comparisons).

Effect of *Artemisia annua* hydroalcoholic extract and artemisinin on intracellular iron

Iron concentrations in D-17 cells were reported in Figure 3. Intracellular iron concentration in untreated D-17 cells was 45±6 ng/10^6 cells. Cells exposed to pure artemisinin and *A. annua* hydroalcoholic extract presented significantly lower concentrations of intracellular iron than the untreated cells ($p<0.01$) (Fig. 3A and B). The cells exposed to the extract had a lower concentration of iron than those treated with pure artemisinin; this difference was not statistically significant.



B

D-17	Fe ng/1x10^6 cells
Untreated (n = 10)	45.0 ± 6.0
Artemisinin (n = 4)	34.9 ± 5.1
Hydroalcoholic extract (n = 4)	32.3 ± 3.2

Figure 3. Iron concentration in D-17 cells measured by atomic absorption spectrometer. Data are reported as mean ± SD. Different letters above the bars indicate significant differences ($p<0.01$ ANOVA *post hoc* Tukey's test).

Discussion

In the last 30 years, the anticancer activity of artemisinin and its derivatives has been investigated *in vitro* and *in vivo* systems, laying the bases for clinical trials both in human and veterinary medicine (Bhaw-Luximon and Jhurry, 2017; Efferth, 2017). Osteosarcoma (OSA) is the most common primary bone tumour in people and in dogs and is characterized by locally aggressive and highly metastatic

behaviour (Fenger et al., 2014; Gardner et al., 2016).The development of new drugs is necessary to improve therapeutic outcome of OSA, in particular multiple resistance and metastatic OSA. Therefore, the first aim of this study was to investigate the cytotoxic effect of artemisinin by comparison with an hydroalcoholic extract obtained from *A. annua,* on canine osteosarcoma cell line (D-17). Our results demonstrated the cytotoxic effect of both artemisinin and hydroalcoholic extract in a dose-dependent manner on D-17. Efferth et al. (2011) reported a wide range of IC_{50} values of pure artemisinin for a panel of different human cell lines, from 57.1 μM for leukaemia cells to 1602 μM for HeLa cells. In 2014, Jirangkul et al., reported IC_{50} values of pure artemisinin for two human osteosarcoma cell lines, MG63 and 148B, with IC_{50} of 167 μM and 178 μM, respectively. Therefore, the sensitivity of the canine D-17 to the artemisinin (IC_{50} = 548 μM) appears to be intermediate if compared to human tumour cell lines. However, it has been reported that artemisinin by oral administration was poorly bioavailable in dogs and resulted in plasma concentration below 0.1 μM (Hosoya et al., 2014), hence far insufficient to achieve any therapeutic activity. To our knowledge, only dihydroartemisinin (DHA) cytotoxicity was evaluated on canine OSA cell lines. In particular, Hosoya et al. (2008) investigated the cytotoxic effect of DHA on four canine OSA cell lines, D-17, OSCA2, OSCA16, and OSCA50 and IC_{50} values were 8.7, 43.6, 16.8, and 14.8μM, respectively.

In accordance with Efferth et al. (2011), who reported in HeLa cells that the cytotoxic effect of *A. annua* extract is much higher than that of pure artemisinin, our results indicated an IC_{50} for the plant extract one order of magnitude lower than pure artemisinin (65 and 548 μM, respectively). The same authors tested fourteen extracts of seven different *A. annua* plant materials with different phytogeographical origins. They extracted plants in dichloromethane (slightly polar) or methanol (polar) and evaluated the activity of the extracts on HeLa cells, obtaining IC_{50} values ranging from 54.1 to 275.5 μg/ml for dichloromethane extracts and from 276.3 to 1540.8 μg/ml for methanol extracts. A phytochemical investigation of the extracts by GLC–MS revealed the presence of artemisinin, arteanuine B, and scopoletin in all extracts, confirming *in vitro* the synergistic effect of the mixture of compounds that constitute the phytocomplex. Breuer and Efferth

in 2014 reported the successful use of *Herba A. annua Luparte*® as adjuvant therapy for veterinary sarcoma treatment. They found that this extract contained high amount of scopoletin, while artemisinin represented just a minor component. As *A. annua* extracts are generally administered via oral route, it seems unlikely that their effect *in viv*o is due to artemisinin only, but more probably is due to a synergistic effect of the whole phytocomplex.

In the literature, heterogeneous results are reported on the action of artemisinin and its derivatives on the cell cycle arrest. It has been reported that this actives molecules (artemisinin and its derivatives) cause cell cycle arrest mainly in G_1/G_0 phase, through downregulation of cyclin E, cyclin D1 and cyclin-dependent kinases 2 and 4 in several tumour cell lines, including human breast cancer cells (Tin et al., 2012), gallbladder cancer cell lines (Jia et al., 2016), neuroblastoma (Zhu et al., 2014), lung carcinoma cells (A549) and non- small lung cancer cells (H1299) (Tong et al., 2016). In D-17 canine osteosarcoma cell line, pure artemisinin slightly but significantly increased the number of cells in S phase and this has been observed also by Beekman et al. (1997). On the other hand, hydroalcoholic extract caused a significant increase of D-17 cells at G_2/M phase. Other authors found that artemisinin derived drugs induced G_2/M cell cycle arrest. In particular, dihydroartemisinin induced G_2 arrest in osteosarcoma cell line (Ji et al., 2011), ovarian carcinoma cell line (Jiao et al., 2007) and hepatocellular carcinoma cell line (Zhang et al., 2012), while artesunate induced G_2 arrest in breast carcinoma cell lines (Chen et al., 2014), rat pituitary adenoma cell line (Mao et al., 2012) and kidney carcinoma cell lines (Jeong et al., 2015). Kim et al. (2015) evaluated the effect of an *A. annua* extract on human colon cancer cell line HCT116 and found that cell cycle arrest occurred at G_1/S phase mediated by Akt/mTOR pathway. Afterwards, they demonstrated that *A. annua* extract induced apoptosis through the regulation of specific proteins such as Bax, Bak and cytochrome *c* in PDK1/Akt signaling pathways via PTEM/p53-independent manner (Kim et al., 2017). The difference in cell cycle phase arrest may be ascribed to the phytochemical variability of extracts, leading to alternative molecular interactions and hampering one pathway or another. More research is needed to unravel the complex mechanism underlying the effect of *A. annua* extract.

It is well known that cancer cells require higher iron intake than normal cells to deal with their enhanced metabolic demands, thus presenting in most cases an increased number of transferrin receptors (Ho et al., 2014; Torti and Torti, 2011). In the present study, iron concentrations determined in D-17 cell line are similar to those reported by other authors (Cerchiaro et al., 2013), though different analytical methods and measurement system hampered in many cases a direct comparison. Though preliminary, the data reported in the present research indicated that pure artemisinin and *A. annua* extract determined a significant reduction of intracellular iron in D-17 cells. It is well-known that iron has a key role in artemisinin toxicity, as the leading event in the artemisinin-induced cytotoxic cascade is represented by ROS generation via endoperoxide bridge cleavage by ferrous iron (Bhaw-Luximon and Jhurry, 2017; Efferth, 2017). Iron intracellular speciation and redox state are tightly regulated by several proteins and molecular pathways, not all of them fully unravelled. Zhou et al. (2016) identified as artemisinin targets 79 proteins involved mainly in membrane transport, protein trafficking, cell death and survival and nucleic acid metabolism. The authors hypothesized that among these proteins transferrin receptor, that is overexpressed in several tumour cells, could be alkylated by artemisinin leading to a selective iron depletion in cancer cells. A novel mode of iron-dependent cell death, ferroptosis, has been recently described pointing out the role of free iron pool (Dixon et al., 2012). It has been reported that iron enhances cytotoxicity of artemisinin derived drugs towards cancer cells, therefore a role of ferroptosis in cell death cannot be excluded (Efferth, 2017).

Although preliminary, the data obtained in this research showed a more potent cytotoxic activity of the hydroalcoholic extract than pure artemisinin on D-17 canine osteosarcoma cell line, indicating a possible synergistic effect of other bioactive molecules. However, phytochemical characterization of *A. annua* extract will be necessary to accurately quantify artemisinin in the extract and better understand other plant components that act synergistically for the final cytotoxic effect. Furthermore, it will be necessary to investigate the biochemical mechanism of action of *A. annua* extract, in particular the role of iron and ferroptosis. In conclusion, considering the similarities between human and canine osteosarcoma, progress in deepening knowledge and improving therapeutic protocols will

probably be relevant for both species, in a model of reciprocal translational medicine.

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Conclusion

The research reported in this PhD thesis represents an additional step for the development of *in vitro* models to evaluate the biological activity of herbal products utilized in human and veterinary medicine. In particular, three important plants were considered, *Boswellia serrata* and *Cucumis sativus* traditionally used in Ayurvedic medicine and *Artemisia annua* in Traditional Chinese Medicine.

In accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes and the principles of "3Rs", in particular with the "Replacement", *in vitro* models based on cell culture were used.

Scientific evidences on the anti-inflammatory effect of *B. serrata* and *C. sativus* have been obtained on an inflammatory model developed using primary culture of porcine aortic endothelial cells (pAECs) stimulated by LPS, confirming the swine as an excellent animal model for translational medicine.

Although preliminary, the results obtained on *A. annua* confirmed the cytotoxicity of an hydroalcoholic extract in a canine osteosarcoma cell line (D-17), supporting its antitumor properties and indicating a possible synergistic effect of the phytocomplex. Considering the similarities between human and canine osteosarcoma, dog has been confirmed an interesting spontaneous translational model.

Reaffirming the importance of the phytochemical characterization of the extracts and the quantification of most important active molecules, in this study the principal boswellic acids responsible for biological activity of *B. serrata* were quantified and could be used as a tool for the evaluation of extract quality. On the other hand, a limitation of this study was the impossibility of characterizing *C. sativus* extract, while for the hydroalcoholic extract of *A. annua*, LC-MS/MS analysis to quantify artemisinin is currently ongoing.

In conclusion, the *in vitro* models based on animal cell cultures have shown to be a significant preclinical tool, contributing to the application of the "Replacement" principle. Finally, this study confirmed the importance of evidence-based research in phytotherapy for a quality and safe use of herbal medicine, not only in human but also in veterinary medicine.

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