Universidade do Minho
Escola de Ciências

Elisabete Apolinário da Costa

Pharmacological potential of Baccharis dracunculifolia in the treatment of osteoarthritis

Agosto de 2017

Elisabete Apolinário da Costa **Pharmacological potential of Baccharis dracunculifolia in the treatment of osteoarthritis**

Uminho | 2017

Universidade do Minho
Escola de Ciências

Elisabete Apolinário da Costa

Pharmacological potential of Baccharis dracunculifolia in the treatment of osteoarthritis

Dissertação de Mestrado
Em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação do
Professor Doutor Alberto Carlos Pires Dias
Professora Doutora Filipa Pinto-Ribeiro

Agosto de 2017

DECLARAÇÃO

Nome: Elisabete Apolinário da Costa

Endereço eletrónico: eliscosta1991@gmail.com

Número do cartão cidadão: 13931883

Título tese: Pharmacological potential of *Baccharis dracunculifolia* in the treatment of osteoarthritis

Orientador: Professor Doutor Alberto Carlos Pires Dias

Ano de conclusão: 2017

Desiganação do mestrado: Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Universidade do Minho, ___/___/_____

Assinatura: _____

Agradecimentos

A realização desta dissertação de mestrado contou com preciosos apoios de várias pessoas sem os quais não se teria tornado uma realidade e aos quais estarei eternamente grata. A todos eles deixo aqui o meu agradecimento sincero.

Aos meus pais agradeço carinhosamente pela força diária durante esta caminhada, por todo o apoio, pelas palavras de incentivo, paciência, por todo o amor, um muito obrigado pela oportunidade de dar continuidade aos meus estudos e por acreditarem sempre em mim e naquilo que faço e por todos os ensinamentos da vida. Espero que esta etapa, que agora termino, possa, de alguma forma, retribuir e compensar todo o carinho, apoio e dedicação que, constantemente, me oferecem. A eles dedico todo este trabalho!

Aos meus irmãos, a toda a família e amigos agradeço pelo carinho, apoio e força durante esta caminhada.

Ao meu namorado agradeço pelo apoio incondicional, paciência, compreensão, carinho e cumplicidade, por todas as palavras de incentivo e por acreditar sempre em mim.

Agradeço de forma especial à Inês Laranjeira pelo apoio, ajuda, simpatia, colaboração, companheirismo sincero e pela boa amizade que criámos.

Agradeço à Diana Amorim pela ajuda neste trabalho, apoio, simpatia, paciência, e sempre recetiva e disponível para ajudar.

Aos animais experimentais cujas vidas foram sacrificadas em benefício do progresso da ciência, a eles o meu profundo respeito.

Ao meu orientador Professor Doutor Alberto Dias, professor de conhecimento e exemplo de sabedoria, quero agradecer generosamente pela orientação, acolhimento, disponibilidade, incentivo, motivação, preocupação, por todo o apoio, pelas opiniões e críticas, por todos os ensinamentos transmitidos e por me ter aberto as portas para a pesquisa científica.

À minha co-orientadora Professora Doutora Filipa Pinto-Ribeiro, professora de dedicação e alegria contagiante, expresso o meu profundo agradecimento por toda a paciência, pela exigência do trabalho, pela compreensão, pela oportunidade, por me incentivar em todos os momentos, por enriquecer o meu conhecimento científico e pela motivação na área das neurociências.

Obrigada por tudo!

Abstract

Baccharis dracunculifolia D.C. (Asteraceae) (Bd) is a medicinal Brazilian shrub, popularly known as "alecrim do campo" and "vassoura", known for its antioxidant, anti-inflammatory, anti-stress and other activities. Antioxidants are substances that prevent various pathological changes in living cells and medicinal plants are considered potential sources of natural antioxidants.

Inflammation is a protective reaction that is triggered by stimuli in response to tissue infections and injuries, however dermal inflammation can cause acute and chronic diseases depending on the time of persistence. Osteoarthritis (OA) is the most common form of arthritis and the most debilitating, affecting about 40 million people in Europe, especially the elderly. OA is characterized by progressive degeneration of tissues within and surrounding the joints and by recurrent inflammatory episodes that lead to structural changes, including cartilage erosion, fibrillation and decreased thickness of articular cartilage.

In this work, experimental OA was induced in a group of 8 week old female ovariectomized rats (*Rattus norvegicus*, var. *Albinus*, *Wistar*), weighting 210 ± 17 g. Animals were divided in 4 groups (n = 6 per group): (i) SHAM, (ii) ARTH, (iii) ARTH treated with Bd extract (50 mg/kg), and (iv) ARTH treated with Bd extract (100 mg/kg). Four weeks after induction of OA in the animals, Bd extracts were administrated by gavage for a successive period of 5 week. Throughout this period, the animals were assessed for changes in gait (catwalk test), hyperalgesia in the joints (PAM), locomotor ability (OFT), knee circumference, and acute dermal inflammation.

The results of this study showed that *B. dracunculifolia* possesses phenolic compounds and antioxidant and anti-inflammatory activity. Treatments with *B. dracunculifolia* improved motor performance, especially in the group treated with 100 mg/kg of Bd extract. Otherwise, treatment with 50 mg/kg of Bd extract reduced mechanical hyperalgesia. The histology of the joints shows that the treatments with extract of Bd, mainly the treatment of Bd100, inhibit the wear of the cartilage in the joints. In addition, dermal inflammation was also resolved with the topical treatment of *B. dracunculifolia*.

This dissertation should be seen as an aid to the development of new natural drugs, mainly for the treatment of osteoarthritis and its additional consequences to the disease, as well as for dermal inflammations. However, more studies will be needed.

Resumo

Baccharis dracunculifolia D.C. (Asteraceae) (Bd) é um arbusto medicinal brasileiro, popularmente conhecida como "alecrim do campo" e "vassoura", conhecida por suas atividades antioxidantes, anti-inflamatórias, anti-stress, entre outras. Os antioxidantes são substâncias que previnem várias alterações patológicas nas células vivas e as plantas medicinais são consideradas fontes potenciais de antioxidantes naturais.

A inflamação é uma reação de proteção que é desencadeada por estímulos em resposta a infeções e lesões de tecidos, no entanto a inflamação dérmica pode causar doenças agudas e crônicas, dependendo do tempo de persistência. A osteoartrite (OA) é a forma mais comum de artrite e a mais debilitante, e afeta cerca de 40 milhões de pessoas na Europa, especialmente os idosos. OA é caracterizada por degeneração progressiva de tecidos dentro e ao redor das articulações e por episódios inflamatórios recorrentes que levam a mudanças estruturais, incluindo erosão da cartilagem, fibrilação e diminuição da espessura da cartilagem articular.

Neste trabalho, a OA experimental foi induzida num grupo de ratos ovariectomizados fêmeas de 8 semanas de idade *(Rattus norvegicus,* var. *Albinus, Wistar)*, com pesos de 210 ± 17 g. Os animais foram divididos em 4 grupos, (n = 6 por grupo): (i) SHAM, (ii) ARTH, (iii) ARTH tratados com extrato de Bd (50mg/kg), e (iv) ARTH tratados com extrato de Bd (100 mg/kg). Quatro semanas após a indução de OA nos animais, os extratos de Bd foram administrados por gavagem por um período de 5 semanas sucessivas. Ao longo deste período, os animais foram avaliados quanto a alterações na marcha (teste de catwalk), hiperalgesia nas articulações (PAM), habilidade locomotora (OFT), circunferência do joelho, e inflamação aguda dérmica.

Os resultados deste estudo mostraram que a *B. dracunculifolia* possui compostos fenólicos e atividade antioxidante e anti-inflamatória. Os tratamentos com *B. dracunculifolia* melhoraram os desempenhos motores, especialmente no grupo tratado com os 100 mg/kg de extrato de Bd. De outro modo, o tratamento com 50 mg/kg de extrato de Bd reduziu a hiperalgesia mecânica. A histologia das articulações mostra que os tratamentos com extrato de Bd, principalmente o tratamento de Bd100, inibe o desgaste da cartilagem nas articulações. Além disso, a inflamação dérmica também foi solucionada com o tratamento tópico de *B. dracunculifolia.*

Esta dissertação deve ser encarada como um auxílio para o desenvolvimento de novos fármacos naturais, principalmente para o tratamento da osteoartrite e suas consequências adicionais à doença, assim como para inflamações dérmicas. No entanto, mais estudos serão necessários.

Index

Abbreviations

Abs - absorbance

ANOVA – analysis of variance

ARTH - arthritic group

B.C. – before Christ

Bd - *Baccharis dracunculifolia*

Bd100 - treatment group of 100 mg/kg of *Baccharis dracunculifolia*

Bd50 – treatment group of 50 mg/kg of *Baccharis dracunculifolia*

CNS - central nervous system

CO - croton oil

CSF - cerebrospinal fluid

Ct - control

DEX – dexamethasone

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DPPH – 2,2-diphenyl-1-picryl-hydrazyl

EC_{50} - efficient concentration of 50%

Gf – grams of force

HPLC - high performance liquid chromatography

HPLC-DAD - High Performance Liquid Chromatography - Diode Array Detector

H&E - Haematoxylin and Eosin

IASP – International Association for the Study of Pain

ICA - iron chelating activity

ICVS - Life and Health Sciences Research Institute

IL-1β – interleukin-1 beta

i.p. – intraperitoneal injection

K/C - kaolin/carrageenan

LWT - limb withdrawal threshold

NADH - nicotinamide adenine dinucleotide hydride

NBT - nitroblue tetrazolium

NED - *N*-(1-Naphthyl) ethylenediamine

NOS - nitric oxide synthase

OA – osteoarthritis

OFT - open field test

PAM - pressure application measurement

PBS - phosphate buffered solution

PFA – paraformaldehyde

PKC - protein kinase C

PMS - phenazine methosulphate

ROS - reactive oxygen species

Rt - retention time

SC – spinal cord

SEM – standard error of the mean

SNP - sodium nitroprusside

T & CM - Traditional and Complementary Medicine

TPA - 12-*o*-tetracanoilphorbol-13-acetate

UFJF - Federal University of Juiz de Fora

UV-Vis – Ultra Violet-Visible

WHO – World Health Organization

Chapter 1: Introduction

1.1. Medicinal plants in human health

Human populations coexist with a great diversity of plant species, developing particular ways of exploiting them for different purposes, such as shelter, clothing, food, flavors, fragrances and also for therapeutic purposes, namely medicines (Gurib-Fakim, 2006). Medicinal plants have long played important roles in the treatment of diseases around the world (Veiga-Junior, 2008), being one of the most important sources of medicines, mainly used in developing countries as alternative treatments for health problems (Duarte *et al.*, 2005). The WHO defines a medicinal plant as "any plant that possesses in one or more organs substances that can be used for therapeutic purposes or that are precursors of semi-synthetic drugs" (Zhang, 1998).

In the primitive era, mankind turned to nature in order to find alternatives for overcoming desolating harm, whether spiritual or physical (Alvim *et al.*, 2006). Thus, the therapeutic use of medicinal plants in human health is a very old practice, where the writings indicate that therapeutic use of plants is as old as 4000-5000 B.C., and Chinese were the first to use the natural herbal preparations as medicines (Prakash & Gupta, 2005). The late XIXth century and early XXth century emphasized the development of experimental research, causing the knowledge and therapeutic previously used in human health, such as medicinal plants and other popular practices, to be marginalized due to a lack of scientific basis, leading to skepticism on the part of the medical community regarding phytopharmaceutical prescription, since only allopathic drugs (traditional drugs) had pharmacokinetic, pharmacodynamic and toxicology studies and could be safely prescribed for patients and physicians (Alvim *et al.*, 2006; Veiga-Junior, 2008). Nowadays there is a revival of the interest in the consumption of medicinal plants partly due to the search for a better quality of life and the economic situation, associated to the high cost of patentable chemical drugs (Rodrigues & Guedes, 2006). Only in the last decades of the twentieth century has there been a major shift in the attitude of the community and scientists towards medicinal plants with several researchers showing an interest in an interdisciplinary study that investigates scientific foundations for popular beliefs of healing based on plant products (Freire, 2004).

Phytotherapy is considered relatively safe, although there are some controversies and limited scientific evidence for this assumption (Rafieian-Kopaei, 2012). The use of medicinal plants in therapeutic uses for various diseases demonstrates certain advantages, more precisely because they are economical, effective and their relative ease of acquisition, which means that these types of plants have been widely used by traditional doctors in their daily practice (Prakash & Gupta,

2005). In addition, medicinal plants are rich in secondary metabolites, being potential sources of drugs, and essential oils of therapeutic importance, being able to exert biological activity *in vitro* and *in vivo* (Duarte *et al.* 2005; Prakash & Gupta, 2005). However, the indiscriminate use of medicinal plants may pose health risks, depending on the cultural factors and the physical environment in which they develop (pesticide use or soil or water contamination can trigger diseases) (Rodrigues & Guedes, 2006).

The scientific community has always considered the use of medicinal plants for therapeutic purposes with suspicion and disbelief (Freire, 2004); however, there is an upward trend to include phytotherapy in the medical school curriculum, and data on medicinal plants are increasingly being published (Rafieian-Kopaei, 2012). It is important to amplify the knowledge of medicinal plants, how they act, what are their toxic and collateral effects, how their interactions with new allopathic drugs are and what strategies are most appropriate for quality control and phytotherapeutic production, and more multidisciplinary studies involving ethnobotanists, chemists, pharmacologists and agronomists (in this case, in the control of the cultivation of medicinal plants) are required (Junior *et al.*, 2005). Doctors will prescribe more and more phytomedics as many more studies there are about medicinal plants, especially in the field of phytochemistry, ethnopharmacology and toxicity, thus meeting the population's desire to use medicinal plants in their treatments, and opening a golden opportunity to continue making a valid contribution to health (Veiga-Junior, 2008). Pharmacognosy has a very bright future ahead of it, as it will continue to provide new molecules for the various diseases that humanity faces, as long as researchers are sufficiently involved in this area, and clinicians and scientists have the common effort to produce safe, quality and effective products (Gurib-Fakim, 2006).

1.2. *Baccharis dracunculifolia*

The genus *Baccharis* includes more than 500 species, distributed mainly in the tropical areas of South America, and are a group of perennial woody shrubs, which are dioecious with male and female inflorescences, appearing in separate plants (Ferracini *et al.*, 1995). Of the various species of *Baccharis*, *Baccharis dracunculifolia* D.C. (Asteraceae) is a wild shrub that is born wildly in Brazil, where it is native, and is popularly known as "alecrim do campo" and "vassoura" (Da Silva Filho *et al.*, 2009; Missima *et al.*, 2006). This plant is widely used in folk medicine as an infusion to prevent certain diseases, due to its anti-inflammatory, antipyretic, antiseptic properties, for

treatment of skin wounds, hepatic disorders, stomach ulcers, among others (Lemos *et al.*, 2007; Parreira *et al.*, 2010). In addition, this plant also produces essential oils, presenting commercial value due to its exotic and long-lasting aroma and its potential range of applications, in the food industry, pharmaceuticals, agrochemicals, perfumery and cosmetics (Park *et al.*, 2004; Rezende *et al.*, 2014).

Figure 1: Adult shrub of *Baccharis dracunculifolia* (Sousa, 2007).

B. dracunculifolia is the main botanical source of propolis in South Eastern Brazil, which due to its color is called green propolis, and is recognized for its antiseptic and antiprotozoal activities (Missima *et al.*, 2006; Parreira *et al.*, 2010). Propolis is a natural resinous substance collected from plants through honeybees (*Apis mellifera*) purposely to be used as a protective barrier in the beehives (Rezende *et al.*, 2014).

Currently, due to their biological activities, Brazilian green propolis and *Baccharis dracunculifolia* leaves are used worldwide in food and beverages, especially in Brazil and Japan, aiming to improve health and to prevent several diseases due to its numerous medicinal properties, such as hepatoprotective, antibacterial, anti-viral, antifungal, antioxidant, anti-tumor, anti-inflammatory, anticancer, among others (Da Silva Filho *et al.*, 2009; Guimarães *et al.*, 2012; Missima *et al.*, 2006; Rezende *et al.*, 2014).

The numerous properties of propolis and *B. dracunculifolia* leaves derive from the presence of a wide variety of compounds, such as polyphenols (mainly flavonoids), cinnamic acid and phenolic acids (especially prenylated *p*-coumaric acid derivatives) in their composition (Funari *et al.*, 2007; Guimarães *et al.*, 2012; Lemos *et al.*, 2007; Missima *et al.*, 2006).

1.3. Antioxidant activity

Antioxidants are compounds that retard, inhibit or prevent the oxidation of oxidizable substances present in living cells (including proteins, lipids, DNA and carbohydrates), by elimination and/or capture of reactive oxygen species (ROS) and reduction of oxidative stress (Al-Jaber *et al.*, 2011).

ROS are several forms of activated oxygen, which are associated with a process of cell injury and aging, which can develop various human diseases, such as neurodegenerative diseases, coronary heart disease, cancer, diabetes, inflammation, viral infections, atherosclerosis, pathologies autoimmune, disorders of the digestive system, among others (Al-Jaber *et al.*, 2011; Huda-Faujan *et al.*, 2009). ROS are generated through the normal metabolism of drugs (Al-Jaber *et al.*, 2011), environmental chemicals (Al-Jaber *et al.*, 2011) and other xenobiotics (Al-Jaber *et al.*, 2011), as well as stress hormones (adrenalin and noradrenalin) (Al-Jaber *et al.*, 2011), and evidence proves that ROS can be eliminated or avoided by using natural antioxidant compounds present in foods and medicinal plants (Al-Jaber *et al.*, 2011).

In recent years, there has been great interest in the research and identification of possible sources of natural antioxidants from medicinal plants (Huda-Faujan *et al.*, 2009), which are found in different parts of the plant, such as seeds, fruits and leaves (Al-Jaber *et al.*, 2011).

Although it is not known what the most active compounds of plants, the natural antioxidants present in plants may be polyphenols, alkaloids, carotenoids or vitamins (ascorbic acid or tocopherol) (Al-Jaber *et al.*, 2011). Polyphenols are the most important secondary metabolites in the plant kingdom, raising interest in scientists, food manufacturers (because they delay the oxidative degradation of lipids and thus improve the quality and nutritional value of food) and consumers due to food with specific health benefits (Huda-Faujan *et al.*, 2009).

There is a considerable number of chemical and biochemical tests for the identification and quantification of antioxidant activities *in vitro* of plant extracts and other compounds, being important for the screening of bioactive compounds that can be used in drugs and for other purposes (Alves *et al.*, 2010). Some of these existing tests are the method diphenylpicrilhydrazyl (DPPH, 1,1-diphenyl-2-picrylhydrazyl), iron chelating activity (ICA), inhibition of nitric oxide production, superoxide scavenging activity, and high performance liquid chromatography (HPLC).

Several studies, such as Funari *et al.* (2007), Guimarães *et al.* (2012), Rezende *et al.* (2014), show that *B. dracunculifolia* exhibits potent antioxidant activity.

1.4. Inflammation

Inflammation is a tissue reaction of the organism to the wound, with the aim of restoring homeostasis, and is characterized by an increase in the amount of circulating blood in a given site (hyperemia) and exudation from blood vessels to the inflammatory focus (Okoli *et al.*, 2003). Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury, leading to the release of exogenous and endogenous chemical mediators, which in turn give rise to the primordial signs of inflammation such as redness, heat, swelling and pain (Okoli *et al.*, 2003; Serhan *et al.*, 2008).

Inflammatory pathways consist of inducers, sensors, mediators and effectors (Medzhitov, 2008). Inducers are the signals that initiate the inflammatory response and they activate specialized sensors, which trigger the production of specific sets of mediators (Medzhitov, 2008). The mediators, in turn, alter the functional states of tissues and organs (which are the effectors of inflammation) in a way that allows them to adapt to the conditions indicated by the particular inducer of inflammation (Medzhitov, 2008).

The mediators of inflammation may be exogenous or endogenous, and exogenous mediators include microbial peptides that act as chemoattractants to recruit neutrophils to the challenge site, where they phagocytose invading microorganisms and cellular debris (Medzhitov, 2008; Serhan *et al.*, 2008). Neutrophils also have the capacity to produce reactive oxygen species (ROS) to kill trapped microorganisms or degrade cellular debris (Serhan *et al.*, 2008). Thus, the initial inflammatory response works to protect the host (Serhan *et al.*, 2008). However, sometimes the neutrophil granule contents can spill into the extracellular medium, inadvertently or when phagocytes encounter foreign surfaces they do not ingest, which leads to local tissue damage and the amplification of acute inflammatory signals within minutes to hours after the initial challenge (Serhan *et al.*, 2008).

A successful acute inflammatory response results in the elimination of the infectious agents, followed by a repair phase, that allows the return to homeostasis, and it is very important that this inflammatory reaction is resolved to avoid that the inflammation spreads, becoming chronic (Medzhitov, 2008; Serhan *et al.*, 2008). Thus, in acute inflammation, depending on the type and location of injury and host response, complete resolution of the inflammation may occur or may progress to chronic inflammation (Serhan *et al.*, 2008).

On the other hand, if the acute inflammatory response cannot eliminate the pathogen, the inflammatory process persists and acquires new characteristics (Medzhitov, 2008).

Thus, the neutrophil infiltrate is replaced by macrophages, and in the case of infection also with T cells, and even if the combined effect of these cells is still insufficient, a chronic inflammatory state occurs, involving the formation of granulomas and tertiary lymphoid tissues (Medzhitov, 2008). In addition to persistent pathogens, chronic inflammation may result from other causes of tissue damage, such as excessive and/or unresolved inflammatory responses, and the presence of non-degradable foreign bodies (Serhan *et al.*, 2008).

Therefore, a controlled inflammatory response is normally thought to be beneficial (for example, in protection against infection), but may become detrimental if disregulated (for example, causing septic shock) (Medzhitov, 2008).

1.5. Pain

Pain is a universal experience that alerts to an imminent threat, allowing the body to know if homeostasis has been disrupted and that a change in behavior is needed (Linley *et al.*, 2010; Meyr & Steinberg, 2008). According to the International Association for the Study of Pain (IASP), pain can be defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (McGuire & Kennedy, 2013). The strong stimulation of most body tissues causes unpleasant sensations of pain, however pain is beneficial because it has a protective role in driving behaviors that eliminate or minimize the threat (Dubin & Patapoutian, 2010; Marchand *et al.*, 2005).

Nociception is the neural process of encoding and processing noxious stimuli and includes the detection and localization of a potentially damaging stimulus or a site of injury ((Dubin & Patapoutian, 2010; Linley *et al.*, 2010). Nociception is a function of the nervous system in which there is transmission of electrical and chemical signals, which originate from the peripheral nerve endings, called nociceptors, passing through the spinal cord (SC) and finally reaching the brain for interpretation (Cheng, 2010). Although nociception is a protective mechanism, when there is a persistent injury, nociception may start to misrepresent sensory stimulation, causing pain that is no longer beneficial to the organism (Linley *et al.*, 2010).

When the stimuli that the nervous system detects are intense, they generate acute pain, and when there is a persistent injury, the components of the central and peripheral nervous system of the pain transmission pathway exhibit enormous plasticity, increasing pain signals and producing hypersensitivity (Basbaum *et al.*, 2009). In this way, plasticity can facilitate protective reflexes, being a beneficial act, but when the alterations persist, it can lead to a chronic pain condition, causing damage to the body (Almeida *et al.*, 2004; Basbaum *et al.*, 2009).

In the dorsal horn of the spinal cord, nociceptors project to distinct laminae, as shown in the following figure:

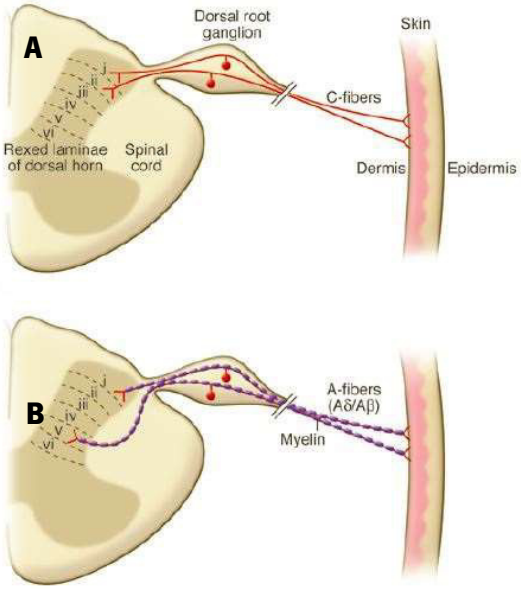


Figure 2: (A) Most nociceptors are unmyelinated with small diameter axons (C-fibers, red), and their peripheral afferent innervates the skin (dermis and/or epidermis) and central process projects to superficial laminae I and II of the dorsal horn. (B) A-fiber nociceptors are myelinated and usually have conduction velocities in the Aδ range (red). A-fiber nociceptors project to superficial laminae I and V (the Aδ fibers projects to superficial laminae I and V, and the Aβ fibers projects to superficial laminae V) (Dubin & Patapoutian, 2010).

After the primary afferent nociceptors convey nociceptive information to spinal projection neurones forward the nociceptive signal to the brain through several ascending tracts: spinothalamic, spinoreticular, spinomesencephalic, cervicothalamic and spinohypothalamic tracts (Almeida *et al.*, 2004). Then, from the interaction of the sensory impulses in the spinal cord, the nociceptive afferent pathways give origin to the different models of projection to subcortical and cortical

structures, thus occurring the supraspinal pain processing (Almeida *et al.*, 2004). Finally, occur the pain descending modulatory systems (Cheng, 2010).

1.5.1. Acute pain

Acute pain, or protective pain, is an alert function characterized by the fact it is limited in time, lasting from seconds to minutes, and disappearing with the resolution of the pathological process (Almeida *et al.*, 2004). The acute pain pathway, although well understood, remains a complex physiologic process and can be thought of as a serial bottom-up systems in which a peripheral stimulus leads to a staged series of reactions heading up the central nervous system (Meyr & Steinberg, 2008).

In order to treat the source of pain there are four important points of occurrence: stimulus, transmission, modulation and perception (Meyr & Steinberg, 2008). Then, in acute pain, a stimulus first occurs and is detected by cells with appropriately tuned receptive properties (Julius & Basbaum, 2001). Then, there is the transmission of this peripherally produced noxious stimulus to the spinal cord and the central nervous system, through the activation of nociceptors (Julius & Basbaum, 2001; Meyr & Steinberg, 2008). The modulation point corresponds to all the activity that occurs at the dorsal horn of the spinal cord as the peripheral noxious signal carried by the primary afferent synapses with the neuron of the central nervous system (Meyr & Steinberg, 2008). And finally, the perception represents another complex zone of mixed ascending and descending excitatory and inhibitory signals, as well as the taking of a quantitative central nervous system stimulus and translating it into a qualitative physical and emotional response (Meyr & Steinberg, 2008).

Primary sensory neurons that can be excited by noxious heat, intense pressure or irritant chemicals, but not by innocuous stimuli such as warming or light touch (Julius & Basbaum, 2001).

Pain is transmitted throughout the body to the brain through fibres that innervate these regions and that arise from cell bodies in trigeminal and dorsal root ganglion (DRG), and can be categorized into three main groups based on anatomical and functional criteria, being the fibres Aβ, Aδ, and C (Julius & Basbaum, 2001; Linley *et al.*, 2010). However, only the Aδ and C fibres are involved in acute nociceptive transmission (Julius & Basbaum, 2001; Meyr & Steinberg, 2008). These two types of fibres correspond to most nociceptors, and are characterized by being medium and small diameter cell bodies, and lightly myelinated or unmyelinated, respectively (Julius & Basbaum,

2001). In addition, it has long been assumed that Aδ and C nociceptors mediate "first" and "second" pain, respectively (Julius & Basbaum, 2001).

Complete removal of the stimulus is an important factor for the resolution the acute pain pathway and avoiding sensitization and activation of chronic pain cycles (Meyr & Steinberg, 2008).

1.5.2. Chronic pain

The standard definition of chronic pain endorsed by the International Association for the Study of Pain states that it is pain that persists past the healing phase following an injury (Apkarian *et al.*, 2009. Therefore, chronic pain can be associated with injury or disease (such as diabetes, arthritis, or tumor growth) and can result from alterations in the properties of peripheral nerves, leading to increased spontaneous firing or alterations in conduction properties or neurotransmitters (Basbaum *et al.*, 2009).

Acute pain becomes chronic and debilitating when pain often outlives its usefulness as a warning system, and long-term changes occur at the periphery and within the central nervous system (CNS), namely in primary sensory neurons, spinal cord and brain (Julius & Basbaum, 2001; Voscopoulos & Lema, 2010).

Chronic pain may also result from an uncontrolled inflammatory response that may lead to peripheral sensitization (Meyr & Steinberg, 2008), with the common clinical signs accompanying the development of chronic pain being hyperalgesia, allodynia and spontaneous pain (Julius & Basbaum, 2001). These chronic pain symptoms are a consequence of increased responsiveness from nociceptors, plasticity of central nociceptive neurons and peripheral Aβ fibres that normally signal innocuous sensation applied to the skin, muscle, and joints (Julius & Basbaum, 2001).

In chronic pain, neuropathic pain is the result of a nerve injury or disease in the peripheral or central nervous system (Persson *et al.,* 2009), whereas nociceptive pain (like in arthritis) is a consequence of the prolonged activation of nociceptors by injury and is usually accompanied by inflammation (Julius & Basbaum, 2001).

1.5.2.1. Inflammatory pain

The inflammatory pain results from increased excitability of the peripheral nociceptive sensory fibres due to the action of inflammatory mediators, and whose excitatory effect is a result of the altered activity of the ion channels within the affected sensory fibres (Linley *et al.*, 2010).

The inflammatory responses are composed of several chemical factors that are released into the extracellular space, these chemical agents are often referred to as "inflammatory mediators" (Linley *et al.*, 2010). The results of these inflammatory mediators are depolarisation, lowering of the threshold for action potential firing, delayed repolarisation and as a result, hypersensitivity and over-excitability of sensory neurons (Linley *et al.*, 2010).

In states of chronic inflammatory pain, nociceptive function can be altered to induce abnormal sensory phenomena such as hyperalgesia, allodynia and spontaneous pain (Cheng, 2010). While spontaneous pain is characterized by an intense painful sensation of short duration with no apparent cause, allodynia is defined as the increase of nociception in normally innocuous stimuli that are perceived as painful (for example, light touch or heat) (Basbaum *et al.*, 2009) (Cheng, 2010). On the other hand, hyperalgesia is defined as an excessive painful response to a painful stimulus (e.g., pinprick) (Cheng, 2010), and can be differentiated into primary hyperalgesia (increased pain perception in the area of the lesion or exposure to noxious stimuli) and secondary hyperalgesia (increased pain perception in the region around the area exposed to noxious stimulus) (Cheng, 2010; Dubin & Patapoutian, 2010). Spontaneous pain, allodynia, and hyperalgesia can develop under certain pathophysiological conditions, such as inflammation or nerve injury, and may last for months or years, for example in arthritis and neuropathic pain, causing suffering and distress (Linley *et al.*, 2010).

Chronic inflammatory disorders include diseases involving musculoskeletal disorders, the most common being rheumatoid arthritis and osteoarthritis (Merskey & Bogduk, 1994).

1.6. Osteoarthritis

OA is a common chronic degenerative joint disease, affecting a large portion of the population and occurring mainly in middle-aged and elderly people (Ji *et al.*, 2016). OA is one of the most prevalent and debilitating joint diseases associated with reduced quality of life, and whose cause is not yet fully understood (Ji *et al.*, 2016; Şükür *et al.*, 2016). In humans, OA is associated with multiple risk factors that contribute to its development, such as age, sex, obesity, malalignment, genetic

factors and joint injuries caused by the practice of high impact exercises (Ozeki *et al.*, 2016; Rojas-Ortega *et al.*,, 2015). The clinical condition of OA consists of a series of symptoms, such as pain, stiffness, swelling, deformity, loss of function, limitations in the patient's activity and low quality of life, affecting the whole joint, particularly the hips, knees and part lower back (Ji *et al.*, 2016). The symptoms of OA are different between individuals and between different stages of the disease, and may also change over time, these being more intense after inactivity or excessive use of physical movement, being generally worse after the activity or at the end of the day (Bultink e Lems, 2013). OA is divided into primary OA or secondary OA (Cui *et al.*, 2015), and primary osteoarthritis evolves spontaneously such as postmenopausal OA, while secondary OA results from traumatic injuries and others (Cui *et al.*, 2015).

According to the World Health Organization, OA is already one of the ten most disabling diseases in developed countries, estimating that 9.6% of men and 18.0% of women over 60 years of age have symptomatic OA (World Health Organization, 2014).

OA is characterized by biochemical, enzymatic and morphological changes, affecting several tissues and neighbouring joints with the most significant alterations being observed in the proximal tibial bone of knee OA (Bultink e Lems, 2013; Wang *et al.*, 2015). These changes are characterized by damage to the articular cartilage (fibrillation, erosion and gradual loss of hyaline cartilage, and development of cysts at the margins of the joints); bone changes (formation of osteophytes and subchondral bone sclerosis); synovia damage (synovial membrane); chondrocyte death (by apoptosis or necrosis) and gaps formation; destruction of other periarticular soft tissue structures, such as ligaments and tendons; and also changes in the architectural microstructure of trabecular bone, such as the formation of thick, sparsely placed and vertically oriented trabeculae (Bultink e Lems, 2013; Moriyama *et al.*, 2008; Podsiadlo *et al.*, 2008; Wang *et al.*, 2015). In addition, studies have shown that ROS, which are capable of leading to oxidative stress, are also part of the factors that cause OA, since oxidative stress triggers inflammatory responses, causing degradation of extracellular matrix and inhibiting its synthesis, thus triggering OA (Cheuk *et al.*, 2016).

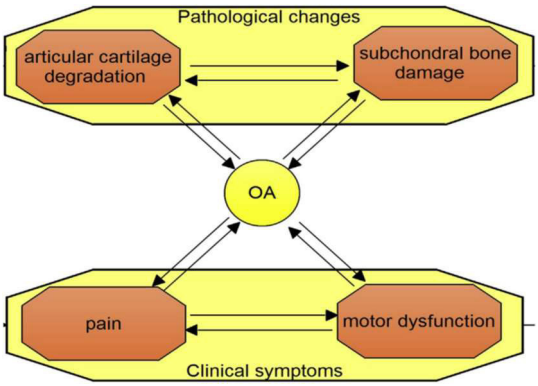


Figure 3: OA is characterized by pathological changes on articular cartilage and subchondral bone, with clinical symptoms of pain and motor dysfunction (Ji *et al.*, 2016).

Although OA is constantly increasing due to ageing, there is still no promising drug capable of altering or slowing the progression of this disease, largely due to the complex disease pathology that affects not only the cartilage but also the subchondral bone and synovium (Ozeki *et al.*, 2016). However, there are medical therapies available, such as traditional analgesics and non-steroidal anti-inflammatory drugs, capable of only alleviating the symptoms of this disease (Şükür *et al.*, 2016).

1.6.1. Animal models of experimental osteoarthritis

OA is a complex disease whose singular mechanisms are more easily studied in animal models (Bendele, 2001). Several animal models of OA have been developed in an attempt to imitate all aspects of human disease, in order to facilitate discoveries in science and thus improve health and human well-being (Gregory *et al.*, 2012). Thus, the animal model chosen should try to imitate the human pathology (Gregory *et al.*, 2012). The animal models most commonly used in the studies include various species, such as, mice, rats, guinea pigs, Syrian hamsters, primates, dogs, rabbits, sheep, goats and horses (Bendele, 2001; Gregory *et al.*, 2012).

As a rule, animal models of OA are divided into two main categories: spontaneous joint models degeneration and experimentally induced OA models (Wang *et al.*, 2015). The spontaneous joint models degeneration are characterized by OA occurring naturally, however the rats have very little spontaneous degeneration in their knee joints, occurring more commonly in mice, guinea pigs, Syrian hamsters and nonhuman primates (Bendele, 2001; Wang *et al.*, 2015). The experimentally

induced OA models is further subdivided into three types: mechanically induced OA caused by joint instability through partial meniscectomy combined with transection of collateral and/or cruciate ligaments; chemically induced OA through intra-articular injection of compounds that cause joint pathology and damage to ligaments and tendons; and genetically induced OA through the overexpression of some compounds (Little and Zaki, 2012).

In this work, for the induction of OA, a type of chemical model was used that is characterized by an intra-articular injection of a solution of kaolin and carrageenan (K/C model) into the synovial cavity of the knee joint. As with human osteoarthritis, this model causes cartilage damage, synovial inflammation, and synovial fluid exudate, and arthritis develops within hours and persists for weeks (Neugebauer *et al.,* 2007).

Chapter 2: Objectives

2.1. General objective

Considering OA is the most common form of arthritis and the most debilitating, causing discomfort and pain in patients, and that *Baccharis dracunculifolia* is a medicinal plant with potential anti-inflammatory and anti-oxidant activities, the general objective of this work was to evaluate the pharmacological potential of *Baccharis dracunculifolia* extract in the treatment of osteoarthritis.

2.2. Specific objectives

- To analyze possible antioxidant activities of *Baccharis dracunculifolia*;
- To identify and quantify compounds of *Baccharis dracunculifolia* extract by high performance liquid chromatography (HPLC) analysis;
- To compare the locomotor ability, hypersensitivity and edema of the joints, between healthy animals and animals with experimental osteoarthritis with treatment and without treatment;
- To compare the knee joints between healthy animals and animals with experimental osteoarthritis with treatment and without treatment;
- To evaluate the possible anti-inflammatory activity of the Baccharis dracunculifolia extract in the control of dermal inflammations and consequently in the inflammations of the knee joints.

Chapter 3: Materials and Methods

3.1. Extract of Baccharis dracunculifolia

The top part of *Baccharis dracunculifolia* plants were collected in the garden of the Faculty of Pharmacy of the Federal University of Juiz de Fora (UFJF), Brazil, in January 2013. The aerial parts were cleaned and dried at room temperature. Afterwards they were crushed with the aid of a knife mill until obtaining pulverized vegetable material. Then, the resulting powder was subjected to a maceration process with hydroalcoholic solution (8:2 v/v) (Ethanol P.A., Vetec) for a period of 3 days. Thereafter, the hydroalcoholic solution was filtered by a filter paper and concentrated in a rotary evaporator (Buchi® RII) under reduced pressure (Buchi® V-700 pump), at 50°C. Finally, a concentrated crude hydroalcoholic extract of aerial parts of *Baccharis dracunculifolia* was obtained.

This concentrated extract was placed in a lyophilizer (Christ® Alpha 2-4, B. Braun) and freeze-dryed, thus obtaining a *Baccharis dracunculifolia* powder. This powder of *Baccharis dracunculifolia* was duly stored in a closed falcon in a dry place and protected from light, til further uses.

3.2. HPLC

Baccharis dracunculifolia powder extract was subjected to HPLC analysis to separate, identify and quantify the major phenolic compounds of this extract. This analysis was performed using a Hitachi-Merck HPLC-DAD system (high performance liquid chromatography with diode array detection), controlled by Merck pc- software (Merck, USA).

The chromatographic separation was performed on Hitachi ELITE LaChrom (Merck-Hitach, Tokyo, Japan), equipped with an L-2130 pump, an L-2200 autosampler, an L-2300 column oven and an L-2455 DAD, operating at 30°C. To perform the separation of compounds was injected 20 μl of Bd extract (concentration 3 mg/ml) solubilized in methanol. The solvent system was a methanol gradient (Merck, Darmstadt, Germany), using solvent A (0.1% formic acid in methanol P.A. (Merck, Darmstadt, Germany)) and solvent B (ultra-pure water with 0.1% of formic acid (Merck, Darmstadt, Germany)). The elution consisted of a six step gradient: starting with 15% of solvent A and mantaining the same concentration (15% A) for 3 min, increasing to 90% A at 35 min, maintaining 90% A at 45 min, decreasing to 15% A at 50 min and maintaining 15% A at 60 min. Detection was performed with a diode array detector, and spectral data of all peaks were accumulated in the wavelength range 245-530 nm and the chromatograms were recorded at 260, 280 and 350 nm.

All of these procedures were performed based on the protocol described by Dias *et al.* (1999).

Elution was performed using a gradient specifically developed for analysis of the extracts. Identification of the compounds was performed according to their UV-Vis spectra, retention time (Rt), and compared to standard reference compounds.

3.3. Antioxidant activity of *Baccharis dracunculifolia*

In this work, in order to study the antioxidant potential of the *Baccharis dracunculifolia* extract, four different antioxidant model systems were used: DPPH method, Iron Chelating Activity (ICA), Inhibition of Nitric Oxide production and Superoxide scavenging activity.

3.3.1. DPPH method

Initially, to carry out all the following antioxidant tests, 150 mg/ml of *Baccharis dracunculifolia* extract in DMSO was diluted. In this assay the antiradical activity of the Bd extract was evaluated using a dilutions series, in order to obtain a spectrum of concentrations: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.0315 mg/ml, 0.01563 mg/ml. 10 μl of Bd extract was mixed with 140 μl of a DPPH (Sigma, Lisbon, Portugal) stock solution (400 μM, ethanol 100%). Then, the plate was placed in the microplate reader (SpectraMax Plus 384) and read at 515 nm by SoftMax program, with punctual readings at t = 0, 3, 6, 9, 12, 15, 20, 30, 60. For the blank, 10 μl of the above solutions and 140 μl of ethanol were used. For the negative control, 10 μl of solutions and 140 μl of DPPH were used. The DPPH solution was prepared daily, and kept protected from the light at 4˚C when not in use between analyses. All measurements were performed in triplicate.

The 50 minutes was considered as a time of reactive steady-state discoloration for all the plant extracts, since at this moment the reaction reached the plateau state for all the samples used. Thus, the absorbance data obtained at this time point was recorded, and the percentages of inhibition of discoloration for each plant extract concentration were calculated. To calculate the percentage of reduced DPPH, the following formula was used:

$$\%\ \mathrm{DPPHr} = \frac{\mathrm{Abs\ control} - (\mathrm{Abs\ t60} - \mathrm{Abs\ blank})}{\mathrm{Abs\ control}} \times 100$$

In this way, the EC50 was obtained from the reduced DPPH curve percentage and represents the sample concentration required to quenching DPPH free radicals which produces 50% decreasing

in absorbance. All the parameters were calculated graphically using the software GraphPad Prism 5 (GraphPad Software Inc, LaJolla, CA, USA). All of these procedures were based on the protocol of Silva *et al.* (2008), with some adaptations.

3.3.2. Iron Chelating Activity (ICA)

The iron chelating activity assay (ICA) estimates the ability of antioxidants to chelate Fe^{2+} (Riemer *et al.*, 2004). The ferrozine forms a complex with Fe^{2+} (Fe^{2+} - FZ) providing a purple color, however in the presence of chelating agents the formation of the complex is interrupted and there is a decrease of the purple color, thus allowing the colorimetric quantification of iron (Riemer *et al.*, 2004). To evaluate the iron chelating activity of *Baccharis dracunculifolia*, several solutions were prepared daily and kept protected from light. This activity was assessed using a series of dilutions of the Bd extract so as to obtain a broad spectrum of plant concentrations as follows: 50 µl of Bd extract (the extracts were dissolved in 100% ethanol to give different concentrations: 3 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.375 mg/ml, 0.1875 mg/ml e 0.09375 mg/ml), 50 µl FeSO4 stock solution (0.12 mM, ultrapure water; (Sigma, Lisbon, Portugal)) and 50 µl of Ferrozine stock solution (0.6 mM, ultrapure water; (Sigma, Lisbon, Portugal)). For blank, the above mentioned solutions were used, whereas for negative control such solutions and water were used. The readings were performed after 10 minutes of dark incubation at room temperature, at 515 nm with a microplate reader (SpectraMax Plus 384) via SoftMax software. In the presence of chelating agents the formation of the Ferrozine-Fe^{2+} complex is inhibited and the color of the initially purple complex declines and thus with the measurement of the color reduction it is possible to estimate the chelator activity (Riemer *et al.*, 2004). The different solutions were prepared daily, and kept protected from the light. All measurements were performed in triplicate, and all parameters were calculated graphically using GraphPad Prism 5 software (GraphPad Software Inc., LaJolla, CA, USA). All of these procedures were based on the protocol of Russo *et al.* (2005), with some adaptations.

To calculate the chelating activity, the following formula was used:

$$\%\ \text{ICA} = \frac{\text{Abs control} - (\text{Abs sample} - \text{Abs blank})}{\text{Abs control}} \times 100$$

in which Abs control corresponds to the absorption of ferrozine with iron, without extract; Abs sample corresponds to ferrozine absorbency with iron and extract; and Abs blank corresponds to the absorbance of the extract without ferrozine. EC50 was obtained from the percent ICA plot

against the concentration of Bd extract and represents the concentration necessary to reduce ferrozine decolorization by 50%.

3.3.3. Inhibition of Nitric Oxide production

Sodium nitroprusside (SNP) is a potent nitric oxide (NO) donor (Pandareesh & Anand, 2014). NO react with molecular oxygen, superoxide anion and transition metals to produce reactive nitrogen species such as nitrosonium (NO^+), nitroxyl (NO^-), N_2O_3, NO_2, NO_2^-, NO_3^-, peroxynitrite ($OONO^-$) and metal-nitrosyl adducts (Pandareesh & Anand, 2014). Thus, one of the reactive species produced by NO in the presence of molecular oxygen is nitrite (NO_2^-), which reacts with the Griess reagent and produces a pink chromophore that can be measured at 562 nm (Korhonen *et al.*, 2005) (Pandareesh & Anand, 2014).

For the determination of nitric oxide concentration, the extract of *Baccharis dracunculifolia* was initially diluted in phosphate buffer to obtain different concentrations of Bd extract (2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml). The Bd extract concentrations (50 µl) and the control (phosphate buffer, pH 7.4) were placed in quadruplicate in 96-well plates for reading on the plate reader (Spectra_max Plus 384). In each well was added 50 µl of 20 mM SNP (Sigma, Merck) and 50 µl of Bd extract dissolved in phosphate buffer. The plate was left at room temperature under light for 1h. Then, 50 µl of Griess reagent (except for the sample blank and control) was added. The Griess reagent was composed by 50% of 1% sulfanilamide in 5% H3PO4 NED (0.1%, ultrapure water). For the control (blank) 50 µl phosphate buffer and 50 µl SNP were used, and for the negative control 50 µl phosphate buffer, 50 µl SNP and 50 µl Griess were used.The plate was incubated in a microplate reader (Spectra_max Plus 384) at room temperature for 10 minutes, protected from light and the absorbance determined at 560 nm, through the SoftMax program. All measurements were performed in triplicate, and all parameters were calculated graphically using GraphPad Prism 5 software (GraphPad Software Inc., LaJolla, CA, USA).

Results are expressed as percent inhibition of nitrite production relative to the control. Therefore, if the extracts have a good ability to eliminate NO, the nitrite production decreases as well as the absorbance. All these procedures were based on the protocol Colle *et al.* (2011), with some adaptations.

In order to calculate the percent inhibition of nitric oxide, the following formula was used:

$$\%\ \text{Inhibition of NO} = \frac{\text{Abs control} - (\text{Abs sample} - \text{Abs blank})}{\text{Abs control}} \times 100$$

3.3.4. Superoxide scavenging activity

In this study, the non-enzymatic assay was used to evaluate the superoxide radical scavenging activity, and the superoxide radicals were generated by the NADH/PMS system (Valentão *et al.*, 2002b). In addition, superoxide reacts with NBT, producing a color change from light yellow to dark purple, which can be measured by spectrophotometer at 560 nm (Hanson *et al.*, 2006). Therefore, the antioxidant capacity of a particular extract to eliminate superoxide radicals can be determined by the inhibition of NBT reduction (Hanson *et al.*, 2006).

For the evaluation of superoxide anion scavenging activity, *Baccharis dracunculifolia* extract was diluted in phosphate buffer to obtain different concentrations of Bd extract (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml). Subsequently, 50 µl of Bd extract dissolved in phosphate buffer, 50 µl of 197 µM NADH solution, 150 µl of 81,5 µM NBT solution (Roche Diagnostics), and 50 µl of 3.26 µM PMS solution (ACROS Organics) were added to each well of the plate. For the blank 50 µl of the above mentioned solutions, 50 µl of NADH and 150 µl of NBT were used. For the negative control, 50 µl of phosphate buffer, 50 µl of NADH, 150 µl of NBT and 5 µl of PMS were used. The assays were performed at room temperature, and the anti-radical activity was determined spectrophotometrically on a 96-well plate reader (Spectra_max Plus 384) at 560 nm through the SoftMax program, for 2 minutes. This reading was started with the addition of the PMS solution. All measurements were performed in triplicate, and all parameters were calculated graphically using GraphPad Prism 5 software (GraphPad Software Inc., LaJolla, CA, USA).

In order to calculate the percentage of superoxide anion scavenging activity, the following formula was used:

$$\%\ \text{Superoxide scavenging} = \frac{\text{Abs control} - (\text{Abs sample} - \text{Abs blank})}{\text{Abs control}} \times 100$$

Results are expressed as percent inhibition of NBT production relative to the control. Therefore, in the presence of extracts capable of eliminating O_2, the rate of NBT reduction decreases and consequently there is a decrease in absorbance. All these procedures were based on the protocol of Valentão *et al.* (2002a), with some adaptations.

3.4. Animals and ethical issues

In this experimental work, 24 adult female rats were used, *Rattus norvegicus,* var. *Albinus, Wistar*, provided by the ICVS vivarium of the University of Minho. The animals were housed in a maximum of three animals per cage (45.4 x 25.5 x 20 cm), and always with water and food available *ad libitum*, in the vivarium of the Life and Health Sciences Research Institute (ICVS). The animals were kept under a controlled light cycle of 12 h light/12 h dark cycle (with lights on at 08:00 am), at a controlled temperature of 22°C and 55% relative humidity.

All experimental procedures were performed in accordance with ethical guidelines for animal experiments for scientific purposes, with all appropriate measures to minimize pain and discomfort in animals. In addition, in this study all efforts were also made to use only the number of animals necessary to produce reliable scientific data.

3.5. Surgical Procedures

In this work the ovariectomy and the induction of OA were performed on week 3. The induction of the ovariectomy preceded the induction of OA, taking advantage of the same anesthesia and anti-sedation, thus avoiding to expose the animals to two anaesthesia procedures.

3.5.1. Ovariectomy

All the female rats underwent ovariectomy to avoid interferences of the hormonal cycle and also to reduce estrogen levels, thus mimicking the postmenopausal period in women (Monteiro *et al.*, 2005).

Prior to surgery, all animals were weighed and doses of anesthesia and anti-sedative were administered according to the weight of the animal. Afterwards, all animals were given general anesthesia with a combined solution of ketamine (0.75 mg/kg, i.p.; Imalgene, Merial Lyon, France)

and medetomidine (0.5 mg/kg, i.p.; Dorbene, Esteve Veterinaria, Léon, Spain), by intraperitoneal route (i.p.), using a standard hypodermic syringe (Terumo, Neolus, 0.6 × 25 mm, Belgium) (David-Pereira *et al.*, 2016). As soon as the animals failed to show any blink reflex or any other type of reflex, the surgery was started.

Surgery was performed on a sterile operating table where the animal was placed on ventral recumbency. The lumbar area was first shaved to remove hairs, and the skin was disinfected with chlorhexidine disinfectant. Then, with the use of a scalpel, a midline dorsal skin incision was made, with an average extension of 1 to 2 cm, half way between the middle of the back and the base of the tail, as represented in the following figure 4.

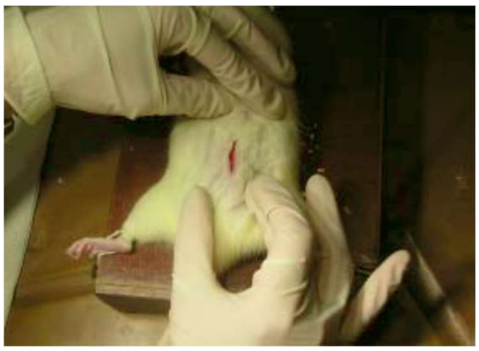


Figure 4: Longitudinal, dorsal midline skin incision (Lasota & Danowska-Klonowska, 2004).

Thus, the peritoneal cavity was accessed, the ovaries were found, surrounded by a variable amount of fat, and were exposed with the aid of a forceps. The ovaries were removed and baked with non-absorbable silk yarn (3.0 mm, Mersilk, W5714, Ethicom, B. Braun, Tuttlingen, Germany). The remaining tissue were repositioned in the peritoneal cavity, and then, the muscle and the skin were baked with absorbable silk yarn (2.0 mm, Monocryl, W3448, Ethicom, B. Braun, Tuttlingen, Germany). Finally, each animal was disinfected with braunol disinfectant (B. Braun, Melsungen AG, Tuttligen, Germany). All these procedures were based on the protocol of Lasota & Danowska-Klonowska (2004).

Throughout the surgical procedure, great care was taken to minimize tissue damage to ensure optimal conditions for functional recovery.

3.5.2. Induction of experimental osteoarthritis

The following protocol was based on Amorim *et al.* (2014b). In all animals (ARTH), with the aid of a standard hypodermic syringe (Microlance, 0.3 × 13 mm), the animals were injected with a solution of 3% kaolin and 3% carrageenan (Sigma-Aldrich, St. Louis, MO, USA) dissolved in distilled saline solution (0.9% NaCl), in the synovial cavity of the right knee joint of the hind paw, to a total volume of 0.1 ml. Finally, in each animal 10 flexion-extension movements of the knee joint were performed. This model produces mechanical hyperalgesia, which begins just a few hours after the injection of kaolin/carrageenan (K/C) and lasts for several weeks (Radhakrishnan *et al.*, 2003). In the SHAM group, the animals were injected with the vehicle solution, with the same saline concentration and a total volume of 0.1 ml, also in the synovial cavity of the right knee joint of the hind paw, also followed by 10 flexion-extensions of the right hind paw.

After the surgical procedures, anesthesia was reversed by the intraperitoneal administration of the anti-sedative atipamezole hydrochloride (1 mg/kg, i.p.; Antisedan, Orion Pharma, Orion Corporation, Espoo, Finland), using a standard hypodermic syringe (Terumo, Neolus, 0.6 × 25 mm, Belgium) (David-Pereira *et al.*, 2016). The animals were then monitored until they were fully recovered (awake and in good condition to carry out their hygiene and to be able to feed themselves).

OA progressed for one month, from week 3 to week 7.

3.6. Administration

For administration with Bd extract, Bd powder was diluted in 10 mM PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g of KH_2PO_4, 1% tween 20, pH 7.4) at a concentration of 50 mg/ml weighed on a precision scale (Mettler Toledo AG 245). Subsequently, this solution was placed in a sonicator (Branson 2510) for 30 minutes, until completely dissolved and stored in the dark. In the SHAM group only 10 mM PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g of KH_2PO_4, 1% tween 20, pH 7.4) was administered.

The administration was performed for 5 successive weeks, from week 7 to week 12. One week before starting the administration, the animals were trained by the researcher, inserting an orogastric cannula into the animal's stomach.

Administration was given by gavage every day, every 24 hours. Prior to each administration the drug was placed in the sonicator (Branson 2510) for 15 minutes to dissolve the extract completely. The treatment consisted of administration by gavage of 1 ml and 1.5 ml (according to the treatments of Bd50 and Bd100, respectively) of content according to the experimental group:

a) SHAM: 10 mM PBS

b) ARTH: 10 mM PBS

c) Bd50: 50 mg/kg Bd extract

d) Bd100: 100 mg/kg Bd extract

The concentration of the extract administered in each animal was adjusted weekly according to the weight of the animal, so that the final volumes to be administered (1 ml and 1.5 ml) contained 50 mg of Bd/kg and 100 mg of Bd/kg body weight of the animal in the Bd50 and Bd100 treatments, respectively. These doses administered in the treatments and the described procedure of the gavage were based on the protocol of Rezende *et al.* (2014) with some changes.

3.7. Behavioural tests

Pain is the main symptom of OA and is an important factor of study, because in the clinical situation patients are evaluated in the way they deal with pain during normal life (Gabriel *et al.*, 2007). Since the types of treatments available for this disease are not the most desirable (very invasive) for patients seeking medical care, animal studies are necessary to elucidate mechanisms underlying OA-induced pain and to evaluate drug efficacy. (Ferreira-Gomes *et al.*, 2012). Thus, in this study, several pain and locomotion tests were performed on animals, such as Catwalk, Open Field Test and PAM.

3.7.1. Catwalk

The catwalk is a behavioral test relative to the locomotor ability that allows a thorough and detailed analysis of the gait of the animal, being used to measure paw position and gait pattern (Ferreira-Gomes *et al.*, 2012; Gabriel *et al.*, 2007). In addition, the catwalk is a method that with parameters related to individual paws can be used to assess mechanical allodynia in a model of acute inflammatory pain. (Gabriel *et al.*, 2007).

The apparatus consists of a closed corridor (below and sides), with a width of 7 cm to prevent the animals from straying. The entire apparatus is made of wood and covered by plastic (for easy cleaning) and has the following dimensions: L × W × H: 100 × 7 × 10 cm^3.

The animals were trained to cross the catwalk the day before the test, and were motivated to cross the corridor to their cages located at the end of the catwalk. On the day of the test, the rat's plantar surfaces were painted with non-toxic red acrylic paint for the front paws and non-toxic blue acrylic paint for the hind paws. The animals were then allowed to walk freely along the corridor, with white paper strips at the base, and following the same procedure used for training. Finally, the paper strips with the prints of the rat paws were marked according to the corresponding animal. The strips of paper with the prints of the paws were affixed to the wall of the vivarium and allowed to dry for 7 days. All this procedure was done based on the protocol described by Redondo-Castro *et al.* (2013), with some adaptations.

The catwalk test was performed on all animals three times throughout the experiment. The first test was performed prior to induction of OA or saline at week 2, the second was performed after induction of OA at week 5, and the last was performed during treatment at week 9.

In this test the following parameters related to the individual paws were analyzed in detail:

- Print area (expressed in cm^2): total floor area contacted by the paw during the stance phase.
- Box length (expressed in cm): length of the print area (distance between the paw and the third digit). This parameter can also be considered as related to the development of hyperalgesia or allodynia in the limb (Gabriel *et al.*, 2007).
- Box width (expressed in cm): width of the print area. This parameter can be considered as related to the development of hyperalgesia or allodynia in the limb (Gabriel *et al.*, 2007).
- Stride length (expressed in cm): distance between the placement of the front and rear legs on the same side of the animal in each step cycle.
- Intermediate toe spreading (expressed in cm): distance between the second and fourth digits.
- Total toe spreading (expressed in cm): distance between the first and fifth digits.

These measurements were made following the protocols adapted from Gabriel *et al.* (2007) and Bozkurt *et al.* (2011). For each animal, three ipsilateral and contralateral hind paw prints were measured and the means for all parameters were calculated. Measurements of all these

parameters were performed using the public domain image processing program IMAGE J (National Institutes of Health, Bethesda, MD).

The same protocol was used in all catwalk evaluations. Identically, the same researcher performed all evaluations in order to minimize variability.

3.7.2. Pressure Application Measurement (PAM)

The pressure application measurement (PAM) is a behavioral and preclinical test, widely used in rats, which consists in the application of an increasing pressure, which allows to evaluate the hypersensitivity of the joints in animals (Barton *et al*. 2007).

In this research, the protocol was based on Amorim *et al*. (2014a) and Di Giminiani *et al.* (2016).

The PAM apparatus (PAM, Ugo Basile, Comerio, Italy) consists of a force transducer mounted on a unit fitted to the operator's thumb, which is connected to a recording base unit containing the control panel and digital read-out display. The results are presented in grams of force (gf) and correspond to the force peak immediately applied at the time of the animal's discomfort or suffering behavior. In this study, the device was programmed to apply a maximum pressure of 1500 gf, because this cut-off value was recommended by the manufacturer to ensure the best level of stability of the force transducer and to minimize the risk of damage to the tissues of the animals. In this device, the force transducer is calibrated by a function of the force applied by the researcher and the resistance offered by the knee joint of the animals.

This test was performed prior and after administration (week 6 and week 10). During testing, the animals were removed from the cages and held by the researcher. Next, the transducer with the help of the researcher's thumb was placed on the side of the animal's knee joint and the index finger on the other side. Then, the pressure on the joint was gradually increased until the animal showed any behavioural signs of discomfort, such as vocalization, paw withdrawal or squirming. Therefore, stimulation at the knee joint of the animal was interrupted when a behavioral response occurred or when the shear force of 1500 gf was reached. The peak gram force (gf) applied immediately prior to limb withdrawal was recorded by the base unit, and this value was designated the limb withdrawal threshold (LWT). Finally, the animals were returned to their respective cages after finishing the test session.

This test was performed on both hind limbs of each animal, twice on each of the knees. However, any movement by the animals to try to withdraw from the device, as well as movements of pulling or twisting out of the operator's fingers, were common to happen, so each movement of those was taken as an indication of the end point of the test and this value was rejected and a new attempt was made until a correct test value was obtained. All LWTs on both knees of the animals were recorded. For the analysis of the results, the means of the second test were subtracted from the means of the first test.

3.7.3. Open Field Test (OFT)

There are several behavioral tests used to measure locomotion and emotionality in animals, however, one of the most used is the open field test (OFT), which became popular as a test of exploratory behavior.

The OFT is used to analyze activity profiles, and indirectly based on it, anxiety in rodents (Schmitt & Hiemke, 1998). This test does not require training or motivation time, and behaviour in OFT is determined by the behaviour of the animal and can be highly variable among individual rats (Schmitt & Hiemke, 1998).

Wilson *et al.* (2000) conducted a study that showed the exploratory behavior exhibited by animals during exposure to the open field tends to decrease as this animal is re-exposed to the same test, so the test should only be performed once per animal. Distinctly, Lamprea *et al.* (2008) also carried out a study in which they showed rats prefer to walk along adjacent walls and avoid entering the central area, since they consider it safer areas.

In this experimental study, the OFT was performed to evaluate the locomotion and the exploratory activity of the rats. The apparatus consisted of a square wooden box (L × W × H: 100 × 100 × 40 cm3) (large enough to not inhibit the locomotion of the animal), with solid floor and surrounded by lateral walls of wood. All this was painted black and surrounded by opaque walls, from which they could not escape. Above the central region of the arena was a 40 cm video camera, capable of recording the image of the whole apparatus, which recorded the ambulation patterns both in the center and in the peripheral regions (near the walls). The experiment room was lit in dim light, and the device was brighter in the center than in the periphery. All animals were subjected to this test, with only one rat being placed at the center of the apparatus floor. Prior to each test, to prevent

animals from using olfactory clues from previously tested animals, the arena was cleaned with a 90% alcohol solution, and wiped with a dry cloth.

The entire experiment was based on the protocol of Amorim *et al.* (2014b), and some adaptations were made.

All animals were tested in 5 minute sessions, counted from the time they were placed in the center of the arena. Each animal was tested only once. This experiment was performed at week 10, that is 3 weeks after initiation of administration.

The behavior in the arena (active time, speed, time spent in the center and time spent in the periphery) was quoted through video analysis. In the analysis of results, a transparent sheet of acetate was used, placed on the monitor screen of the computer where the test videos were analyzed, with squares drawn on the sheet that divided the image of the floor of the arena in 16 equal squares, allowing the precise location of the animal's displacements and the exact location of the behaviors displayed in each of the squares.

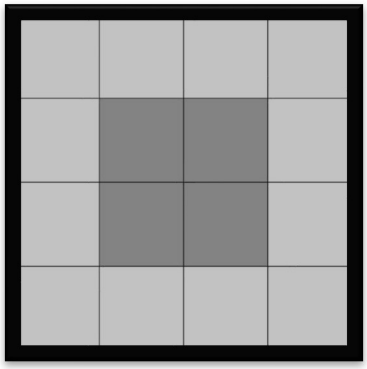


Figure 5: Schematic representation of the arena of the open field test. The darker squares represent the center of the arena (without walls), while the remainder represent the peripheral zone (with walls).

In order to analyze the behavioral events occurring in each square, the data were grouped according to regions: (1) central area with 4 squares without any wall and more illuminated, and (2) peripheral area with 12 squares with adjacent wall and less light.

To analyze the locomotion of animals in this test, the parameters recorded were:

- Number of times the rodent crossed a square line with front paws and ears;
- Duration of the time spent moving and immobile;
- Duration of time spent on the periphery and in the center.

After the analysis of the videos, the stationary and moving times, and the times spent in the center or the periphery, were calculated for each animal. With this data it was possible to obtain for each animal the active time, the ambulatory speed, the time spent in the center and the time spent in the periphery.

3.7.4. Perimetry of the knees

Edema of the knee joint in rats was evaluated by measuring the perimetry in both knees of each rat. The measurement was performed shortly after euthanasia, using a tape measure and measuring in the area of the kneecap.

These measurements were intended to deduce the swelling of the joints resulting from the intra-articular injection of kaolin/carrageenan (K/C) (ARTH) or saline (SHAM) in the joints of the ipsilateral knees, according to the study groups.

The edema results are expressed in millimeters and are presented as the difference between the contralateral and ipsilateral knee perimetry of each animal, since the contralateral knee can be considered the control of the same animal since it was not injected.

These procedures were based on the protocol of Adães *et al.* (2015).

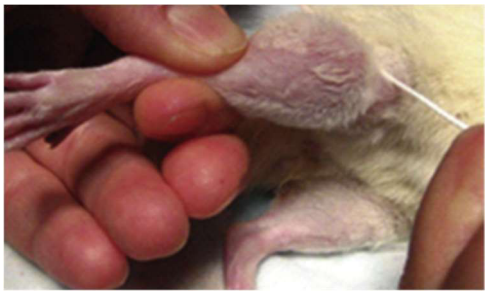


Figure 6: Measurement of the knee joints of rats (Sükür *et al.*, 2016).

3.8. Acute dermal irritation/corrosion test

Skin diseases can be treated either topically or systemically with various compounds available on the market, however, these products are usually aggressive and not effective in all cases (Silva *et al.*, 2015). Therefore, it is relevant to study extracts and isolated compounds from herbal medicine to discover new effective and safe topical anti-inflammatory drugs (Silva *et al.*, 2015). The aim of

this study was to investigate the possible *in vivo* acute topical anti-inflammatory power of *Baccharis dracunculifolia* on rat's ears using croton oil as an irritant.

The acute dermal corrosion/irritation study was carried out 6h prior to euthanasia. This test was carried out according to the methodology described by Silva *et al.* (2015) with some modifications.

Thus, to perform this dermal test, 34 rat ears were subjected to topical applications of different compounds on the inner surface of the ear:

Initial application	**Application 15 minutes later**	**Nº of ears**
Control	—	7
Bd extract	—	6
Croton oil	Dexamethasone	7
Croton oil	Bd extract	7
Croton oil	—	7

Edema was induced in 21 rat ears by topical application of 20 µl of croton oil 5% (Sigma, Lisbon, Portugal) dissolved in acetone, and on the other hand, in 6 ears 20 µl of Bd extract dissolved in acetone (100 mg/ml) was topically applied, and in 7 ears nothing was applied (control). After 15 minutes, 20 µl of Bd extract dissolved in acetone (100 mg/ml) was topically applied to 7 of the ears that initially received croton, 20 µl of dexamethasone (Decadron 0.5 mg, Climed, Portugal) dissolved in acetone (1 mg/ml, used as reference drug) was applied to 7 of these ears, and 7 of these ears were not applied at all.

The edema was expressed as ear thickness variation, which was measured using a manual micrometer (Analog external micrometer, 0 to 25 mm, resolution 0.001 mm, Mitutoyo, America). After euthanasia, 6 mm diameter biopsies of the ear were collected using a metal punch and subjected to histopathological analysis, and other ear samples were snap frozen in liquid nitrogen and stored at −80°C to eventually be processed further for biochemical testing.

3.9. Euthanasia

After completing all the experimental sessions, the animals were euthanized at the end of week 11.

Outside the euthanasia room, the animals received a lethal dose of sodium pentobarbital (200 mg/kg animal, i.p., eutasil, OrionPharma, Espoo, Finland) intraperitoneally, (Altwegg-Boussac *et al.*, 2016). Then, inside the euthanasia room, as soon as the animal was already dead, showing no blinking reflex or any other kind of reflex, a necropsy was performed and the internal organs and hindpaws of all animals were excised. Thus, left and right legs (for knee analysis), left and right ear, thymus, lung, liver, left and right supra renal, left and right kidney, heart and spleen were removed. All of these internal organs were immediately weighed on a precision balance (Sartorius), and the values of each were recorded as they were removed from each animal.

Some samples were stored in 4% paraformaldehyde (PFA) (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain) for later use, namely the legs, all internal organs and some ears. While, some ears and a liver sample from each animal were frozen immediately in liquid nitrogen and kept frozen at -80°C for later use.

3.10. Experimental design

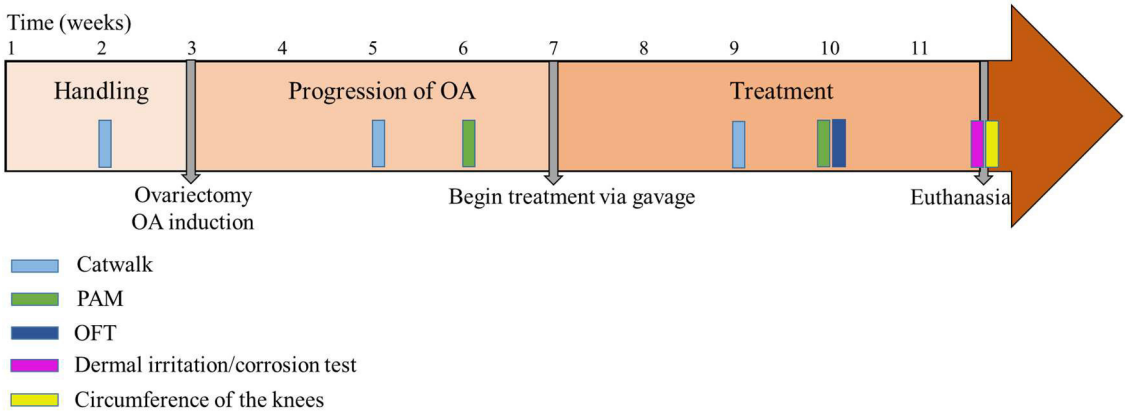
A total of 24 adult female rats (*Rattus norvegicus*, var. *Albinus*, *Wistar*), were divided into 4 groups, 6 animals per group for the development of the osteoarthritis study:

Groups	Surgery	Administration
SHAM	Saline injection	PBS
ARTH	Injection of osteoarthritis	PBS
Bd50	Injection of osteoarthritis	50 mg/Kg Bd extract
Bd100	Injection of osteoarthritis	100 mg/Kg Bd extract

Before starting the experiment, all the animals went through a period of habituation, being placed daily in the experimental room and handled by the researcher for 2 weeks, in order to become accustomed to the presence of the researcher.

It should be noted that the general health and hygiene rules in this experience were taken into account, with the animals being evaluated weekly by the resident veterinarian and weighed weekly by the researcher. Every effort has been made to minimize the number of animals as much as possible, and to avoid their suffering. The experiment lasted for 11 weeks, and 2 weeks were spent to habituation of the animals to the researcher.

The following diagram briefly shows the various procedures performed throughout the experiment:



All rats underwent ovariectomy, followed by the induction of OA. In the SHAM group, only the vehicle was injected into the joint. While the ARTH, ARTH-Bd50 and ARTH-Bd100 groups received kaolin-carrageenen in the knee joint to cause osteoarthritis.

After 4 weeks of disease progression, administration was started. Administration of the drug was by gavage, and was performed every day at the same time in all animals for 5 successive weeks. The SHAM and ARTH group received PBS, while the ARTH-Bd50 and ARTH-Bd100 groups were given 50 mg/kg and 100 mg/kg Bd extract dissolved in PBS, respectively.

Throughout this experiment, several locomotor and pain tests were also carried out on the animals, namely Catwalk, PAM and Open Field Test.

Several hours prior to the sacrifice, various compounds (Bd extract, croton oil and dexamethasone) were topically applied to the ears of the animals in order to test whether the extract of *Baccharis dracunculifolia* displayed an anti-inflammatory effect. After all these procedures all animals were sacrificed, and the perimeter of the knees of all animals on both knees was measured to see the swelling in the joint. Then, left and right legs, left and right ear, thymus, lung, liver, left and right

supra renal, left and right kidney, heart and spleen were removed to calculate the relative weight of the organs, and for further analysis.

3.11. Histological processing

Each sample was placed separately in cassettes (Biocassette, Bio Optica, Italy) and duly identified for the corresponding animal, and placed again in 4% PFA (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain) at room temperature.

Samples were then dehydrated in ascending grade ethanol/water solutions (from 70 to 100%) using an automatic sample processor (Leica AutoStainer XL, USA) for 19 h followed by washing three times with xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group). Afterwards, the specimens were immersed in paraffin (Thermo Scientific, UK) at 62°C and allowed to solidify at -5°C. Slides (25 × 75 × 1 mm, Superfrost Plus, Thermo Scientific) and cut into 4 µm thick sections, and mounted in a micro-slide glass (Matsunami glass Ind. Ltd., Japan), and then, paraffin (Thermo Scientific, UK) was melted by placing the slides (25 × 75 × 1 mm, Superfrost Plus, Thermo Scientific) in the oven at 71°C for 20 minutes and allowed to cool down at a room temperature. The remnant paraffin (Thermo Scientific, UK) was then washed off with hexane for 5 min (S.T. Chemical, Japan), followed by dipping into an ethylene/propylene mixture (Clear Plus, Falma Co., Tokyo, Japan) for 3 min. Then, slides (25 × 75 × 1 mm, Superfrost Plus, Thermo Scientific) were immersed three times in 100% ethanol for 2 minutes. Each slide (25 × 75 × 1 mm, Superfrost Plus, Thermo Scientific) was duly identified according to the sample and the corresponding animal. All these procedures were performed based on the protocol described by Oliveira *et al.* (2010).

After the respective staining of the slides the microscopic analysis was begun. The slides of the internal organs and ears (epidermal and dermal thickness measurements) were analyzed using an optical microscope (Olympus, BX61) interconnected with a digital color camera (Olympus, DP70) through the Olympus program (CellSens Dimension), with a 10× microscope objective. Slides of the knee joints and ear (number of infiltrations) were analyzed using a Carl Zeiss optical microscope (Axioplan 2 Imaging, Zeiss, Olympus) interconnected with the computer through a camera (Sony, Color Video Camera, 3 CCD, Exwave HAD) through the Stereo Investigator program (for knee joints and ear) with a 2.5× microscope lens (for joint cartilage and bone) and 20× (for trabeculae). Finally, all slides were photographed using the Olympus (CellSens Dimension) program on an optical

microscope (Olympus, BX61) with a digital color camera (Olympus, DP70). For each animal all replicates of slides were analyzed, and all measurements were averaged.

3.11.1. Histological processing and analysis of internal organs

Histological analysis of the internal organs is important to analyze the state of health of animals during the experimentation process. The internal organs analyzed were the thymus, lung, heart, spleen, liver, left and right supra renal, left and right kidney.

All internal organs were initially cut into sections in transverse plane (thymus, heart, spleen, liver, left and right supra renal) and coronal plane sections (lung and left and right kidney), and then subjected to the protocol described above for mounting the slides. The internal organs were cut into sections of 4 μm thickness, and for each internal organ of each animal were made slides (25 × 75 × 1 mm, Superfrost Plus, Thermo Scientific).

All replicates of all internal organs were stained with hematoxylin and eosin as described by Oliveira *et al.* (2010), with some adaptations. In the Haematoxylin and Eosin (H&E) staining procedure, slides were placed in xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group) for 5 minutes, then transferred to absolute ethanol for 3 minutes, ethanol 96 % for 1 minute, and washed with tap water for 1 minute. Subsequently, the slides were immersed in the Harris hematoxicillin (Millipore Corporation, USA) for 1 minute. The washing was run under running water for 3:30 minutes, then the slides were placed in 0.5% ammoniacal water (Sigma Aldrich, Spain) for 10 seconds, and then washed with running water for 1:30 minutes. Then, the slides were placed in 96% ethanol for 45 seconds, and then in eosin Y (Thermo Scientific, UK) for 30 seconds. Finally, the slides were dehydrated with 96% ethanol for 1 minute and absolute ethanol for 4 minutes, and then they were placed in xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group) for 3 minutes. Subsequently, the slides were assembled.

3.11.2. Histological processing and analysis of knee joints

Histological study of the knee joint was performed to examine changes after OA induction, namely in the cartilage and bone structure.

The knee joints after being stored in 4% PFA (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain) were dissected and submerged in a descaling solution (BiodecR, Bio-Optica, Milan, Italy),

during 7 days at room temperature for tissue softening (Amorim *et al.*, 2014b). Then, samples were cut in the sagittal plane with 4 µm of thickness, placed in duly identified cassettes (Biocassette, Bio Optica, Italy) and kept in 4% PFA (DAC, Applichem Panreac, ITW Companies, Barcelona, Spain).

To perform the knee joints staining, the slides were stained using the Fast Green and Safranin-O, Hematoxylin and Eosin (H&E), and Masson's Trichome method. However, the analyzes of the knee joints were done only on Fast Green and Safranin-O staining, but in each analysis of each animal comparisons were made with the remaining stains. Int the Fast Green and Safranin-O stainings three replicates of samples were made, and all were analysed. The protocol for staining the knee joint slides by the method of Fast Green and Safranin-O used was described by Schmitz *et al.* (2010), with some changes; the protocol for the staining of Hematoxylin and Eosin used was described by the protocol of Oliveira *et al.* (2010) as already described above; and Masson's Trichome staining protocol was described by Scipio *et al.* (2008), with some adaptations.

As for the protocol of Fast Green and Safranin-O used, the slides were deparaffinised in an automated machine (Leica AutoStainer XL, USA), and then stained with hematoxylin solution (Millipore Corporation, USA) for 5 minutes (drops) and washed in running tap water for 2 minutes. Afterwards, placed quickly in ammoniacal water 1% (Sigma Aldrich, Spain) and washed in running tap water for 2 minutes. Stained in Safranin-O solution (Sigma, India) for 5 minutes (drops) and washed in running tap water for 1 minute. Next, stained with Fast Green solution (FCF, Sigma Aldrich, UK) for 1 minute and then acetic acid solution (Carlo Erba, Val de Reuil Cedex, Dasit Group) was added for no more than 30 seconds (drops directly on Fast Green solution in the slide). Lastly, dehydrated with 96% ethanol, absolute ethanol, and xylene (C8H10, Carlo Erba, Val de Reuil Cedex, Dasit Group) with a duration of 2 minutes in each solution, and mounted using resinous medium (Entellan, Merck, Germany), and lamellae (24 × 60 mm, Superfrost Plus, Thermo Scientific). After all these procedures, the slides were allowed to dry for 7 days.

With the Fast Green and Safranin-O staining method, cartilage, mucin, and mast cell granules were stained in orange to red tones, the nuclei in dark blue, the cytoplasm in gray-green, and the background turned green (Schmitz *et al.*, 2010).

The area of articular cartilage, bone area and trabeculae thickness were measured in sections stained with Fast Green and Safranin-O. In the analysis of the cartilage the area of worn cartilage was measured. As for the area of the bone tissue, the total area of the bone and the area of all

gaps in the same bone were measured, so the area of the gaps was subtracted from the bone area, thus obtaining the existing area of bone tissue. Finally, to obtain the thickness of the trabeculae the thickness of 10 trabeculae in each bone was measured and the mean values were calculated to obtain a mean trabecular thickness value for each animal.

3.11.3. Histological processing and analysis of the ears

The ears were studied histologically to analyze the acute inflammatory process and the possible anti-inflammatory effect of the *B. dracunculifolia* extract.

The ears were initially cut in a transverse plane and with 4 µm of thickness. The samples were placed in identified cassettes (Biocassette, Bio Optica, Italia). Subsequently, slides were stained using the Fast Green and Safranin-O method.

Then, the slides were allowed to dry in the hote for 7 days until dry, after which the microscopic analysis was started. The number of infiltrations in each ear, the thickness of the epidermis, and the thickness of the dermis were measured. For each animal were measured the 3 existing replicas of the slides of ears, and finally all measurements were averaged to obtain only one value per animal.

3.12. Statistics / Data Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc, LaJolla, CA, USA). To evaluate the antioxidant capacity of the extract, a T-test was used. For the analysis of the data from the locomotion, pain and inflammation tests a one-way analysis of variance (ANOVAone-way) followed by Bonferroni pos-hoc test for comparisons between groups was used. P-values less than 0.05 ($P < 0.05$) were considered statistically significant, and $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P<0.001$. All results are presented as mean ± standard error (SEM).

Chapter 4: Results and Discussion

4.1. Technical considerations

4.1.1. Plant material

The plants belonging to the genus *Baccharis* are reported in the literature as having anti-inflammatory activity, and *B. dracunculifolia* is widely used in folk medicine for the treatment of inflammation, skin wounds, liver disorders, stomach ulcers, among others (Lemos *et al.*, 2007; Parreira *et al.*, 2010).

The present study evaluated the effects of the crude hydroalcoholic extract of *B. dracunculifolia* using various *in vivo* tests of pain, locomotion and inflammation in rodents as well as *in vitro* tests. The results of this present study revealed that the oral administration with the Bd extract (50 mg/kg and 100 mg/kg) was able to significantly decrease the changes related to pain, locomotion and inflammation.

In the literature there are several studies with *B. dracunculifolia* that corroborate with the positive results of the present study. Dos Santos *et al.* (2010) reported the analgesic and anti-inflammatory activities of the crude hydroalcoholic extract of the leaves of *B. dracunculifolia* in different models of pain and inflammation such as edema and inflammatory pain induced by carrageenan. Ribeiro *et al.* (2000) also confirmed the anti-inflammatory power of Bd extract and suggests that it probably exerts its anti-inflammatory effect by interfering with inflammatory components. Also Paulino *et al.* (2008) demonstrated the anti-inflammatory activity of artepilin C, one of the main compounds of *B. dracunculifolia*, in the model of paw edema induced by carrageenan. Dos Santos *et al.* (2010), in addition to confirming the traditional anti-inflammatory indications of *B. dracunculifolia* leaves, also demonstrated for the first time that this plant has relevant oral antinociceptive properties for acute and persistent pain-like behavioural animal model.

According to Massignani *et al.* (2009) the dose of 2000 mg/kg of essential oil of the aerial parts of *B. dracunculifolia* administered orally is toxic, and toxicity and mortality records were observed in the animals.

In the present study, doses of 50 mg/kg and 100 mg/kg of *B. dracunculifolia* were used orally, since in several studies these doses have already been used and shown good results (Rezende *et al.*, 2014; Massignani *et al.* 2009), with no toxicity or death occurring in the animals.

4.1.2. Animal model

Animal models are extraordinarily powerful research tools in the development of knowledge and aiding in the discovery of potential new drugs, but scientists must have an ethical attitude regarding animal experimentation, especially concerning the ethical principle of reverence for life, which requires that a greater gain of knowledge be obtained with a smaller number of animals used with minimal suffering (Rivera, 2002).

Russell and Burch, in 1959, launched the Humanitarian Principle of Animal Experimentation, synthesized in three words beginning with the letter R, being called "The 3 Rs Principle", and being these words *replacement, reduction* and *refinement* (Rivera, 2002). *Replacement* indicates that we should replace animals whenever possible with non-sensitive materials, such as tissue culture or computer models (however, there are certain areas such as behavior, pain, and drug action where it is not possible to use alternatives) (Rivera, 2002). *Reduction* refers to the use in as few animals as possible in certain experiments, only the amount needed to provide significant statistical results (Rivera, 2002). *Refinement* refers to the fact that people should only use animals when well trained to do so, and refine less invasive materials and protocols to minimize suffering (Rivera, 2002).

Currently, most scientists involved in animal experimentation have respect for life, are concerned about conducting their research without causing pain and suffering, and follow the ethical principles of animal experimentation, however, the ethical use of animals in experiments depends greatly on the integrity and conscience of each scientist (Rivera, 2002). Therefore, scientists should be aware of their specific duties, being responsible for the general welfare of animals, calculating means and ends, and always using the "3 Rs" (Rivera, 2002).

There are many important factors to consider in choosing an appropriate animal, such as size, cost, age, gender/reproductive statuses, and the osteoarthritis induction method (Gregory *et al.*, 2012). In general, small animals (mice, rats, rabbits and guinea pigs) are the most advantageous in terms of cost, ease of use, housing, genetic manipulation and public perception, while large animals (dogs, goats, sheep, pigs and horses) are the most advantageous in terms of anatomical and biomechanical similarities to humans (Gregory *et al.*, 2012). Therefore, each animal model has unique advantages and disadvantages, so it is extremely important to fully understand the rationale of the research and the hypothesis being tested in order to choose the most appropriate model (Gregory *et al.*, 2012).

The rat's model is widely used to investigate human-related diseases, including to broadly characterize pain related to osteoarthritis and to investigate its possible treatments (Gregory *et al.*, 2012). The main advantages of using rats in the study of osteoarthritis are its low cost, ease of use, thicker cartilage than can view hole knee can on slides. However, the disadvantages are based on the fact that the joints are small (Gregory *et al.*, 2012).

4.1.3. K/C model

The knee joint arthritis model caused by kaolin/carrageenan has been predominantly used to study the underlying mechanisms of structural joint damage and also to study pain associated with OA (Amorim *et al.*, 2014a; Amorim *et al.*, 2014b). This model of K/C arthritis also mimics the acute inflammatory phase of osteoarthritis, with inflammatory phases being quite frequent in human osteoarthritis (Neugebauer *et al.*, 2007). In addition, intra-articular injection of K/C in rat knee induces nociceptive behaviours associated with movement and loading on the OA joint, mimicking the main complaints of patients, along with histopathological changes that match the description of human AO (Creamer *et al.*, 1998).

The K/C model is extensively used to study several parameters, such as pathological, behavioural and electrophysiological changes in mice, rats, cats and non-human primates (Neugebauer *et al.*, 2007). Intra-articular injections of K/C in the knee produce a monoarthritis which predominantly causes cartilage damage, synovial inflammation, and synovial fluid exudate, and arthritis caused by this model develops rapidly in hours and persists for weeks (Neugebauer *et al.*, 2007). This K/C model was initially defined as a model of acute monoarthritis, however, certain studies have been performed that show that this model can be used as a model of osteoarthritis, because after 4 weeks of induction, the histological analysis showed a clear degeneration of the knee joints (Amorim *et al.*, 2014b), as well as in the present study.

Intra-articular injection of carrageenan alone has been used as a modification of the K/C arthritis model, in which the time course of the carrageenan arthritis is shorter (hours to days) and cartilage damage less pronounced than in the K/C model (Neugebauer *et al.*, 2007). However, among the several experimental models, in this present study the experimental model of osteoarthritis induced by kaolin and carrageenan in the knees of rats was chosen, where the time course of arthritis is longer (weeks), characterized by the induction of acute joint inflammation where several inflammatory and joint degradation aspects are assessed (Gabriel *et al.*, 2007).

The development of osteoarthritis, namely histological changes and the development of hyperalgesia using the K/C model, is directly related to the concentration of kaolin and carrageenan and also to the volume administered (Radhakrishnan *et al.*, 2003). According to Radhakrishnan *et al.* (2003), concentrations lower than 3% of carrageenan in the knee have no effect or cause short-term hyperalgesia. Thus, since in the present study the experiment had a relatively high duration, a concentration of 3% of kaolin and 3% of carrageenan was administered.

Thus, even if further studies are required, the K/C model presents an interesting potential for the study of osteoarthritis.

4.1.4. Administration

Often toxicology and pharmacokinetic studies are performed in rodents in which the test compounds are administered orally by gavage (Brown *et al.*, 2000).

Gavage is a practice that can cause stress in animals, however to know if the animal has felt stress with gavage several analyses should be done, such as analysing physiologic changes (may include changes in gastric secretion, motility, and increases in heart rate and of blood pressure), biochemical changes (include a marked increase in glucocorticoids), and also to note that exposure to a "stressor" can cause adrenal secretion of corticosterone through activation of the hypothalamic-pituitary-adrenal axis (Brown *et al.*, 2000). Although in the present study did not carry out these analyses, the weighing of the animals throughout the experiment are also an important factor capable of showing whether the animal felt stress or not.

In addition to stress, there are other important experimental variables to consider which may interfere with study results, as well as the dose volume that may affect the absorption characteristics of orally administered compounds (Nickerson *et al.*, 1994), the dose concentration of the test compound (Ferguson, 1962), as well as the vehicle type (Rezende *et al.*, 2014). The dose volume and the concentration of the test compound may be additional factors in vehicle selection, since the dose volume chosen for administration depends on the solubility of the compound in the vehicle and the dose concentration to be administered (Brown *et al.*, 2000). Brown *et al.* (2000) states that the dosage of gavage causes stress in a vehicle-dependent manner. In the study by Brown *et al.* (2000) concluded that the administration of oils as a vehicle by gavage stimulated the hypothalamic-pituitary-adrenal axis, resulting in secretion of corticosterone, resulting

in a volume-dependent stress response. However, this response was not due to aspiration, but may be related to the administration of large amounts of lipids (Brown *et al.*, 2000).

Certain studies state that in rats that were fasted the administration of gavage administration may occur in large volumes (> 20 mL/kg) (Ferguson, 1962), whereas in studies the dosage occurs daily for a defined period of time, the animals are fed *ad libitum*, resulting in foods that reside in a considerable portion of their upper intestinal tract, and consequently, the administration of large volumes can lead to aspiration of the test compound, leading to pulmonary injury and/or stress (Brown *et al.* , 2000). Since in the present study treatment lasted for several weeks and the animals were fed *ad libitum*, the volume of gavage administered was low (1 ml and 1.5 ml), thus avoiding aspiration in the animals.

Otherwise, aspiration of a test compound, in addition to causing stress, can cause pulmonary and airway injuries, and subsequently can have serious consequences (Brown *et al.*, 2000). In the study by Brown *et al.* (2000), vehicle reflux occurred immediately after administration of the gavage in dose volumes of ≥ 30 mL/kg, and was more prevalent with water, and aspiration of the vehicle to the lungs occurred, and this was probably due to the low viscosity of the water. In the present study administration by gavage in addition to having a dose lower than 30 mL/kg, water was not used as vehicle, and there were no aspirations of the vehicle to the lungs, corroborating with the good state of the internal organs of the animals. With this, Brown *et al.* (2000) suggests that the dose volumes for gavage administration in rats should generally not exceed 10 mL/kg, a factor that was observed in the present study.

Regarding the choice of vehicle type in the gavage, in the present study the vehicle used for the administration of the drug was PBS, assuming that it does not cause stress, aspiration and edema in the animal due to its inherent properties, when compared with other studies. Brown *et al.* (2000) demonstrated problems using water as a vehicle because of its low viscosity, and also demonstrated stress problems using oils as vehicle by gavage. Rezende *et al.* (2014), underwent oral administration with ethanol and showed that it causes hyperemia, edema, necrosis and submucosal hemorrhage, as well as circulatory disorders.

Therefore, certain factors in the gavage may affect the final results of the experiment, namely the vehicle, the dose volume and the dose concentration (Brown *et al.*, 2000). In addition, the duration of drug application and the frequency of treatment also affect treatment outcomes (Cheuk *et al.*, 2016).

4.2. HPLC

High performance liquid chromatography (HPLC) is a technique used in analytical chemistry to separate, identify and quantify the constituents of a mixture, in this case a vegetable extract (Chen & Pawliszyn, 1995; Hayes *et al.*, 2014). Among different types of chromatography, HPLC has been most widely used as an essential analysis tool for research, manufacturing, clinical tests and diagnostics, due to its universal applicability and remarkable assay precision (Hayes *et al.*, 2014). HPLC is composed of several advantages, such as a fast, cheap and efficient sampling technique (Chen & Pawliszyn, 1995). In addition, many classes of semi or non-volatile organic compounds are nowadays used, such as pharmaceutical products, drugs, proteins, among others, which are best separated by HPLC method (Chen & Pawliszyn, 1995). In this way, the HPLC method is of great importance and widely used for various purposes, for example in pharmaceuticals, food, life sciences, environment and also the daily analysis in research laboratories (Hayes *et al.*, 2014).

The hydroalcoholic powder extract of aerial parts of *Baccharis dracunculifolia* was subjected to HPLC-DAD analysis (Figure 7).

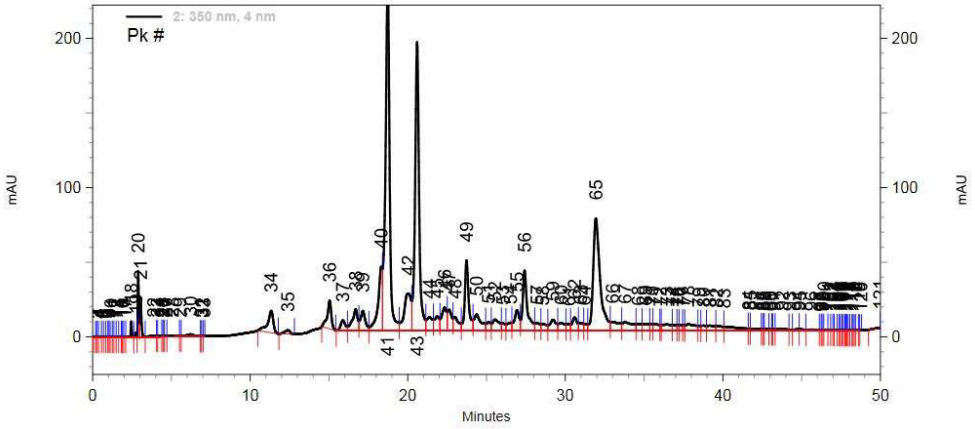


Figure 7: Chromatogram obtained by high performance liquid chromatography (350 nm) of the extract of the aerial parts of *B. dracunculifolia.*

Identification of phenolic compounds was performed by direct comparison of HPLC with authentic standards and was based on retention time, and UV-Vis absorption spectra. Thus, through the chromatogram it was possible to observe that caffeoylquinic acid derivatives and an apigenin derivative are present in *B. dracunculifolia* extract.

The quantification of the compounds was performed through the peak area, compared to several pure compounds, used as external standards. To determine the caffeoylquinic acid, chlorogenic acid was used as the standard, and to determine apigenin, luteolin was used as the standard (Table1).

Table 1: Phenolic composition of *B. dracunculifolia* extract (µg/mg).

Peak	**Rt (min)**	**Compound**	**Area**	**µg/mg**
34	11,333	Caffeoylquinic acid	1458345	8,248287
36	15,047	Apigenin	1360477	1,504333
37	15,88	Caffeoylquinic acid	558928	3,161254
38	16,693	Caffeoylquinic acid	1335437	7,553129
41	18,727	Caffeoylquinic acid	17256071	97,59901
42	20,053	Caffeoylquinic acid	3031484	17,14584
43	20,593	Caffeoylquinic acid	13713009	77,55972
46	22,34	Caffeoylquinic acid	1345468	7,609863
49	23,733	Caffeoylquinic acid	3251138	18,38819
56	27,42	Not identified	3262413	-
65	31,94	Not identified	9265090	-
Total compounds:				238,7696

B. dracunculifolia has chemical composition and internationally recognized biological activities (Lemos *et al.*, 2007, Missima *et al.*, 2007; Massignani *et al.*, 2009). Several studies have reported that *B. dracunculifolia* has high levels of phenolic compounds and these are related to its antioxidant properties (Funari *et al.*, 2007; Guimarães *et al.*, 2012; Lemos *et al.*, 2007; Missima *et al.*, 2007).

Analysis by HPLC-DAD of the crude hydroalcoholic extract of the aerial parts of *B. dracunculifolia* allowed to observe the presence of several compounds in the present study. Thus, the individual phenolic compounds were determined, and caffeoylquinic acids and apigenin were found to predominate in the composition of the Bd extract (the caffeoylquinic acids are the major compounds). These findings are in agreement with the phytochemical studies found in the

literature, which report the complexity of this extract and the presence of these types of compounds in extracts of *B. dracunculifolia* (Rezende *et al.*, 2014; Kumazawa *et al.*, 2003; Park *et al.*, 2004).

There are several factors that can affect the chemical composition of plants, since in these biosynthetic pathways responsible for the production of secondary metabolites are sensitive to several factors, such as environmental, climatic variations and, especially, interaction with insects and predators (Sforcin *et al.*, 2012).

Besides these, seasonality is an important factor to be taken into account during cultivation and biomass production, since the availability of rainfall and nutrients, relative humidity of the air, temperature, herbivorous or pathogen attack are factors which may influence the biosynthesis of secondary metabolites of the cultivated plant (Sforcin *et al.*, 2012). Confirming this fact, the study by Sforcin *et al.* (2012) showed that the constituents of *B. dracunculifolia* vary according to the seasonality that are collected.

Otherwise, Park *et al.* (2002), through HPLC analyses demonstrated that the chemical compositions of hydroalcoholic extracts of shoots, young leaves and mature leaves of *B. dracunculifolia* are significantly different, both qualitatively and quantitatively, thus demonstrating that the chemical composition of the extract of Bd can vary considerably according to the part of the plant collected.

Thus, the HPLC used to identify the secondary metabolites present in the Bd extract were satisfactory, and besides demonstrating the presence of phenolic compounds, it also allowed the identification and quantification of the main components. In addition, it should be noted that the chemical composition of this Bd extract used in this study differs from other Bd extracts used in other studies, probably due to differences in seasonality, part of the plant chosen, climate, altitude and latitude, pathogens , among others.

4.3. Antioxidant activity of *Baccharis dracunculifolia*

Increasingly it has been concluded that synthetic antioxidants need to be replaced with natural antioxidants because of their potential health risks and toxicity (Dudonné *et al.*, 2009). Thus, the search for antioxidants from plant species has received much attention and efforts have been made to identify new natural resources for active antioxidant compounds and anti-inflammatory properties, however *in vitro* assays are needed to have a more accurate assessment of the

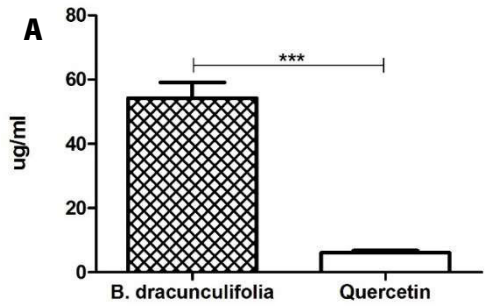
potentiality of the agents antioxidants and anti-inflammatories of plant extracts (Dudonné *et al.*, 2009; Silva *et al.*, 2005). In order to fully elucidate a complete profile of the antioxidant capacity of a particular extract, it is necessary to perform different antioxidant capacity tests because no single assay will accurately reflect all the antioxidants of this extract (Ebrahimzadeh *et al.*, 2008).

4.3.1. DPPH method

Antioxidants intended to prevent the presumed harmful effects of free radicals in the human body are of great interest, and for this there is a method which is based on the use of the stable free radical diphenylpicrilhydrazyl (DPPH, 1,1-diphenyl-2-picrylhydrazyl) (Molyneux, 2004). DPPH can be reduced by an antioxidant through the donation of hydrogen to form the stable DPPH-H molecule (Molyneux, 2004; Silva *et al.*, 2005). Therefore, in this DPPH assay the Bd extract was measured in terms of hydrogen donation or radical scavenging ability using the stable DPPH radical (Sigma, Lisbon, Portugal), which is purple at room temperature (Silva *et al.,* 2005).

In the analysis of the DPPH method, the results show there are significant differences between *B. dracunculifolia* and Quercetin ($t_{(16)}$=9.870, P<0.0001). In the DPPH method*, post hoc* tests indicate that *B. dracunculifolia* extract has EC_{50} of 54.26 (µg/ml), and quercetin (used as a standard reference) showed an higer activity (Figure 8).

A



B

	EC_{50} (µg/ml)
B. dracunculifolia	54.26 ± 14.48
Quercetin	6.12 ± 1.98

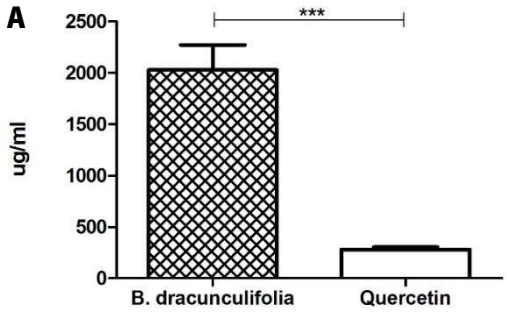
Figure 8: Comparison of the antioxidant activity of *B. dracunculifolia* with Quercetin, by the DPPH method (A), and their respective EC_{50} values (B). Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

4.3.2. Iron Chelating Activity (ICA)

Iron is essential for life, because it is necessary for oxygen transport, respiration, and the activity of many enzymes (Nehir & Karakaya, 2004). However, the accumulation of toxic amounts of iron can adversely affect biological systems, since in the active redox form it catalyzes the generation of highly reactive oxygen species, leading to several complications such as heart failure, diabetes, hypothyroidism, hepatic insufficiency and, ultimately, early death (Ebrahimzadeh *et al.*, 2008; Nehir & Karakaya 2004; Riemer *et al.*, 2004). In addition, iron is capable of generating free radicals from peroxides by Fenton reactions, and minimizing iron concentration in the Fenton reaction provides protection against oxidative damage, improving quality of life and overall survival (Ebrahimzadeh *et al.*, 2008; Nehir & Karakaya, 2004). For this, iron chelators are required that mobilize tissue iron to form soluble and stable complexes are then excreted in feces and/or urine, thus reducing iron-related complications (Ebrahimzadeh *et al.*, 2008). Antioxidant agents derived from plants are increasingly sought after and for iron chelation is no exception, and scientists are increasingly making great efforts to find any potentially useful sources to get the most benefit possible with the least possible harm (Ebrahimzadeh *et al.*, 2008).

In the analysis of the Iron Chelating Activity (Figure 9), the results show there are significant differences between *B. dracunculifolia* and Quercetin ($t_{(12)}$=6.142, *P*<0.0001). *Post hoc* tests in the ICA indicate that *B. dracunculifolia* extract has EC_{50} of 2026 (µg/ml), and quercetin (used as a standard reference) showed an higer activity.

A



B

	EC_{50} (µg/ml)
B. dracunculifolia	2026 ± 687.44
Quercetin	281.17 ± 56.00

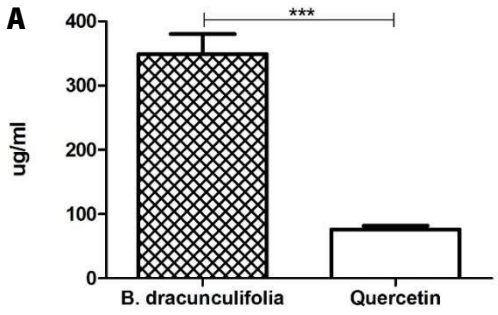
Figure 9: Comparison of the antioxidant activity of *B. dracunculifolia* with Quercetin, by the Iron Chelating Activity (A), and their respective EC_{50} values (B). Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

4.3.3. Inhibition of Nitric Oxide production

Inflammation is a protective reaction of tissues against injury or infection, and is mediated by several signalling molecules produced by macrophages and others (Saha *et al.*, 2004). In response to inflammation, macrophages can produce pro-inflammatory molecules, such as nitric oxide (NO), which regulates various physiological and pathophysiological responses in the human body, such as lesions, infections, and others (Korhonen *et al.*, 2005; Saha *et al.*, 2004). NO and reactive oxygen species (ROS) when produced at high levels, have been associated with dysregulation of signaling events and immune response and, ultimately, cause cell and tissue injury, leading to the initiation and/or propagation of the inflammatory processes (Jahanshahi *et al.*, 2004). Thus, inflammatory stimuli cause an overproduction of NO that contain pro-inflammatory and destructive effects, however, NO has also protective effects in some inflammatory responses (Korhonen *et al.*, 2005). NO is synthesized from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzymes (Korhonen *et al.*, 2005). The NOS is responsible for the production of large amounts of NO and has been reported as a mediator of inflammation and appears to be involved in both acute and chronic inflammation (Saha *et al.*, 2004).

In the analysis of the Inhibition of Nitric Oxide production, the results show there are significant differences between *B. dracunculifolia* and Quercetin ($t_{(18)}$=5.597, P<0.0001). *Post hoc* tests indicate that *B. dracunculifolia* extract has EC_{50} of 348 (µg/ml), and quercetin (used as a standard reference) showed an higer activity (Figure 10).

A



B

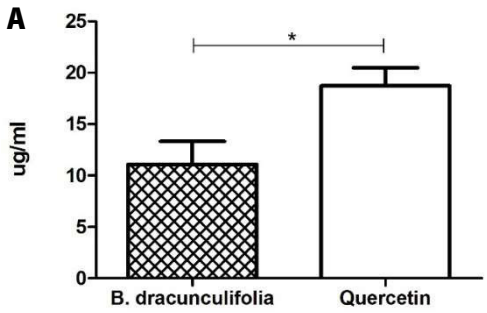
	EC_{50} (µg/ml)
B. dracunculifolia	348.99 ± 117.22
Quercetin	76.12 ± 13.89

Figure 10: Comparison of the antioxidant activity of *B. dracunculifolia* with Quercetin, by the Inhibition of Nitric Oxide production (A), and their respective EC_{50} values (B). Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

4.3.4. Superoxide scavenging activity

Superoxide radicals generated *in vivo* are normally converted to hydrogen peroxide and, like other free radicals, attack biological molecules and can damage lipids, proteins and DNA, leading to cellular and/or tissue damage that can cause various diseases (Hanson *et al.*, 2006). Thus, antioxidants from plants with free radical scavenging activities are increasingly relevant in diseases in which oxidants or free radicals are implicated (Hanson *et al.*, 2006; Valentão *et al.*, 2002b).

In the analysis of the Superoxide scavenging activity (Figure 11), the results show there are significant differences between *B. dracunculifolia* and Quercetin ($t_{(15)}$=2.637, *P*=0.0187). *Post hoc* tests indicate that *B. dracunculifolia* extract has EC_{50} of 11 (µg/ml), and showed an higher activity than quercetin (used as a standard reference).



B

	EC_{50} (µg/ml)
B. dracunculifolia	11.09 ± 6.71
Quercetin	18.73 ± 4.96

Figure 11: Comparison of the antioxidant activity of *B. dracunculifolia* with Quercetin, by the Superoxide scavenging activity (A), and their respective EC_{50} values (B). Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

B. dracunculifolia is described in the literature as possessing antioxidant and anti-inflammatory properties (Funari *et al.*, 2007; Guimarães *et al.*, 2012; Rezende *et al.*, 2014). Phenolic compounds (analyzed by HPLC) may be related to the antioxidant potential of the sample (Guimarães *et al.*, 2012; Rezende *et al.*, 2014), and for this in this study the antioxidant potential of the same extract of *B. dracunculifolia* was evaluated through *in vitro* tests. To study the antioxidant potential of the *B. dracunculifolia* extract, four different systems of antioxidant models were used: DPPH method, Iron Chelating Activity (ICA), Inhibition of Nitric Oxide production and Superoxide scavenging activity.

Quercetin is a standard compound and in this study was used as a positive control, however it is important to mention that the EC_{50} value for quercetin is determined for a pure compound, unlike the Bd extract which is composed of a complex series of several compounds, which means that this comparison is not on the same basis (Guimarães *et al.*, 2012). Quercetin has already been shown to be an excellent antioxidant that has *in vitro* anti-inflammatory capabilities, and its antioxidant and anti-inflammatory effects have also been shown *in vivo* (Boots *et al.*, 2008). The EC_{50} corresponds to the half maximal inhibitory concentration, and the lower the value of EC_{50}, higher antioxidant power the sample has (Guimarães *et al.*, 2012).

In all the antioxidant assays it is verified that the Bd extract has EC_{50} values, which means that this extract exhibits antioxidant activity, which is promising for *in vivo* tests (Guimarães *et al.*, 2012). In the DPPH method, ICA and inhibition of nitric oxide production it is found that quercetin exhibits better antioxidant power than the Bd extract, which may be due to the fact that quercetin is only a pure compound whereas the Bd extract is a sum of various compounds. On the other hand, in the method of the superoxide scavenging activity it is found that Bd extract exhibits better results than quercetin, indicating that the Bd extract is a good $O_2^{\cdot\cdot}$ eliminator. This indicates that Bd extract besides possessing antioxidant properties is also a good eliminator of ROS, which are not only involved in the occurrence of oxidative stress, but also in the proliferation of inflammatory processes and certain diseases, such as osteoarthritis (Boots *et al.*, 2008; Cheuk *et al.*, 2016). Thus, elimination of ROS (namely $O_2^{\cdot\cdot}$) not only avoids the occurrence of oxidative stress, but also helps mitigate inflammation and consequently the proliferation of osteoarthritis (Boots *et al.*, 2008; Cheuk *et al.*, 2016). The fact that the Bd extract showed different behaviors in the four *in vitro* assays may be due to the different mechanisms involved in the steps of the oxidation process (Pacheco *et al.*, 2014).

With this, as it has been shown in the literature that *B. dracunculifolia* has phenolic compounds and antioxidant activity, and that phenolic compounds are probably the main contributors to this antioxidant potential (Funari *et al.*, 2007; Guimarães *et al.*, 2012; Lemos *et al.*, 2007; Missima *et al.*, 2006), in the present study it can be deduced that the phenolic compounds present in this extract (previously analysed by HPLC) may be the main contributors to these antioxidant activities of this extract.

Taking into account all the antioxidant tests performed, it can be said in general that the Bd extract has good antioxidant activity *in vitro*, since in addition to this extract is to be compared with a pure

compound, it also has an IC 50 value for all tests. Moreover, as the higher EC_{50} value indicates a lower antioxidant power, a higher concentration of Bd extract may be required to inhibit or eliminate 50% of the initial free radical concentration in the DPPH method, ICA and inhibition of nitric oxide production. Furthermore, the differences between these results and some literature results may be due to the variations found in the extracts of *B. dracunculifolia* according to the locality of the collection of the plant material, seasonality and the way of obtaining the extract that can change its composition quantitatively and qualitatively (Park *et al.*, 2002; Sforcin *et al.*, 2012).

4.4. Animal Welfare

4.4.1. Body weight

As for the weekly weight gain of the animal (Figure 12A), the analysis of the results shows there are no significant differences between groups (ANOVAone-way: $F_{(3,35)}$= 0.005597; *P*=0.9994). Figure 12B shows the total weight gain per group throughout the experiment, and statistical analysis shows there are significant differences between the ARTH and Bd100 groups (ANOVAone-way: $F_{(3,19)}$= 4.797, *P*=0.0144). *Post hoc* tests show that ARTH animals gain the most body weight throughout the experiment, unlike the Bd100 animals that gain less body weight.

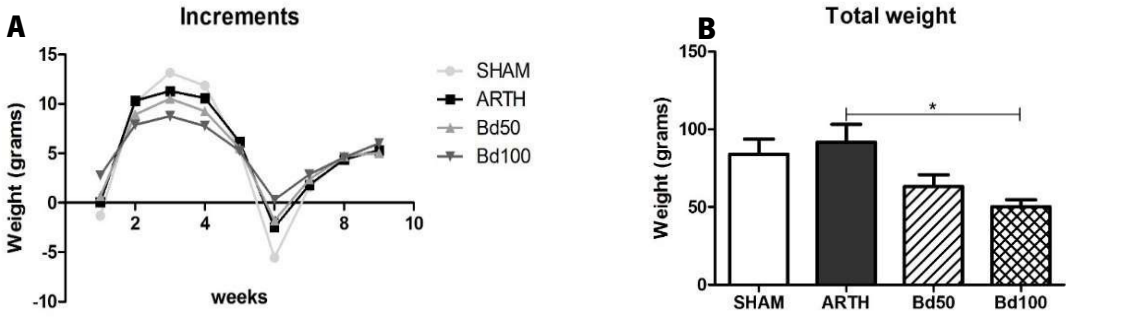


Figure 12: Increments in body weights of animals from week 1 to 9 (A). Total body weight gained by animals throughout the experiment (B). The peak at week 6 corresponds to the beginning of gavage treatment. At the total body weight it was verified that the Bd100 group did not increase as much of weight in relation to the groups SHAM, ARTH and Bd50. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

As for the increments of animal weights over the weeks, the statistical analysis of the results shows that there are no significant differences between the groups. However, at week 7, at the beginning

of gavage administration, there was a general decrease in weight of all groups. This decrease may be due to the change in the environment in which the animals were inserted, namely to experimental manipulation performed for the first time (administration by gavage). According to Brown *et al.* (2000), administration by gavage causes stress in the animals, and this is probably the factor responsible for the decrease of their weights in the week of initiation of administration by gavage. After week 6 and until the last experimental day, the animals of all groups gradually and individually recovered the weight.

On the other hand, in the total weight (Figure 12B) acquired by the animals during the whole experiment (weight of the last week - weight of the first week) it is verified that there are significant differences between the ARTH and Bd100 groups. Several studies have shown that several species of the genus *Baccharis* have diuretic power (Goleniowski *et al.*, 2006; Rodriguez *et al.*, 2010), and *Baccharis dracunculifolia* may also have some diuretic power and may have contributed to a decrease in the weight of the animals after administration of this extract (as shown in Figure 12B, the higher the extract concentration, the greater the weight decrease of the animals). The fact that the ARTH animals gained more weight than the rest of the animals during the whole experiment could also be due to the fact that they had osteoarthritis and did not move much (confirmed by the active time in the open field test), thus contributing for weight gain.

4.4.2. Internal organs

Figure 13 is relative to the weight of the internal organs (with adjustment to the weight of the animal), the analysis of the results shows there are no significant differences between groups in any of the evaluated organs (Thymus: ANOVAone-way: $F_{(3,19)}$ =0.9750, P=0.4290; Supra renal: ANOVAone-way: $F_{(3,19)}$ =0.3499, P=0.7897; Kidneys: ANOVAone-way: $F_{(3,19)}$ =1.004, P=0.4166; Lungs: ANOVAone-way: $F_{(3,19)}$ =1.186, P=0.3464; Liver: ANOVAone-way: $F_{(3,19)}$ =1.069, P=0.3901; Heart: ANOVAone-way: $F_{(3,19)}$ =1.280, P=0.3150; Spleen: ANOVAone-way: $F_{(3,19)}$ =0.2077, P=0.8896). *Post hoc* tests allow us to state that there was no macroscopic or microscopic alteration of the organs and no change in organ weight induced by the treatment.

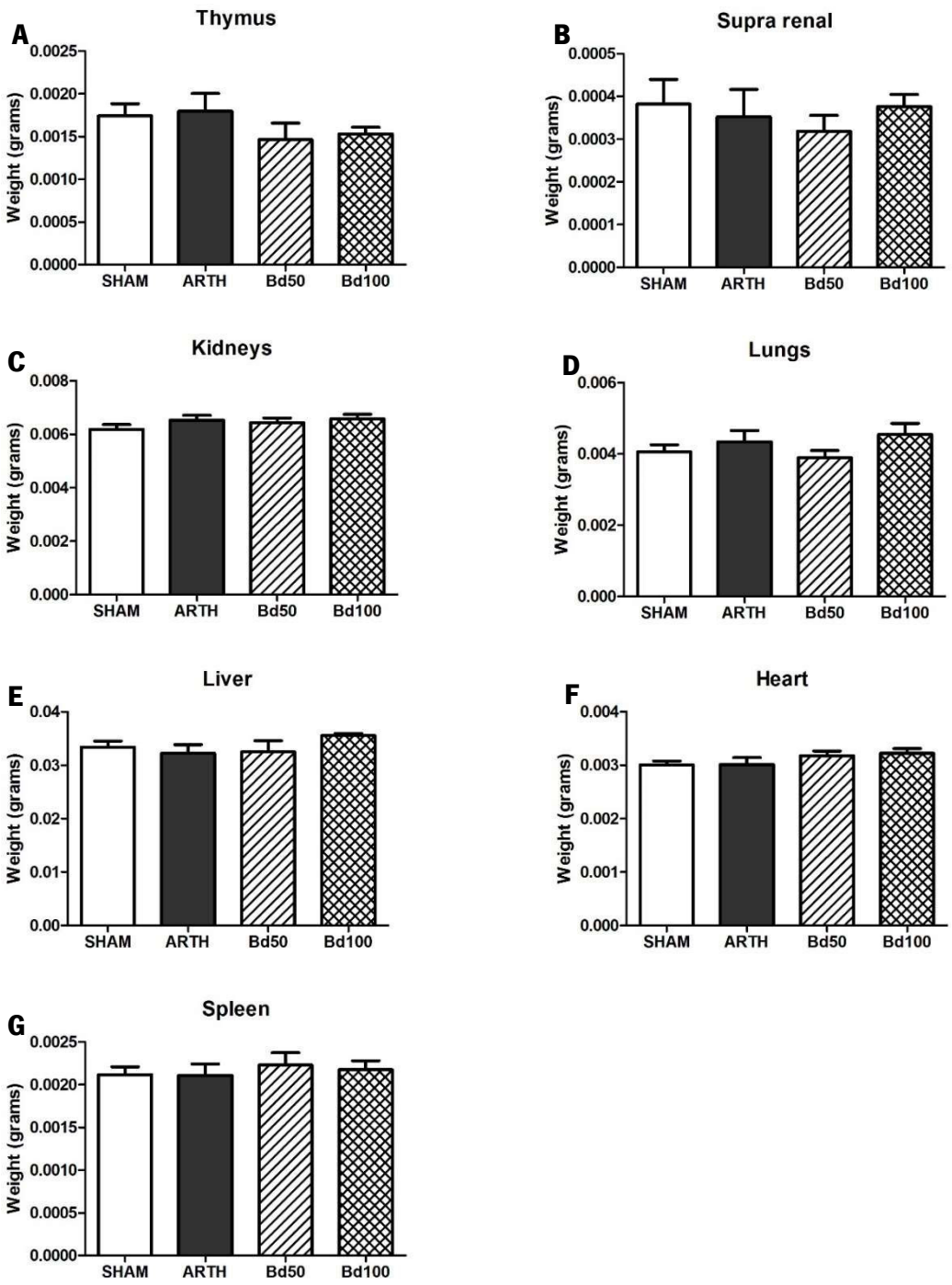


Figure 13: All internal organs (Thymus (A), Supra renal (B), Kidneys (C), Lungs (D), Liver (E), Heart (F), Spleen (G)), did not show significant differences between the SHAM, ARTH, Bd50 and Bd100 groups. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

In internal organs, their weights were analyzed at the time of euthanasia, and histopathological samples were also analyzed. Regarding the weight of the internal organs (with adjustment to the weight of the animal), the statistical analysis of the results shows that there are no significant

differences between the groups in any of the evaluated organs. Thus, it can be affirmed that there were no macroscopic or microscopic alterations of the organs and no changes in weight or normal architecture of the organs induced by the treatment, thus deducing that the animals presented themselves in good health. In addition, the reason that there are no significant differences in the weight of the individual organs may be due to the fact that the effect of stress induced by the gavage was not strong enough to produce a decrease in weight of these organs. In other hand, Ishikawa *et al.* (1995) with their study showed that stress induces adrenal hypertrophy, and since in this study the adrenals were in perfect condition, it can be deduced that the animals were not stressed (at least in excess). There are also several studies that show that Bd extract is harmless to the internal organs, as well as Rezende *et al.* (2014), which shows that Bd extract has a hepatoprotective effect at the same concentrations of Bd extract of 50 mg/kg and 100 mg/kg.

With all these data of the weighings during the experiment and with the analysis of the internal organs, it can be affirmed that the animals in this study were always in good health.

4.5. Behavioural tests

4.5.1. The Catwalk test

An extensive and reasonably simple set of tests is available in the human clinic to analyse locomotion, however, in animals the locomotion analysis is not so simple (Hamers *et al.*, 2006). Several different tests to analyse locomotion in laboratory animals have been developed over the years, such as tests that evaluate specific performances, such as the catwalk, among others (Hamers *et al.*, 2006).

In the catwalk the rats are encouraged to walk continuously characterizing this test by the procedure of habituation, the motivated runway crossing and the uni-directionality of locomotion caused by the narrow passage with vertical walls on either sides (Koopmans *et al.*, 2005).

Catwalk offers a powerful tool to asses more than 15 gait related parameters in a single test, and allows the quantification of individual parameters as well as parameters related to coordination between members (Gabriel *et al.*, 2007). One of the advantages of catwalk and OFT is that animals do not need to undergo any complex training process before each test (Gabriel *et al.*, 2007).

In the catwalk, static parameters of the animals were analysed, corresponding to the print area, stride length, box length and width, and toe spreading.

In the statistical analysis of the print area, the results show there are significant differences between SHAM and ARTH ($t_{(8)}$=2.312, *P*=0.0495) and between ARTH and Bd100 (ANOVAone-way: $F_{(3,19)}$= 5.705, *P*=0.005) (Figure 14A). *Post hoc* tests indicate that ARTH animals are those with a lower print area compared to the other groups, and that the treatment of 100 mg/kg reverses the situation. Figure 14B corresponds to the stride length, and the results (ANOVAone-way: $F_{(3,19)}$= 11.76, *P*=0.0003) show there are significant differences between SHAM and Bd100, ARTH and Bd50, and ARTH and Bd100. *Post hoc* tests allow us to affirm that the treatment animals are those with the highest stride length.

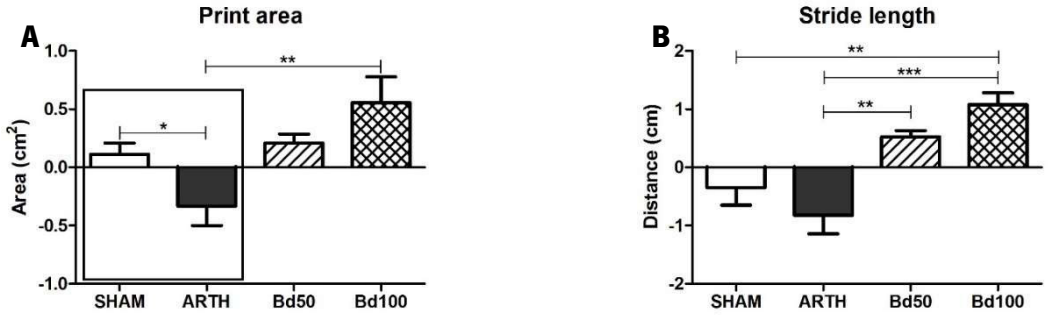


Figure 14: Total floor area contacted by the ipsilateral paw during the stance phase (expressed in cm^2) (A). Distance between the placement of the front and back paws on the ipsilateral side of the animal, in each step cycle (expressed in cm) (B). In the print area the ARTH animals are those with a lower print area compared to the SHAM, Bd50 and Bd100 animals, and that the treatment of 100 mg/kg reverses the situation. In the stride length the Bd50 and Bd100 animals are those with the highest stride length, when compared to SHAM and ARTH animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

In the parameter of the print area it is verified that there are significant differences between the animals SHAM and ARTH, and between animals Bd100 and ARTH, being that the ARTH animals are those that present smaller print area with respect to the other groups. The paw print area is an estimate of the pressure exerted by the paw in contact with the floor, since less printing area means less pressure applied in the paw (Ferreira-Gomes *et al.*, 2008). Thus, these data show that ARTH animals minimize contact with the floor and exert less pressure on the painful limb during walking, thus showing a decreased weight bearing in the osteoarthritic limb. This is probably due to an increased sensitivity to mechanical stimuli and avoidance of noxious mechanical stimulation resulting from damage of the knee joint induced by OA, and this is correlated with the daily situation

of patients with OA, in which walking causes the most severe mechanical stimulation leading to a change in weight in the affected leg (Messier, 1994). With these data it can be stated that the treatment of Bd100 reverses the situation, and these data are also in agreement with several studies, such as Ferreira-Gomes *et al.* (2008), Ferreira-Gomes *et al.* (2012), and Gabriel *et al.* (2007).

The stride length did not show any significant difference between the SHAM and ARTH groups, that is, the K/C model in this parameter did not develop differences between the two groups. This situation may be due to the K/C induced injury not causing any observable disagreement in this aspect, or an adaptation of the animal in the relationship between the anterior and posterior limbs, or this may also be related to stronger postural adjustment due to the animals are quadrupeds (Gabriel *et al.*, 2007, Wang *et al.*, 2012). However, the treatment animals present significant differences with the ARTH animals, having a greater stride length.

The analysis of the box length (Figure 15A) and box width (Figure 15B) shows that there are significant differences between SHAM and ARTH, ARTH and Bd50, and ARTH and Bd100, and in the box length there is more between Bd50 and Bd100 ((ANOVAone-way : $F_{(3,19)}$=15.44, P<0.0001) and (ANOVAone-way: $F_{(3,19)}$=22.09, P<0.0001), respectively). *Post hoc* tests indicate that the treatments of 50 mg/kg and 100 mg/kg revert both situations.

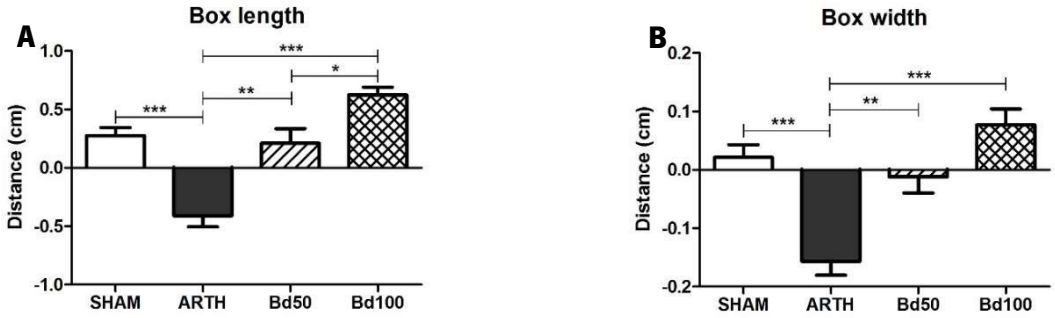


Figure 15: Length of the print area (A) and width of the print area (B). The ARTH group presented shorter box length and width when compared to the SHAM, Bd50 and Bd100 groups. Treatments of Bd50 and Bd100 revert both situations. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

Regarding the length and width of the box, the K/C model caused significant differences between the SHAM and ARTH groups. In addition, both in the length and width of the box there are significant

differences between the ARTH animals and the two treatments, Bd50 and Bd100, it being possible to affirm that both types of treatments revert both situations. Correlated by the studies of Gabriel *et al.* (2007), the results of these parameters prove that Bd extract is a possible good treatment for allodynia and/or hyperalgesia.

Figure 16A corresponds to the total toe spreading, and the results show there are significant differences between SHAM and ARTH ($t_{(8)}$=2.558, *P*=0.0338) and between SHAM and Bd100 (ANOVAone-way: $F_{(3,19)}$=5.363, *P*=0.0095). In the intermediate toe spreading, figure 16B, the results show (ANOVAone-way: $F_{(3,19)}$=7.945, *P*=0.0018) there are significant differences between ARTH and Bd50 and between ARTH and Bd100.

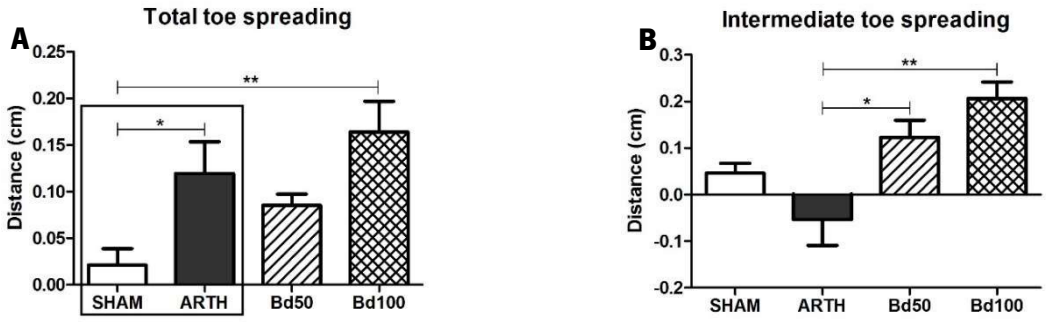


Figure 16: Distance between the first and fifth digits (A) and between the second and fourth digits (B). In the total toe spreading the ARTH and Bd100 groups showed a greater distance between the first and fifth digits. In the intermediate toe spreading the Bd50 and Bd100 groups showed a greater distance between the second and fourth digits. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

As for the total toe spreading, the K/C model caused significant differences between SHAM and ARTH animals, with ARTH animals further opening the total toe spreading to try to decrease body weight on the paw and thus decrease the pain caused by osteoarthritis. Regarding the intermediate toe spreading, there are no significant differences between healthy animals and ARTH animals, and it may be due to the K/C model induced injury that does not cause any observable disagreement in this aspect. However, there are significant differences between the ARTH animals and the treatment animals, showing that the ARTH animals most close these toes probably because of trying to better withstand the pain caused by osteoarthritis, but the treatment animals did not, which means that both treatments have been able to reverse this situation, and potentially indicate improved mechanical properties and/or less pain (Korntner *et al.*, 2017). These results can be

confirmed by the study of Bozkurt *et al.* (2011), which states that with osteoarthritis, the rat almost exclusively uses its heel to contact the ground rather than its toes (as is the case for uninjured rats).

Figure 17 corresponds to two strips of paper with animal foot prints of the ARTH and Bd100 groups. It is found that the ARTH animal has a much lower ipsilateral paw contact in relation to its contralateral paw, due to disease. On the other hand, in the treatment animal Bd100 it is found that the animal touches the floor in an identical manner between both paws, and has a more regular stride length than the ARTH animal.

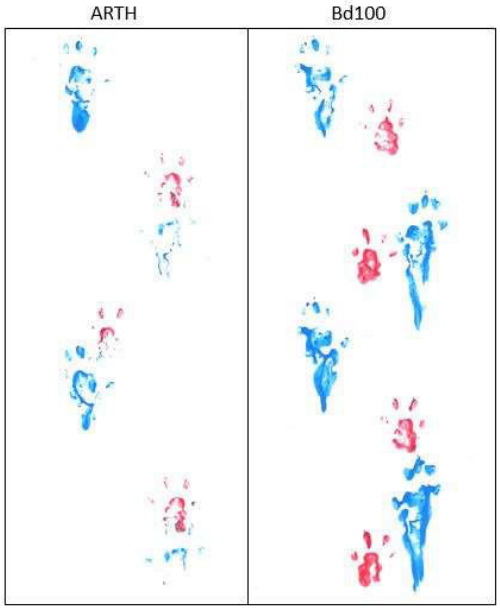


Figure 17: Example of two foot prints from ARTH and Bd100 animals.

Since the catwalk parameters related to single paws are strongly correlated to the development of mechanical hyperalgesia and allodynia, it is possible that in this study the ARTH animals developed mechanical allodynia (Gabriel *et al.*, 2007).

This study, as well as others (Wang *et al.*, 2012), shows that the catwalk is low-stress methods to analyse the motor functions of rats, which may be applied to monitor gait variability related to disease progression and treatment strategy. This study also showed that the catwalk were able to sensitively assess gait disorder in a K/C induced osteoarthritis model.

4.5.2. The Pressure Application Measurement (PAM) test

PAM is a behavioural tool capable of assessing joint pain in a rat model of experimental chronic inflammation of the joints, aiming to align pre-clinical measures to those used clinically and help the translation of animal studies to human conditions, since there is no objective measure of joint hypersensitivity to evaluate the pain of experimental arthritic joints in laboratory rodents (Barton *et al.,* 2007). Measurements of PAM are more accurately expressed as a force, in grams, and not as a force per unit area (pressure), as it cannot be assumed that the entire surface of the disc is in contact with the joint, moreover in Inflamed joints the deformation of the joint onto the disc is different to that in SHAM animals (Barton *et al.*, 2007).

Several studies have indicated that PAM provides a reliable and quantitative measure of localized and induced mechanical hypersensitivity of kaolin/carrageenan (K/C) injection in the rat knee joint (Amorim *et al.*, 2014a, Amorim *et al.*, 2014b). To assess mechanical hyperalgesia, PAM is more advised than other tests, such as calibrated forceps and von Frey test.

One of the advantages of PAM is that it provides a visual feedback on the instrument's monitor of the force applied by the operator and directly records the precise force placed on the knee joint to the point of withdrawal of the joints, thereby reducing deviations from the intended rate, unlike the other tests (Di Giminiani *et al.*, 2016). In contrast, with the forcep style of device (used as a means of mechanical stimulation during electrophysiological recording or to measure knee joint pain), authors report several problems with this device, including variation caused by the operator due to the placement of the finger on the forceps and inaccurate values of the withdrawal thresholds, as the calibrated reading given is a function of the force applied by the experimenter and the resistance offered by the joint (Barton *et al.*, 2007). On the other hand, PAM is preferable to the forcep style of device and the von Frey test, since the pressure exerted on the skin with a punctuate object, such as a needle, may activate nociceptive afferents in several tissues, and may exclusively activate nerve endings in the skin, in particular C fibres (Treede *et al.*, 2002). Furthermore, since skin deformation can be achieved with very small forces, these stimuli have little effect on the afferents that innervate the deeper tissues, as opposed to a pressure exerted on a larger area of the skin and the contact surface is rounded or padded where a preferential activation of deep afferents is possible (Treede *et al.*, 2002). This evidence adds support for the use of PAM as a measure of nociceptive activity in deeper tissues, such as the joint itself, rather than overlying skin. This contrasts with von Frey hairs and the forcep style of device, which as a punctuate stimuli activate

skin afferents rather than innervate the joint capsule and surrounding tissues. An additional advantage of PAM is the ability to change the size of the pressure application surface and the force range of the transducer, allowing the device to be used in several species, as well as the possibility of assessing other joints and paws (Barton *et al*., 2007).

Figure 18 corresponds to the PAM test and the results show there are significant differences between SHAM and ARTH ($t_{(8)}$=4.164, *P*=0.0031). *Post hoc* tests indicate that the induction of experimental OA significantly increased mechanical hyperalgesia in ARTH animals when compared to SHAM animals. Moreover, the results also show there are significant differences between ARTH and Bd50 animals (ANOVAoneway: $F_{(3,19)}$=4.066, *P*=0.0252. *Post hoc* tests allow us to affirm that the treatment of Bd50 significantly decreases mechanical hyperalgesia.

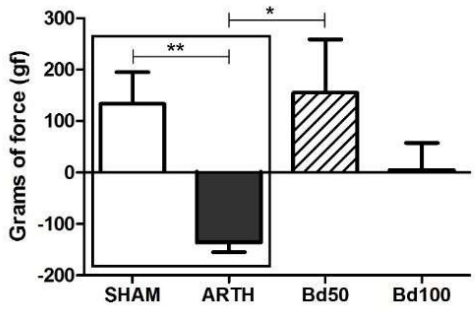


Figure 18: Evaluation of mechanical hyperalgia in SHAM, ARTH, Bd50 and Bd100 animals, using the pressure application measurement. The SHAM and Bd50 animals significantly decreases mechanical hyperalgesia, unlike ARTH animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

The results of this study show that PAM was able to detect hypersensitivity induced by kaolin/carrageenan injection, observed as a significant decrease of LWTs in the joints of ARTH animals compared to SHAM and Bd50 animals. Thus, these results show that ARTH animals exhibit deep mechanical hyperalgesia in the ipsilateral knee and that PAM also managed to detect the analgesic action of *Baccharis dracunculifolia* at a concentration of 50 mg/kg. On the other hand, the fact that there are no significant differences between the ARTH and Bd100 animals may be due to the dose of 100 mg/kg being detrimental or toxic to the analgesic effect.

PAM proved to be simple and easy-to-use test that allowed rapid and reproducible measurements, proving to be a valuable addition to current measurements to assess hypersensitivity.

4.5.3. The Open Field Test (OFT)

The observation of spontaneous locomotion in the OFT is one of the oldest and conceptually simpler ways of assessing locomotor performance, however a serious problem with this test is that the different functional levels of the animals are seldom clearly defined nor equally spaced (Hamers *et al.*, 2006).

In the open field, a rat is free to go where it wants within the borders of the open field, so each movement is suggested to be completely voluntary (Koopmans *et al.,* 2005). However, the square open field is used to analyse anxiety function in the rat, in which the rats act aversively towards open spaces in a way that they will spend most of the time close to the wall or in a corner, so this test for analysis of locomotion becomes more limited (Koopmans *et al.*, 2005).

The analysis of the results of active time (Figure 19A) and ambulatory speed (Figure 19B) shows that there are significant differences between SHAM and ARTH ((ANOVAone-way: $F_{(3,19)}$= 3.265, *P*=0.0489) and ($t_{(8)}$= 5.965, *P*=0.0003), respectively). The *post hoc* tests indicate that the induction of experimental OA performed significant changes in the locomotion when comparing ARTH animals with SHAM animals.

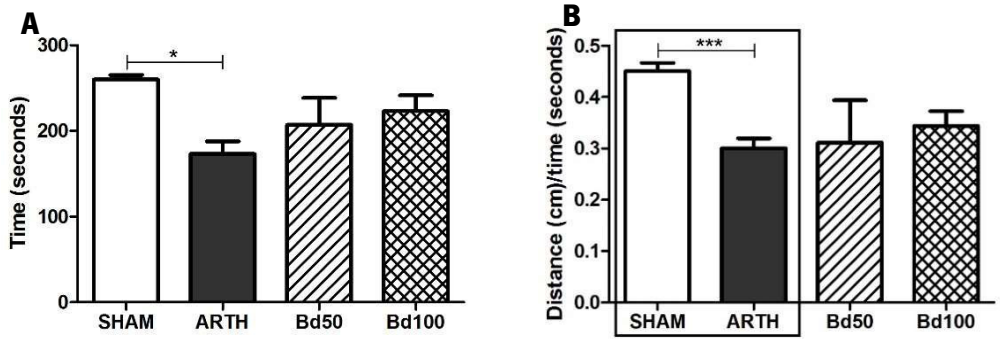


Figure 19: Active time (A) and ambulatory speed (B) in the OFT, in SHAM, ARTH, Bd50 and Bd100 animals. ARTH animals show lower active time and ambulatory speed than the remaining groups of animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

The following figures correspond to two ratios that provide additional information to the ambulatory speed data. Statistical analysis of the center/periphery ratio (Figure 20A) results shows there are significant differences between SHAM and ARTH, and ARTH and Bd100 (ANOVAone-way: $F_{(3,19)}$=5.171, *P*=0.0109). *Post hoc* tests indicate that SHAM and Bd100 animals move faster than ARTH animals. Analysis of the center/total ratio (Figure 20B) results shows there are significant differences between SHAM and ARTH ($t_{(8)}$=2.783, *P*=0.0238), and *post hoc* tests allow to affirm that SHAM animals move faster than the ARTH animals.

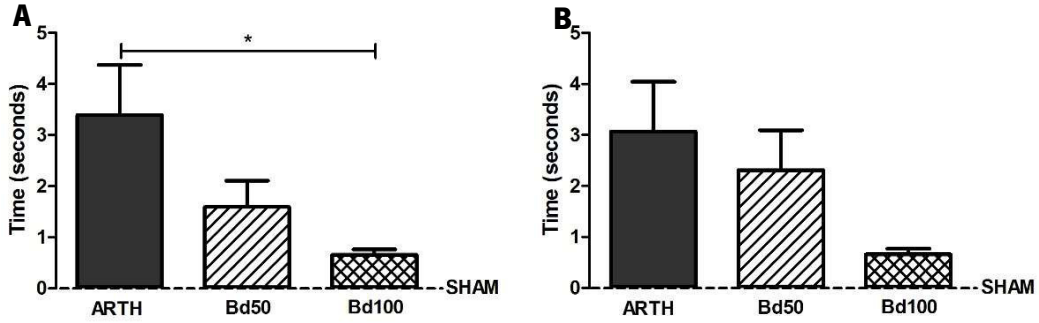


Figure 20: Center/periphery ratio (A) and center/total ratio (B) in the OFT, in SHAM, ARTH, Bd50 and Bd100 animals. SHAM and Bd100 animals move faster than ARTH animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

In the open field test, the induction of experimental OA made significant changes between the SHAM and ARTH groups, both in active time and ambulatory speed, which proves locomotor changes in the animals between groups, and that healthy animals move more and faster. In addition, studies have shown that animals spend less time in the center of the OFT because of the anxiety they feel when they are too exposed (Koopmans *et al.,* 2005), so in this study it is verified that ARTH animals spend more time in the center than the SHAM animals, which indicates that the ARTH animals were not more often in the center of the arena, but rather moved more slowly than SHAM animals each time they passed through the center of the arena. Therefore, these two ratios related to the relationship between center and periphery confirm that SHAM animals and treatment animals are more in movement time and have a higher velocity than ARTH animals.

In summary, OFT analysis in the K/C induced inflammation model shows that all these results show better locomotion and higher velocity in healthy animals and treatment animals compared to ARTH animals.

4.5.4. Perimetry of the knees

The induction of osteoarthritis in the knee joint in addition to causing joint damage and mechanical hyperalgesia, also causes the formation of edema (Barton *et al.*, 2007). Thus, measurement of the joint perimeter to measure edema can be of great value as a simple and rapid parameter of the clinical evolution of the AO, and this joint measurement test is a relevant complement to the PAM test, since edema is the main cause of pain (Barton *et al.*, 2007).

Figure 21 corresponds to the perimetry of the knees and the analysis of the results shows there are significant differences between knee perimeters of the SHAM and ARTH animals ($t_{(8)}$=3.024, *P*=0.0165), but there are no significant differences between the remaining groups (ANOVA one-way: $F_{(3.19)}$=2.525; *P*=0.0944). *Post hoc* tests indicate that induction of experimental OA caused swelling in the knee joints, and that treatments with concentrations of 50 and 100 mg/kg partially reverse the swelling of the knee joint.

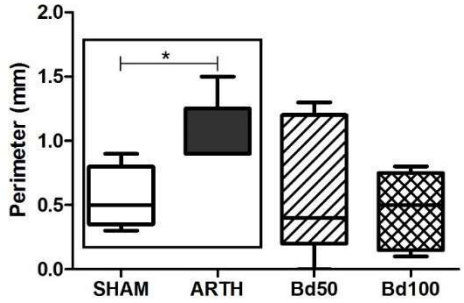


Figure 21: Measurement of the knee joint perimeter in the SHAM, ARTH, Bd50 and Bd100 groups. The ARTH animals present a larger perimeter in the knee joint relative to the remaining groups of animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

Measurement of knee perimeter showed that the induction of osteoarthritis in the joints caused a significant increase of edema in the ARTH animals when compared to SHAM animals. However, the administration animals do not show significant differences in edema compared to ARTH animals, but through the figure 21 can see the means of each administration and it is verified that

these resemble the SHAM group, indicating that the perimeter of the joint of the treatment animals is identical to healthy animals. This indicates that the administration animals do not show significant differences with respect to the ARTH animals because the values of the treatment animals are very discrepant (high standard deviation). With this, it can be affirmed that the Bd extract was able to prevent joint edema, which may indicate antiedematogenic action of *Baccharis dracunculifolia.*

Thus, these results demonstrate that intra-articular injection of K/C induced a characteristic inflammation with edema. In addition, in this study, knee perimetry proved to be a simple and easy-to-use test that allowed rapid and reproducible measurements, proving to be a valuable addition to current measurements to assess joint edema.

4.6. Acute dermal irritation/corrosion test

Ear edema models induced by phlogistic agents have been extensively used as pharmacological tools for the investigation of new topical anti-inflammatory drugs, including natural products which are useful in the treatment of inflammatory skin disorders (Phanse *et al.*, 2012). However, this acute dermal irritation/corrosion test was used primarily to test the possible anti-inflammatory effect of *B. dracunculifolia*, and to correlate with the results obtained in the analysis of the knee joints.

Croton oil contains 12-*o*-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents, and once topically applied, results in an increased vascular permeability, migration of polymorphonuclear leukocytes (mainly neutrophils), liberation of histamine and serotonin, and a moderate synthesis of eicosanoids (Badilla *et al.,* 2007). Croton oil is able to activate protein kinase C (PKC), which can lead to other activations and leads to the release of various metabolites, as well as cytokines, which are mediators of inflammatory pathways and are responsible for triggering and maintaining inflammation (Stanley *et al.*, 1991).

On the other hand, dexamethasone is a glucocorticoid that binds to specific intracellular receptors that activate or repress transcription of genes to inhibit the actions of the immune system as a whole (Silva *et al.*, 2015). Dexamethasone is able to inhibit the expression of several compounds, as well as cytokines, and induces the expression of other proteins that inhibit the synthesis of prostanoids (eicosanoids), reducing inflammation (Silva *et al.*, 2015).

Figure 22 corresponds to the thicknesses of the ears of the animals measured after topical application of various compounds (Bd extract, croton oil and dexamethasone) on the day of the acute dermal irritation/corrosion test. Analysis of ear thickness results shows there are significant differences between Ct and CO, and Bd and CO (ANOVAone-way: $F_{(4,34)}$=5.847, *P*=0.0013). *Post hoc* tests indicate that croton oil causes a significant increase in ear thickness.

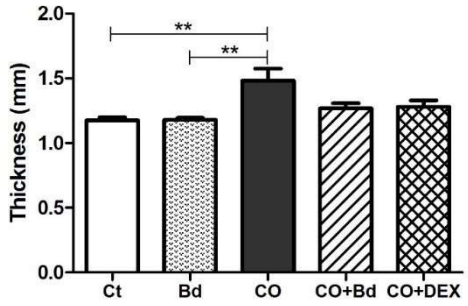


Figure 22: Measurement of animal ear thickness after the acute dermal irritation/corrosion test. Ct: control, nothing was applied to the ear; Bd: *Baccharis dracunculifolia* extract; CO: croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone and after 15 minutes application of *Baccharis dracunculifolia* extract; CO+DEX: croton oil 5% dissolved in acetone and after 15 minutes application of dexamethasone. The ears with croton oil presented greater thickness than the remaining ones. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

Figure 23 corresponds to a rat that was subjected to the acute dermal irritation/corrosion test. It is verified that the right ear has a larger dimension (was applied croton oil) than the left ear (was applied Bd extract) due to the inflammation caused by croton oil.

Figure 23: Acute dermal irritation/corrosion test performed on a rat. In the left ear was applied Bd extract and in the right ear was applied croton oil.

The following figures correspond to microscopic analysis of animal ears using the acute dermal irritation/corrosion test.

The following figure 24 corresponds to the analysis of ear epidermis thickness shows there are significant differences between Ct and CO, and Bd and CO (ANOVAone-way: $F_{(4,9)}$=14.50, *P*=0.0059). The *post hoc* tests indicate that the epidermis of the ears with application of croton oil have a greater thickness due to inflammation. On the contrary, in the control ears and the ears that were applied Bd it is verified that the thicknesses of the epidermis were smaller, showing no inflammation.

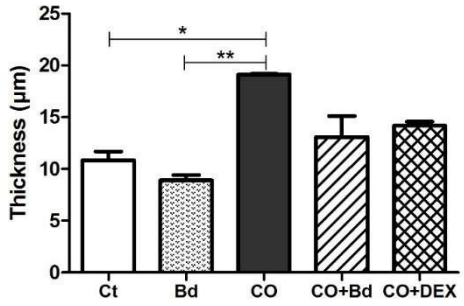


Figure 24: Measurement of the thickness of the animal's ear epidermis after the acute dermal irritation/corrosion test. Ct: control, nothing was applied to the ear; Bd: *Baccharis dracunculifolia* extract; CO: croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone and after 15 minutes application of *Baccharis dracunculifolia* extract; CO+DEX: croton oil 5% dissolved in acetone and after 15 minutes application of dexamethasone. The ears with croton oil presented greater thickness of the epidermis than the others. The control ears and the ears with Bd are the ones that present smaller thickness. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

The analysis of ear dermis thickness (Figure 25) shows there are significant differences between Ct and CO, Bd and CO, and CO and CO+Bd (AN1OVAone-way: $F_{(4,9)}$=10.45, *P*=0.0121). *Post hoc* tests indicate that the thickness of the ear dermis with application of croton oil is higher due to inflammation. However, in the control ears and in the ears that were applied Bd, it is verified that the thicknesses of the dermis were smaller, showing no inflammation. In addition, in the ears that had croton oil and subsequent application of Bd, *post hoc* tests indicate that the thickness of the dermis was also significantly lower, indicating that the application of Bd reverses the inflammation caused by croton oil.

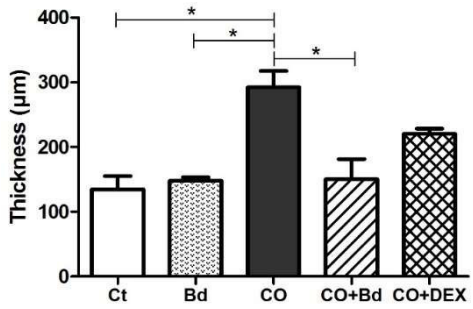


Figure 25: Measurement of the thickness of the animal's ear dermis after the acute dermal irritation/corrosion test. Ct: control, nothing was applied to the ear; Bd: *Baccharis dracunculifolia* extract; CO: croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone and after 15 minutes application of *Baccharis dracunculifolia* extract; CO+DEX: croton oil 5% dissolved in acetone and after 15 minutes application of dexamethasone. The ears with croton oil presented greater thickness of the dermis than the others. The control ears, the ears with Bd and the ears with initial application of croton oil and later application of Bd are those that present smaller thickness of the dermis. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

The analysis of the number of inflammatory cells in the ears (Figure 26) shows there are significant differences between Ct and CO, Ct and CO+DEX, Bd and CO, Bd and CO+DEX, CO and CO+Bd, and CO and CO+DEX (ANOVAone-way: $F_{(4,29)}$=19.95, *P*<0.0001). *Post hoc* tests indicate that the number of inflammatory cells in the ears with application of croton oil is greater than in the remaining ears due to inflammation. In the ears that had croton oil and subsequent application of Bd and dexamethasone, *post hoc* tests also indicated that the number of inflammatory cells was significantly lower, indicating that the application of Bd and dexamethasone reverses the inflammation caused by croton oil.

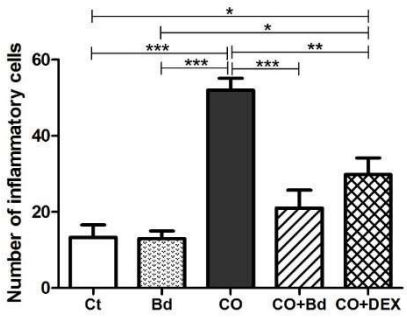


Figure 26: Measurement of the number of inflammatory cells in the ears after the acute dermal irritation/corrosion test. Ct: control, nothing was applied to the ear; Bd: *Baccharis dracunculifolia* extract;

CO: croton oil 5% dissolved in acetone; CO+Bd: croton oil 5% dissolved in acetone and after 15 minutes application of *Baccharis dracunculifolia* extract; CO+DEX: croton oil 5% dissolved in acetone and after 15 minutes application of dexamethasone. The ears with croton oil are those with the highest number of inflammatory cells in comparison to all the others. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

In the present study, histopathological analysis of ear tissue after application of croton oil revealed a significant increase in ear thickness, dermis and epidermis thickness, and number of inflammatory cells, associated with edema formation, vasodilation and infiltration of inflammatory cells when compared to non-inflamed ears (control) and ears with application of Bd extract. This event can be explained by the fact that during the inflammatory process (when croton oil is applied), some inflammatory factors (like cytokines) are released or by the cells of the altered tissue either by the inflammatory infiltrates, which act as mediator factors that can influence the dermal growth and the physiological characteristics of the tissues (Hübner *et al.*, 1996; Pacheco *et al.*, 2014). Otherwise, in non-inflamed ear tissues (control and with application of Bd extract) the physiological characteristics were preserved.

The results observed in these histopathological analyses of sections of the ear tissues obtained from croton oil-induced edema demonstrate the anti-edematous and anti-inflammatory effect of *B. dracunculifolia*, suggesting that Bd has a similar effect to dexamethasone (glucocorticoids) once which demonstrated reduced ear thickness (edema), vasodilation and infiltration of inflammatory cells.

Otherwise, the flavonoids present in the Bd extract identified by HPLC are probably interacting with important inflammatory pathways that impede the actions of mediators implicated in the formation of edema and may be potential targets for the development of new topical anti-inflammatory therapeutic agents (Rodrigues *et al.*, 2016). Moreover, through the various antioxidant tests performed in this study, it was also verified that Bd extract has antioxidant and free radical scavenging activity, and since ROS also play an important role in the formation of edema, it is assumed that antioxidant activity of Bd may have contributed to the anti-inflammatory activity observed in this study (Pacheco *et al.*, 2014).

As first evidence, the results of this study showed that Bd extract was active against croton oil-induced ear edema, reducing the inflammatory parameters triggered by this agent. In summary, the results of this study showed the efficacy and safety of Bd extract as a topical anti-inflammatory agent, which significantly reduced ear edema (namely in the thickness of the ear and the number of inflammatory cells), and which may serve as a source for the development of drugs effective for inflammatory skin disorders. In addition, these findings also contribute to the international policy strategies proposed by World Health Organization related to Traditional and Complementary Medicine (T & CM). However, additional studies are needed to identify other mechanisms and chemical constituents that may be contributing to this activity.

Since one of the characteristics of osteoarthritis is the presence of inflammation in the joint, and as this acute dermal irritation/corrosion test shows that *B. dracunculifolia* exhibits an anti-inflammatory effect, it is concluded that the anti-inflammatory effect of *B. dracunculifolia* can help protect the joints of the knees, confirming also by the histological analyzes of cartilage in this present study (Adães *et al.*, 2015).

4.7. Histology of joints

The kaolin/carrageenan model has been predominantly used to study the mechanisms underlying structural joint damage and also to study pain associated with OA (Amorim *et al.*, 2014a; Amorim *et al.*, 2014b). As shown here, intra-articular injection of kaolin/carrageenan into the knee of rats induces nociceptive behaviours associated with movement and loading on the OA joint, mimicking the main complaints of patients, along with histopathological changes that match the description of human AO (Creamer *et al.*, 1998).

The histopathological changes in the knee joints induced by injection of kaolin/carrageenan were analysed to confirm the development of OA and to verify the power of the extract of *Baccharis dracunculifolia.*

The induction of experimental OA caused significant changes in the knee joint cartilage (Figure 27) as the analysis of the results shows there are significant differences between experimental groups (ANOVAone-way: $F_{(3,15)}$= 11.26, P=0.0008). *Post hoc* tests indicate that ARTH animals are the ones with the greatest loss of cartilage, and that treatments with 50 mg/kg and 100 mg/kg reverted the situation of cartilage loss.

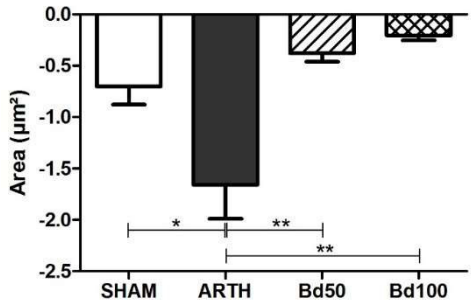


Figure 27: Area of worn cartilage in the tibias. The animals ARTH are the ones that present greater loss of cartilage, unlike the animals SHAM, Bd50 and Bd100 that revert the situation of loss of cartilage. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

Figure 28 corresponds to photomicrographs of sections of the knee joint, visualizing the different cartilage states constituting the groups of animals. It has been found that in ARTH animals there is a greater loss of cartilage than in the other groups. SHAM animals and Bd50 and Bd100 treatments appear to have no loss of cartilage.

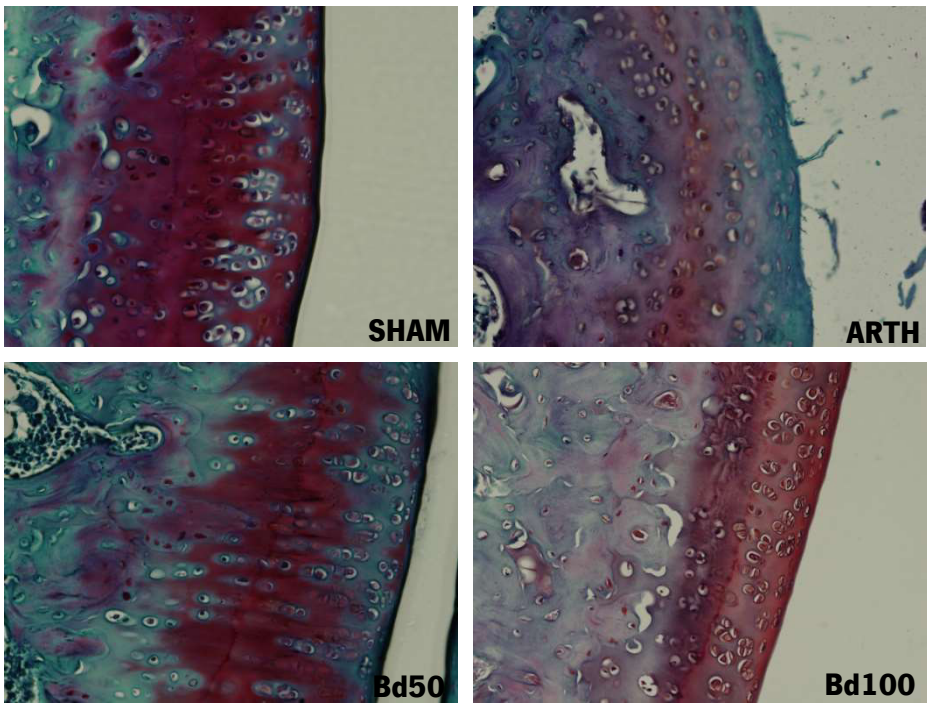


Figure 28: Photomicrography of knee joint sections in SHAM, ARTH, Bd50 and Bd100 animals – Fast Green and Safranin-O staining.

Analysis of the total trabecular bone area in the tibia (Figure 29) shows there are no significant differences between the groups (ANOVAone-way: $F_{(3,15)}$= 0.8420, *P*=0.4968). *Post hoc* tests indicate there were no macroscopic or microscopic changes in the area of total trabecular bone.

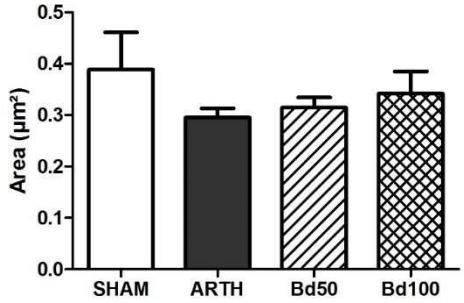


Figure 29: Total trabecular bone area in the tibia. There were no macroscopic or microscopic changes in the area of total trabecular bone between the SHAM, ARTH, Bd50 and Bd100 animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

Analysis of the results shows the induction of experimental OA caused significant changes in the trabecular width (Figure 30), since there were significant differences between SHAM and ARTH, ARTH and Bd50, ARTH and Bd100, and Bd50 and Bd100 groups (ANOVAone-way : $F_{(3,15)}$=29.10, *P*<0.0001). *Post hoc* tests indicate that the treatments of 50 mg/kg and 100 mg/kg reverted the situation.

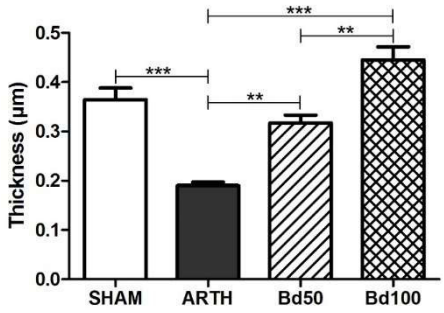


Figure 30: Thickness of the trabeculae in the tibia. The ARTH animals present a smaller trabecula thickness than the SHAM, Bd50 and Bd100 animals. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.005, ***P<0.001.

As kaolin/carrageenan induced OA progresses, articular destruction becomes severe. Control animals injected with saline (SHAM) presented normal cartilage and subchondral bone structure, with no measurable severe damage to the knee joints. On the other hand, rats injected with kaolin/carrageenan developed histopathological changes that included extensive degeneration of hyaline cartilage with areas of marked surface erosion, indicated by the marked reduction of the Safranin-O stain, as well as pronounced chondrocyte depletion. Degeneration was confirmed in all cases of OA animals, and histopathological changes were similar in all OA animals. In contrast, it is found that in the treatment animals the results are favourable and promising for the Bd extract. In the Bd50 and Bd100 treatments the cartilage degradation was minimal, and the chondrocytes appeared to be of a benign structure.

There is usually a close anatomic relationship between the damaged cartilage and changes in the subchondral bone, these changes being an increase in osteoclastic and osteoblastic activity in the trabecular bone immediately underlying the areas of cartilage loss and degeneration (Guzman *et al.*, 2003). In this study, regarding the total trabecular bone, there were no significant differences between the various groups. This may be due to a number of factors, such as the concentration of drug administered and the duration of administration (5 weeks) were not sufficient to have caused significant differences in this structure of the knee joint. In addition, as changes in trabecular bone may be induced by increased load on the subchondral bone due to advanced cartilage loss (Guzman *et al.*, 2003), and in this study there are no marked changes in trabecular bone between the different groups of animals, this may be due to the fact that the animals are quadruped and avoid overloading the ipsilateral joint, due to the pain felt in this joint, and do not cause the formation of so many osteoblasts and osteophytes.

Generally, in joints affected by OA, the horizontal trabeculae do not change, as opposed to the vertical trabeculae that increase the number of trabecular structures due to the wear of the cartilage in these joints (Messent *et al.*, 2005). However, the way the trabeculae are analysed can influence the width of these, and in this study the trabecular width was obtained by microscopically analysing the histology slides. However, there are more reliable methods such as macro-radiographs analysis of trabeculae that allow to distinguish a greater degree of trabecular detail between knees OA and non-OA knees, and also the detection of structures that lie behind one other within the plane of the object imaged in the macro-radiograph (Messent *et al.*, 2005). It should also be pointed out that the perspective from which the trabecula was cut can influence the measurement of the trabecular width, if it was cut in the zone of larger or smaller diameter.

In this study, a substantial loss of tibial bone structure was observed in all knees with AO, corroborating a decrease in trabecular width. Messent *et al*. (2005), with his study states that the thickened cortical plaque, flattening and increased congruity between the articular elements (patients with OA) lead to greater absorption of local stress, reducing load transmission to the underlying subchondral bone, and this reduction of load applied to the medial compartment leads to osteoporosis. Thus, in the present study as the ARTH animals are the ones with the lowest trabecular width, it can be hypothesized that it is due to a bone loss caused by osteoporosis located inside the tibia of rats with OA in the knee, due to the greater absorption of local stress in the joint.

It should also be noted that the contralateral knees of rats injected with kaolin/carrageenan did not present alterations in any of the groups and in none of the parameters. The absence of a significant differences between the ipsilateral and contralateral joints in SHAM animals indicated that measurements of cartilage wear, total trabecular bone and trabecular width could use the contralateral joint as an internal control, an idea originally proposed by Randall & Selitto (1957).

Currently, there is still a lack of evidence supporting that any of the pharmaceuticals used can achieve promising effects of osteoarthritis modification, although some treatments are effective in reducing pain but associated with side effects (Zhang *et al*., 2010). The extract of Bd has natural antioxidants, which have fewer side effects and may be the answer to this problem, however more studies will have to be done.

In summary, the results of this study showed that administration by gavage of Bd extract had promising results regarding knee joint structures when compared to ARTH animals, especially in the absence of cartilage wear.

Chapter 5: Conclusions

With this dissertation some pharmacological potentials of *Baccharis dracunculifolia* have been demonstrated, namely in the treatment of osteoarthritis and dermal inflammation.

With data from animal weighing and histological analysis of internal organs it is concluded that Bd extract at the doses of 50 mg/kg and 100 mg/kg are not orally toxic and apparently do not cause health problems in animals, and all 24 animals remained alive until the end of the experiment.

The HPLC and antioxidant tests were satisfactory with the presence of phenolic compounds in this extract of *B. dracunculifolia*, and in general, due to the presence of these compounds, it was verified that this extract has good antioxidant and anti-inflammatory activity *in vitro*.

After ovariectomy and injection of K/C on the rats' right knee, no complications were observed in the animals.

In this study it was also confirmed that the K/C model presents an interesting potential for the study of osteoarthritis in rats, with cartilage degradation and inflammation, with hypersensitivity and edema. With this, administration by gavage of the extract of *B. dracunculifolia* has been shown to improve locomotion and decrease hypersensitivity in ARTH animals, it being concluded that this plant has anti-inflammatory and analgesic properties. In addition, the extract of *B. dracunculifolia* also exerted a positive influence on the protection of OA development in rats, reflecting a prevention of the extent of abrasion and injury of the cartilage.

B. dracunculifolia extract has also been shown to decrease and prevent inflammation, both in the dermal analyses of the ears, and in the inflammation caused by osteoarthritis by measuring knee joint edema, respectively.

In summary, the results obtained in this study demonstrate that *B. dracunculifolia* exhibits good activity in the treatment of pain, locomotion and inflammation. Thus, this extract may be promising for the treatment of osteoarthritic disorders and dermal inflammations, which corroborates the use of this plant in popular medicine, contributing to its pharmacological validation. However, more detailed *in vivo* studies are needed to establish the safety and clinical relevance of *B. dracunculifolia* leaf extract.

Chapter 6: Future perspectives

This dissertation approach a current and quite common theme, and this study may constitute a working basis for future research. Taking into account certain limitations that were found throughout this work, it is now possible to present a series of suggestions for possible future work, they are interesting ways to deepen and improve the presented study.

As already mentioned, it was verified that the treatment of Bd100 was almost always more satisfactory than the treatment of Bd50, however in some parameters this was not observed, possibly due to the concentration of 100 mg/kg being too excessive for certain parameters. It would be interesting to test an intermediate dose of 75 mg/kg to be feasible to estimate a possible progression relative to the doses administered. In addition, it would also be important to evaluate the effect of administration on longer periods of administration and its impact on the various parameters studied in this research.

The SHAM animals in this experimental work received an intra-articular injection with saline solution, however nothing guarantees that the way in which the intra-articular injection is applied does not damage the knee joint, and consequently impairs the locomotion and nociception of the animal, impairing the test results. Thus it would be interesting to have a group of animals without any intra-articular injection to compare the results of these with the other experimental groups.

Several behavioural tests were performed on the animals in this study, however there are other tests that would also be pertinent to have performed, as well as the rotarod test to analyse the locomotion and speed of the animals, as well as the flexion-extension test of the knee joint to verified if the rats that developed osteoarthritis vocalized due to nociception felt.

Otherwise, it would also be interesting to have analysed the cerebrospinal fluid (CSF) to verify if the properties of *B. dracunculifolia* extract were able to cross the blood-brain barrier or not, and thus could affect the interpretation of the results. It would also be important to have analysed the animals' blood to see if there were any toxic properties or other compounds that could have affected the animal in any way.

Another interesting parameter for future study would be to perform tests with interleukin-1 beta (IL-1β) on the tissues of the ears to better analyse the inflammation, like tests of enzymatic activity to analyse cutaneous inflammation, as well as the tissue myeloperoxidase assay (MPO) and the tissue N-acetyl-β-D-glucosaminidase assay (NAG). In the ear edema tests, it would be interesting to perform dermal irritations with different irritant agents (such as arachidonic acid, phenol, ethyl phenylpropiolate and capsaicin) to better analyse the differences between compounds and the

properties and mechanisms of action of Bd extract. Furthermore, in the ear edema tests it would also be relevant to use several doses of Bd extract in order to verify whether the concentration of Bd may influence the inflammatory responses or not, and what is the best dose of this extract in the dermal anti-inflammatory process.

In short, it is of interest that this dissertation should be seen as an aid to the development of new natural drugs, mainly for the treatment of osteoarthritis and its additional consequences to the disease, as well as for dermal inflammations.

Chapter 7: References

- Adães, S., Ferreira-Gomes, J., Mendonca, M., Almeida, L., Castro-Lopes, J. M., & Neto, F. L. (2015). Injury of primary afferent neurons may contribute to osteoarthritis induced pain: an experimental study using the collagenase model in rats. *Osteoarthritis and Cartilage*, 23 (6), 914-924.
- Al-Jaber, N. A., Awaad, A. S., & Moses, J. E. (2011). Review on some antioxidant plants growing in Arab world. *Journal of Saudi Chemical Society*, 15 (4), 293-307.
- Almeida, T. F., Roizenblatt, S., & Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain research*, *1000* (1), 40-56.
- Altwegg-Boussac, T., Mahon, S., Chavez, M., Charpier, S., & Schramm, A. E. (2016). Induction of an Isoelectric Brain State to Investigate the Impact of Endogenous Synaptic Activity on Neuronal Excitability In Vivo. *JoVE (Journal of Visualized Experiments)*, (109), e53576-e53576.
- Alves, C. Q., David, J. M., David, J. P. D. L., Bahia, M. V., & Aguiar, R. M. (2010). Métodos para determinação de atividade antioxidante *in vitro* em substratos orgânicos. *Química Nova*, 33 (10), 2202-2210.
- Alvim, N. A. T., Ferreira, M. D. A., Cabral, I. E., & Almeida Filho, A. J. D. (2006). The use of medicinal plants as a therapeutical resource: from the influences of the professional formation to the ethical and legal implications of its applicability as an extension of nursing care practice. *Revista latino-americana de enfermagem*, 14 (3), 316-323.
- Amorim, D., David-Pereira, A., Marques, P., Puga, S., Rebelo, P., Costa, P., Pertovaara, A., Almeida, A., & Pinto-Ribeiro, F. (2014a). A role of supraspinal galanin in behavioural hyperalgesia in the rat. *PloS one*, 9 (11), e113077.
- Amorim, D., David-Pereira, A., Pertovaara, A., Almeida, A., & Pinto-Ribeiro, F. (2014b). Amitriptyline reverses hyperalgesia and improves associated mood-like disorders in a model of experimental monoarthritis. *Behavioural brain research*, 265, 12-21.
- Apkarian, A. V., Baliki, M. N., & Geha, P. Y. (2009). Towards a theory of chronic pain. *Progress in neurobiology*, *87* (2), 81-97.
- Badilla, B., Cambronero, J., Cicció, J. F., Cordero, T., & Mora, G. (2007). Determination of topical anti-inflammatory activity of the essential oil and extracts of Lippia alba (Mill.) NE Brown (Verbenaceae), using the model of mouse ear edema induced by TPA and AA. *Pharmacognosy Magazine*, *3* (11), 139.

- Baliga, M. S., Jagetia, G. C., Rao, S. K., & Babu, S. (2003). Evaluation of nitric oxide scavenging activity of certain spices in vitro: a preliminary study. *Molecular Nutrition & Food Research*, 47 (4), 261-264.
- Barton, N. J., Strickland, I. T., Bond, S. M., Brash, H. M., Bate, S. T., Wilson, A. W., Chessell, I. P., Reeve, A. J. & McQueen, D. S. (2007). Pressure application measurement (PAM): a novel behavioural technique for measuring hypersensitivity in a rat model of joint pain. *Journal of neuroscience methods*, 163 (1), 67-75.
- Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell*, *139* (2), 267-284.
- Bendele, A. M. (2001). Animal models of osteoarthritis. *J Musculoskelet Neuronal Interact*, 1 (4), 363-376.
- Bonnet, C. S., & Walsh, D. A. (2005). Osteoarthritis, angiogenesis and inflammation. *Rheumatology*, 44 (1), 7-16.
- Boots, A. W., Haenen, G. R., & Bast, A. (2008). Health effects of quercetin: from antioxidant to nutraceutical. *European journal of pharmacology*, *585* (2), 325-337.
- Bozkurt, A., Scheffel, J., Brook, G. A., Joosten, E. A., Suschek, C. V., O'Dey, D. M., Pallua, N., & Deumens, R. (2011). Aspects of static and dynamic motor function in peripheral nerve regeneration: SSI and CatWalk gait analysis. *Behavioural brain research*, 219 (1), 55-62.
- Brown, A. P., Dinger, N., & Levine, B. S. (2000). Stress produced by gavage administration in the rat. *Journal of the American Association for Laboratory Animal Science*, *39* (1), 17-21.
- Bultink, I. E., & Lems, W. F. (2013). Osteoarthritis and Osteoporosis: What Is the Overlap?. *Current rheumatology reports*, 15 (5), 328.
- Chen, J., & Pawliszyn, J. B. (1995). Solid phase microextraction coupled to high-performance liquid chromatography. *Analytical Chemistry*, 67 (15), 2530-2533.
- Cheng, H. T. (2010). Spinal cord mechanisms of chronic pain and clinical implications. *Current pain and headache reports*, *14* (3), 213-220.
- Cheuk, Y. C., Fu, S. C., Mok, S. W., Ho, K. K. W., Hung, L. K., & Chan, K. M. (2016). Intra-articular injection of an antioxidant formulation did not improve structural degeneration in a rat model of post-traumatic osteoarthritis. *Journal of Orthopaedic Translation*, 8, 25-31.
- Colle, D., Arantes, L. P., Rauber, R., de Mattos, S. E. C., Rocha, J. B. T. D., Nogueira, C. W., & Soares, F. A. A. (2012). Antioxidant properties of Taraxacum officinale fruit extract are

involved in the protective effect against cellular death induced by sodium nitroprusside in brain of rats. *Pharmaceutical biology*, 50 (7), 883-891.

- Creamer, P., Lethbridge-Cejku, M., & Hochberg, M. C. (1998). Where does it hurt? Pain localization in osteoarthritis of the knee. *Osteoarthritis and Cartilage*, *6* (5), 318-323.
- Cui, Z., Xu, C., Li, X., Song, J., & Yu, B. (2015). Treatment with recombinant lubricin attenuates osteoarthritis by positive feedback loop between articular cartilage and subchondral bone in ovariectomized rats. *Bone*, 74, 37-47.
- Da Silva Filho, A. A., Resende, D. O., Fukui, M. J., Santos, F. F., Pauletti, P. M., Cunha, W. R., Silva, M.L.A., Gregório, L.E., Bastos, J.K., & Nanayakkara, N. P. D. (2009). In vitro antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* DC (Asteraceae). *Fitoterapia*, 80 (8), 478-482.
- David-Pereira, A., Puga, S., Goncalves, S., Amorim, D., Silva, C., Pertovaara, A., Almeida, A. & Pinto-Ribeiro, F. (2016). Metabotropic glutamate 5 receptor in the infralimbic cortex contributes to descending pain facilitation in healthy and arthritic animals. *Neuroscience*, 312, 108-119.
- Di Giminiani, P., Sandercock, D. A., Malcolm, E. M., Leach, M. C., Herskin, M. S., & Edwards, S. A. (2016). Application of a handheld Pressure Application Measurement device for the characterisation of mechanical nociceptive thresholds in intact pig tails. *Physiology & behavior*, 165, 119-126.
- Dias, A. C. P., Seabra, R. M., Andrade, P. B., & Fernandes-Ferreira, M. (1999). The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from in vivo and in vitro biomass of Hypericum species. *Journal of liquid chromatography & related technologies*, *22* (2), 215-227.
- Dos Santos, D. A., Fukui, M. D. J., Nanayakkara, N. D., Khan, S. I., Sousa, J. P. B., Bastos, J. K., Andrade, S. F., Da Silva Filho, A. A., & Quintão, N. L. (2010). Anti-inflammatory and antinociceptive effects of Baccharis dracunculifolia DC (Asteraceae) in different experimental models. *Journal of ethnopharmacology*, 127 (2), 543-550.
- Duarte, M. C. T., Figueira, G. M., Sartoratto, A., Rehder, V. L. G., & Delarmelina, C. (2005). Anti-Candida activity of Brazilian medicinal plants. *Journal of ethnopharmacology*, 97 (2), 305-311.
- Dubin, A. E., & Patapoutian, A. (2010). Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation*, *120* (11), 3760.

- Dudonné, S., Vitrac, X., Coutière, P., Woillez, M., & Mérillon, J. M. (2009). Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of agricultural and food chemistry*, 57 (5), 1768-1774.
- Ebrahimzadeh, M. A., Pourmorad, F., & Bekhradnia, A. R. (2008). Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. *African Journal of Biotechnology*, 7 (18), 3188.
- Ferguson, H. C. (1962). Dilution of dose and acute oral toxicity. *Toxicology and applied pharmacology*, *4* (6), 759-762.
- Ferracini, V. L., Paraiba, L. C., Leitão Filho, H. F., Da Silva, A. G., Nascimento, L. R., & Marsaioli, A. J. (1995). Essential oils of seven Brazilian Baccharis species. *Journal of Essential Oil Research*, 7 (4), 355-367.
- Ferreira-Gomes, J., Adães, S., & Castro-Lopes, J. M. (2008). Assessment of movement-evoked pain in osteoarthritis by the knee-bend and CatWalk tests: a clinically relevant study. *The Journal of Pain*, *9* (10), 945-954.
- Ferreira-Gomes, J., Adães, S., Mendonça, M., & Castro-Lopes, J. M. (2012). Analgesic effects of lidocaine, morphine and diclofenac on movement-induced nociception, as assessed by the Knee-Bend and CatWalk tests in a rat model of osteoarthritis. *Pharmacology Biochemistry and Behavior*, 101 (4), 617-624.
- Freire, M. D. F. I. (2004). Plantas medicinais: a importância do saber cultivar. *Revista Científica Eletrônica Agronomia,* ano III, (5), 1-9.
- Funari, C. S., de Oliveira Ferro, V., & Mathor, M. B. (2007). Analysis of propolis from Baccharis dracunculifolia DC.(Compositae) and its effects on mouse fibroblasts. *Journal of ethnopharmacology*, 111 (2), 206-212.
- Gabriel, A. F., Marcus, M. A. E., Honig, W. M. M., Walenkamp, G. H. I. M., & Joosten, E. A. J. (2007). The CatWalk method: a detailed analysis of behavioral changes after acute inflammatory pain in the rat. *Journal of neuroscience methods*, 163 (1), 9-16.
- Goleniowski, M. E., Bongiovanni, G. A., Palacio, L., Nuñez, C. O., & Cantero, J. J. (2006). Medicinal plants from the "Sierra de Comechingones", Argentina. *Journal of ethnopharmacology*, *107* (3), 324-341.
- Gregory, M. H., Capito, N., Kuroki, K., Stoker, A. M., Cook, J. L., & Sherman, S. L. (2012). A review of translational animal models for knee osteoarthritis. *Arthritis*, 2012.

- Guimarães, N. S. S., Mello, J. C., Paiva, J. S., Bueno, P. C. P., Berretta, A. A., Torquato, R. J., Nantes, I. L., & Rodrigues, T. (2012). *Baccharis dracunculifolia,* the main source of green propolis, exhibits potente antioxidant activity and prevents oxidative mitochondrial damage. *Food and Chemical Toxicology,* 50 (3), 1091-1097.
- Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine*, 27 (1), 1-93.
- Guzman, R. E., Evans, M. G., Bove, S., Morenko, B., & Kilgore, K. (2003). Mono-iodoacetate-induced histologic changes in subchondral bone and articular cartilage of rat femorotibial joints: an animal model of osteoarthritis. *Toxicologic Pathology*, *31* (6), 619-624.
- Hamers, F. P., Koopmans, G. C., & Joosten, E. A. (2006). CatWalk-assisted gait analysis in the assessment of spinal cord injury. *Journal of neurotrauma*, *23* (3-4), 537-548.
- Hanson, P. M., Yang, R. Y., Tsou, S. C., Ledesma, D., Engle, L., & Lee, T. C. (2006). Diversity in eggplant (Solanum melongena) for superoxide scavenging activity, total phenolics, and ascorbic acid. *Journal of Food composition and Analysis*, 19 (6), 594-600.
- Hayes, R., Ahmed, A., Edge, T., & Zhang, H. (2014). Core–shell particles: Preparation, fundamentals and applications in high performance liquid chromatography. *Journal of chromatography A*, 1357, 36-52.
- Hübner, G., Hu, Q., Smola, H., & Werner, S. (1996). Strong induction of activin expression after injury suggests an important role of activin in wound repair. *Developmental biology*, *173* (2), 490-498.
- Huda-Faujan, N., Noriham, A., Norrakiah, A. S., & Babji, A. S. (2009). Antioxidant activity of plants methanolic extracts containing phenolic compounds. *African Journal of Biotechnology*, 8 (3), 484-489.
- Ishikawa, M., Ohdo, S., Watanabe, H., Hara, C., & Ogawa, N. (1995). Alteration in circadian rhythm of plasma corticosterone in rats following sociopsychological stress induced by communication box. *Physiology & behavior*, *57* (1), 41-47.
- Jahanshahi, G., Motavasel, V., Rezaie, A., Hashtroudi, A. A., Daryani, N. E., & Abdollahi, M. (2004). Alterations in antioxidant power and levels of epidermal growth factor and nitric oxide in saliva of patients with inflammatory bowel diseases. *Digestive diseases and sciences*, 49 (11), 1752-1757.

- Ji, Q., Wang, P., & He, C. (2016). Extracorporeal shockwave therapy as a novel and potential treatment for degenerative cartilage and bone disease: Osteoarthritis. A qualitative analysis of the literature. *Progress in Biophysics and Molecular Biology*, 121 (3), 255-265.
- Julius, D., & Basbaum, A. I. (2001). Molecular mechanisms of nociception. *Nature*, *413* (6852), 203.
- Junior, V. F. V., Pinto, A. C., & Maciel, M. A. M. (2005). Plantas medicinais: cura segura. *Química nova*, 28 (3), 519-528.
- Koopmans, G. C., Deumens, R., Honig, W. M., Hamers, F. P., Steinbusch, H. W., & Joosten, E. A. (2005). The assessment of locomotor function in spinal cord injured rats: the importance of objective analysis of coordination. *Journal of neurotrauma*, *22*(2), 214-225.
- Korhonen, R., Lahti, A., Kankaanranta, H., & Moilanen, E. (2005). Nitric oxide production and signaling in inflammation. *Current Drug Targets-Inflammation & Allergy*, 4 (4), 471-479.
- Korntner, S., Kunkel, N., Lehner, C., Gehwolf, R., Wagner, A., Augat, P., Stephan, D., Heu, V., Bauer, H., Traweger, A & Tempfer, H. (2017). A high-glucose diet affects Achilles tendon healing in rats. *Scientific Reports*, *7*.
- Kumazawa, S., Yoneda, M., Shibata, I., Kanaeda, J., Hamasaka, T., & Nakayama, T. (2003). Direct evidence for the plant origin of Brazilian propolis by the observation of honeybee behavior and phytochemical analysis. *Chemical and Pharmaceutical Bulletin*, *51* (6), 740-742.
- Lamprea, M. R., Cardenas, F. P., Setem, J., & Morato, S. (2008). Thigmotactic responses in an open-field. *Brazilian Journal of Medical and Biological Research*, 41 (2), 135-140.
- Lasota, A., & Danowska-Klonowska, D. (2004). Experimental osteoporosis-different methods of ovariectomy in female white rats. *Rocz Akad Med Bialymst*, 49 (Suppl 1), 129-131.
- Lemos, M., Barros, M. P., Sousa, J. P. B., Filho, A. A. S., Bastos, J. K., & Andrade, S. F. (2007). Baccharis dracunculifolia, the main botanical source of Brazilian green propolis, displays antiulcer activity. *Journal of Pharmacy and Pharmacology*, 59 (4), 603-608.
- Linley, J. E., Rose, K., Ooi, L., & Gamper, N. (2010). Understanding inflammatory pain: ion channels contributing to acute and chronic nociception. *Pflügers Archiv European Journal of Physiology*, *459* (5), 657-669.
- Little, C. B., & Zaki, S. (2012). What constitutes an "animal model of osteoarthritis"–the need for consensus?. *Osteoarthritis and Cartilage*, 20 (4), 261-267.
- Marchand, F., Perretti, M., & McMahon, S. B. (2005). Role of the immune system in chronic pain. *Nature Reviews Neuroscience*, *6* (7), 521-532.

- Massignani, J. J., Lemos, M., Maistro, E. L., Schaphauser, H. P., Jorge, R. F., Sousa, J. P. B., Bastos, J. K. & de Andrade, S. F. (2009). Antiulcerogenic activity of the essential oil of Baccharis dracunculifolia on different experimental models in rats. *Phytotherapy research*, *23* (10), 1355-1360.
- McGuire, B. E., & Kennedy, S. (2013). Pain in people with an intellectual disability. *Current opinion in psychiatry*, *26* (3), 270-275.
- Medzhitov, R. (2008). Origin and physiological roles of inflammation. *Nature*, *454* (7203), 428-435.
- Merksey, H., & Bogduk, N. (1994). Classification of chronic pain. Second ed, Seattle: International association metabotropic glutamate receptors in nerve-injured animals. *Exp Neurol,* 216, 66-74.
- Messent, E. A., Ward, R. J., Tonkin, C. J., & Buckland-Wright, C. (2005). Tibial cancellous bone changes in patients with knee osteoarthritis. A short-term longitudinal study using Fractal Signature Analysis. *Osteoarthritis and cartilage*, *13* (6), 463-470.
- Messier, S. P. (1994). Osteoarthritis of the knee and associated factors of age and obesity: effects on gait. *Medicine and Science in Sports and Exercise*, *26* (12), 1446-1452.
- Meyr, A. J., & Steinberg, J. S. (2008). The physiology of the acute pain pathway. *Clinics in Podiatric medicine and surgery*, *25* (3), 305-326.
- Michalak, A. (2006). Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Polish Journal of Environmental Studies*, 15 (4), 523-530.
- Missima, F., da Silva Filho, A. A., Nunes, G. A., Bueno, P. C. P., Sousa, J. P. B., Bastos, J. K., & Sforcin, J. M. (2006). Effect of *Baccharis dracunculifolia* D.C. (Asteraceae) extracts and its isolated compounds on macrophage activation. *Journal of Pharmacy and Pharmacology*, 59 (3), 463-468.
- Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol*, 26 (2), 211-219.
- Monteiro, S. C., Matté, C., Bavaresco, C. S., Netto, C. A., & Wyse, A. T. (2005). Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. *Neurobiology of learning and memory*, 84 (3), 192-199.
- Moriyama, H., Yoshimura, O., Kawamata, S., Takayanagi, K., Kurose, T., Kubota, A., Hosoda, M., & Tobimatsu, Y. (2008). Alteration in articular cartilage of rat knee joints after spinal cord injury. *Osteoarthritis and Cartilage*, 16 (3), 392-398.

- Muramatsu, Y., Sasho, T., Saito, M., Yamaguchi, S., Akagi, R., Mukoyama, S., Akatsu, Y., Katsuragi, J., Fukawa, T., Endo, J., Hoshi, H., Yamamoto, Y., & Takahashi, K. (2014). Preventive effects of hyaluronan from deterioration of gait parameters in surgically induced mice osteoarthritic knee model. *Osteoarthritis and Cartilage*, 22 (6), 831-835.
- Nehir El, S., & Karakaya, S. (2004). Radical scavenging and iron-chelating activities of some greens used as traditional dishes in Mediterranean diet. *International Journal of Food Sciences and Nutrition*, 55 (1), 67-74.
- Neugebauer, V., Han, J. S., Adwanikar, H., Fu, Y., & Ji, G. (2007). Techniques for assessing knee joint pain in arthritis. *Molecular Pain*, 3 (1), 8.
- Nickerson, D. F., Weaver, M. L., & Tse, F. L. (1994). The effect of oral dose volume on the absorption of a highly and a poorly water soluble drug in the rat. *Biopharmaceutics & drug disposition*, *15* (5), 419-429.
- Okoli, C. O., Akah, P. A., & Nwafor, S. V. (2003). Anti-inflammatory activity of plants. *Journal of Natural Remedies*, 3 (1), 1-30.
- Oliveira, J. M., Kotobuki, N., Tadokoro, M., Hirose, M., Mano, J. F., Reis, R. L., & Ohgushi, H. (2010). Ex vivo culturing of stromal cells with dexamethasone-loaded carboxymethylchitosan/poly (amidoamine) dendrimer nanoparticles promotes ectopic bone formation. *Bone*, 46 (5), 1424-1435.
- Ozeki, N., Muneta, T., Koga, H., Nakagawa, Y., Mizuno, M., Tsuji, K., Mabuchi, Y., Akazawa, C., Kobayashi, E., Matsumoto, K., Futamura, K., Saito, T., & Sekiya, I. (2016). Not single but periodic injections of synovial mesenchymal stem cells maintain viable cells in knees and inhibit osteoarthritis progression in rats. *Osteoarthritis and Cartilage*, 24 (6), 1061-1070.
- Pacheco, N. R., Pinto, N. D. C. C., Mello da Silva, J., Mendes, R. D. F., Costa, J. D. C. D., Aragão, D. M. D. O., Castañon, M. N., & Scio, E. (2014). Cecropia pachystachya: a species with expressive in vivo topical anti-inflammatory and in vitro antioxidant effects. *BioMed research international*, *2014*.
- Pandareesh, M. D., & Anand, T. (2014). Neuroprotective and anti-apoptotic propensity of Bacopa monniera extract against sodium nitroprusside induced activation of iNOS, heat shock proteins and apoptotic markers in PC12 cells. *Neurochemical research*, 39 (5), 800-814.
- Park, Y. K., Alencar, S. M., & Aguiar, C. L. (2002). Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry*, *50* (9), 2502-2506.

- Park, Y. K., Paredes-Guzman, J. F., Aguiar, C. L., Alencar, S. M., & Fujiwara, F. Y. (2004). Chemical constituents in Baccharis dracunculifolia as the main botanical origin of southeastern Brazilian propolis. *Journal of Agricultural and Food Chemistry*, 52 (5), 1100-1103.
- Parreira, N. A., Magalhães, L. G., Morais, D. R., Caixeta, S. C., Sousa, J. P. B., Bastos, J. K., Cunha, W. R., Silva, M. L. A., Nanayakkara, N. P. D., Rodrigues, V., & da Silva Filho, A. A. (2010). Antiprotozoal, Schistosomicidal, and Antimicrobial Activities of the Essential Oil from the Leaves of *Baccharis dracunculifolia. Chemistry & Biodiversity*, 7 (4), 993-1001.
- Paulino, N., Abreu, S. R. L., Uto, Y., Koyama, D., Nagasawa, H., Hori, H., Dirsch, V. M., Vollmar, A. M., Scremin, A., & Bretz, W. A. (2008). Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. *European Journal of Pharmacology*, 587 (1), 296-301.
- Persson, A. K., Gebauer, M., Jordan, S., Metz-Weidmann, C., Schulte, A. M., Schneider, H. C., Pfennigdorff, D. D., Thun, J., Xu, X. J., Hallin, Z. W., Darvasi, A., Fried, K., & Devor, M. (2009). Correlational analysis for identifying genes whose regulation contributes to chronic neuropathic pain. *Molecular pain*, *5* (1), 7.
- Phanse, M. A., Patil, M. J., Abbulu, K., Chaudhari, P. D., & Patel, B. (2012). In-vivo and in-vitro screening of medicinal plants for their anti-inflammatory activity: an overview.
- Podsiadlo, P., Dahl, L., Englund, M., Lohmander, L. S., & Stachowiak, G. W. (2008). Differences in trabecular bone texture between knees with and without radiographic osteoarthritis detected by fractal methods. *Osteoarthritis and Cartilage*, *16* (3), 323-329.
- Prakash, P., & Gupta, N. (2005). Therapeutic uses of *Ocimum sanctum Linn* (Tulsi) with a note on eugenol and its pharmacological actions: a short review. *Indian journal of physiology and pharmacology*, 49 (2), 125-131.
- Radhakrishnan, R., Moore, S. A., & Sluka, K. A. (2003). Unilateral carrageenan injection into muscle or joint induces chronic bilateral hyperalgesia in rats. *Pain*, 104 (3), 567-577.
- Rafieian-Kopaei, M. (2012). Medicinal plants and the human needs. *Journal of HerbMed Pharmacology*, 1 (1), 1-2.
- Randall, L. O., & Selitto, J. J. (1957). A method for measurement of analgesic activity on inflamed tissue. *Archives internationales de pharmacodynamie et de therapie*, *111* (4), 409.
- Redondo-Castro, E., Torres-Espín, A., García-Alías, G., & Navarro, X. (2013). Quantitative assessment of locomotion and interlimb coordination in rats after different spinal cord injuries. *Journal of neuroscience methods*, 213 (2), 165-178.

- Rezende, T. P., Corrêa, J. O. A., Aarestrup, B. J. V., Aarestrup, F. M., Sousa, O. V., & da Silva Filho, A. A. (2014). Protective Effects of *Baccharis dracunculifolia* Leaves Extract against Carbon Tetrachloride- and Acetaminophen-Induced Hepatotoxicity in Experimental Animals. *Molecules*, 19 (7), 9257-9272.
- Ribeiro, R. A., Vale, M. L., Thomazzi, S. M., Paschoalato, A. B., Poole, S., Ferreira, S. H., & Cunha, F. Q. (2000). Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *European journal of pharmacology*, *387* (1), 111-118.
- Riemer, J., Hoepken, H. H., Czerwinska, H., Robinson, S. R., & Dringen, R. (2004). Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Analytical biochemistry*, 331 (2), 370-375.
- Rivera, E. A. B. (2002). Ética na experimentação animal. *Animais de Laboratório: criação e Experimentação,* 30 (1), 9-14.
- Rodrigues, A. C. C., & Guedes, M. L. S. (2006). Utilização de plantas medicinais no Povoado Sapucaia, Cruz das Almas–Bahia. *Revista Brasileira de Plantas Medicinais*, 8 (2), 1-7.
- Rodrigues, K., Chibli, L. A., Santos, B., Temponi, V. S., Pinto, N. C., Scio, E., Del-Vechio-Vieira, G, Alves, M. S. & Sousa, O. V. (2016). Evidence of bioactive compounds from vernonia polyanthes leaves with topical anti-inflammatory potential. *International journal of molecular sciences*, *17* (12), 1929.
- Rodriguez, M. V., MartÝnez, M. L., Cortadi, A. A., Bandoni, A., Giuliano, D. A., Gattuso, S. J., & Gattuso, M. A. (2010). Characterization of three sect. Caulopterae species (Baccharis-Asteraceae) inferred from morphoanatomy, polypeptide profiles and spectrophotometry data. *Plant systematics and evolution*, *286* (3-4), 175-190.
- Rojas-Ortega, M., Cruz, R., Vega-López, M. A., Cabrera-González, M., Hernández-Hernández, J. M., Lavalle-Montalvo, C., & Kouri, J. B. (2015). Exercise modulates the expression of IL-1β and IL-10 in the articular cartilage of normal and osteoarthritis-induced rats. *Pathology-Research and Practice*, 211 (6), 435-443.
- Russo, A., Cardile, V., Lombardo, L., Vanella, L., Vanella, A., & Garbarino, J. A. (2005). Antioxidant activity and antiproliferative action of methanolic extract of Geum quellyon Sweet roots in human tumor cell lines. *Journal of ethnopharmacology*, 100 (3), 323-332.

- Saha, K., Lajis, N. H., Israf, D. A., Hamzah, A. S., Khozirah, S., Khamis, S., & Syahida, A. (2004). Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *Journal of Ethnopharmacology*, 92 (2), 263-267.
- Schmitt, U., & Hiemke, C. (1998). Strain differences in open-field and elevated plus-maze behavior of rats without and with pretest handling. *Pharmacology Biochemistry and Behavior*, 59 (4), 807-811.
- Schmitz, N., Laverty, S., Kraus, V. B., & Aigner, T. (2010). Basic methods in histopathology of joint tissues. *Osteoarthritis and cartilage*, 18, S113-S116.
- Scipio, F. D., Raimondo, S., Tos, P., & Geuna, S. (2008). A simple protocol for paraffin□ embedded myelin sheath staining with osmium tetroxide for light microscope observation. *Microscopy research and technique*, *71* (7), 497-502.
- Serhan, C. N., Chiang, N., & Van Dyke, T. E. (2008). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nature Reviews Immunology*, *8* (5), 349-361.
- Sforcin, J. M., Sousa, J. P. B. D., Silva Filho, A. A. D., Bastos, J. K., Búfalo, M. C., & Tonuci, L. R. D. S. (2012). Baccharis dracunculifolia: Uma das principais fontes vegetais da própolis brasileira.
- Silva, B. A., Malva, J. O., & Dias, A. C. (2008). St. John's Wort (Hypericum perforatum) extracts and isolated phenolic compounds are effective antioxidants in several in vitro models of oxidative stress. *Food Chemistry*, 110 (3), 611-619.
- Silva, C. G., Herdeiro, R. S., Mathias, C. J., Panek, A. D., Silveira, C. S., Rodrigues, V. P., Rennó, M. N., Falcão, D. Q., Cerqueira, D. M., Minto, A. B. M., Quaresma, C. H., Silva, J. F. M., Menezes, F. S., Eleutherio, E. C. A., & Nogueira, F. L. P. (2005). Evaluation of antioxidant activity of Brazilian plants. *Pharmacological Research*, 52 (3), 229-233.
- Silva, J. M., Conegundes, J. L. M., Mendes, R. D. F., Pinto, N. D. C. C., Gualberto, A. C. M., Ribeiro, A., Gameiro, J., Aguiar, J. A. K., Castañon, M. C. M. N., & Scio, E. (2015). Topical application of the hexane fraction of Lacistema pubescens reduces skin inflammation and cytokine production in animal model. *Journal of Pharmacy and Pharmacology*, 67 (11), 1613-1622.
- Sousa, J. P. B. D. (2007). Influência da sazonalidade no perfil químico dos óleos essenciais e das substâncias fixas de Baccharis dracunculifolia cultivada, utilizando-se cromatografia em fases gasosa e líquida. *(Doctoral dissertation, Universidade de São Paulo).*

- Stanley, P. L., Steiner, S., Havens, M., & Tramposch, K. M. (1991). Mouse skin inflammation induced by multiple topical applications of 12-O-tetradecanoylphorbol-13-acetate. *Skin Pharmacology and Physiology*, *4* (4), 262-271.
- Şükür, E., Talu, C., Akman, Y. E., Çirci, E., Öztürkmen, Y., & Tüzüner, T. (2016). Comparison of the chondroprotective effect of a novel hydrogel compound and traditional hyaluronate on rat cartilage in a papain-induced osteoarthritis model. *Acta Orthopaedica et Traumatologica Turcica*, 50 (4), 458-463.
- Treede, R. D., Rolke, R., Andrews, K., & Magerl, W. (2002). Pain elicited by blunt pressure: neurobiological basis and clinical relevance. *Pain*, *98* (3), 235-240.
- Valentão, P., Fernandes, E., Carvalho, F., Andrade, P. B., Seabra, R. M., & Bastos, M. L. (2001). Antioxidant activity of Centaurium erythraea infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. *Journal of Agricultural and Food Chemistry*, 49 (7), 3476-3479.
- Valentão, P., Fernandes, E., Carvalho, F., Andrade, P. B., Seabra, R. M., & Bastos, M. L. (2002a). Antioxidative properties of cardoon (Cynara cardunculus L.) infusion against superoxide radical, hydroxyl radical, and hypochlorous acid. *Journal of Agricultural and Food Chemistry*, 50 (17), 4989-4993.
- Valentão, P., Fernandes, E., Carvalho, F., Andrade, P. B., Seabra, R. M., & de Lourdes Bastos, M. (2002b). Studies on the antioxidant activity of Lippia citriodora infusion: scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid. *Biological and Pharmaceutical Bulletin*, 25 (10), 1324-1327.
- Veiga-Junior, V. F. D. (2008). Estudo do consumo de plantas medicinais na Região Centro-Norte do Estado do Rio de Janeiro: aceitação pelos profissionais de saúde e modo de uso pela população. *Revista Brasileira de Farmacognosia,* 18 (2), 308-313.
- Voscopoulos, C., & Lema, M. (2010). When does acute pain become chronic?. *British journal of anaesthesia*, *105* (suppl_1), i69-i85.
- Wang, X. H., Lu, G., Hu, X., Tsang, K. S., Kwong, W. H., Wu, F. X., Meng, H. W., Jiang, S., Liu, S. W. & Poon, W. S. (2012). Quantitative assessment of gait and neurochemical correlation in a classical murine model of Parkinson's disease. *BMC neuroscience*, *13* (1), 142.
- Wang, Y. X. J., Wang, J., Deng, M., Liu, G., & Qin, L. (2015). In vivo three-dimensional magnetic resonance imaging of rat knee osteoarthritis model induced using meniscal transection. *Journal of Orthopaedic Translation*, 3 (3), 134-141.

- Wilson, J. H., McKinley, S. A., & Young, B. L. (2000). Prolactin levels in juvenile and adult rats following acute restraint and the open field. *Physiology & behavior*, 68 (3), 383-387.
- Zhang, W., Nuki, G., Moskowitz, R. W., Abramson, S., Altman, R. D., Arden, N. K., Bierma-Zeinstra, S., Brandt, K. D., Croft, P., Doherty, M., Hochberg, M., Hunter, D. J., Kwoh, K., Lohmander, L. S., Tugwell, P. & Dougados, M. (2010). OARSI recommendations for the management of hip and knee osteoarthritis. *Osteoarthritis and Cartilage*, *18* (4), 476-499.
- Zhang, X. (1998). Regulatory situation of herbal medicines a worldwide review. *World Health Organization*, 26, 223-228.

ANNEX

Annex 1

Pharmacological potential of *Baccharis dracunculifolia* in the treatment of osteoarthritis and its emotional-like comorbidities

E. Apolinário [1,2,3,#], I. M. Laranjeira [1,2,3,#], D. Amorim [2,3], F. Pinto-Ribeiro [2,3]; Alberto Dias [1]

1 CITAB–Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

2 Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

3 ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

 # Authors contributed equally to the work

Osteoarthritis (OA) is the most common and debilitating form of arthritis, affecting 40 million people in Europe, especially the elderly. OA is characterized by progressive degeneration of tissues within and surrounding weight-bearing joints and recurrent inflammatory episodes leading to structural changes including cartilage erosion, fibrillation and decreased thickness of articular cartilage.

Baccharis dracunculifolia DC (Bd) is a medicinal Brazilian shrub, popularly known as "alecrim do campo", known for its anti-stress and anti-inflammatory activities.

In our work, OA was induced in 8-weeks old female ovariectomized Wistar rats weighting between 167-245 grams. Animals were divided in 4 groups (n=6 each), (i) SHAM animals, (ii) OA animals, (iii) OA treated with Bd extract (50mg/Kg) and (iv) OA treated with Bd extract (100 mg/Kg).

Four weeks after OA induction, Bd extracts (50 and 100 mg/Kg) were administrated by gavage for a period of 30 days. At the end of this period animals were assessed for changes in gait (catwalk test), locomotor ability (open-field test), knee circumference, anxiety- (open-field test), and depressive-like (forced swimming and sucrose preference tests) impairments.

Our results showed treatment with Bd improved animals motor performances, especially in the group treated with the 100 mg/kg. Emotional-like comorbidities were also partially reversed.

Poster presented in:

- XIX National Congress of Biochemistry; Guimarães, Portugal.

Pharmacological potential of *Baccharis dracunculifolia* in the control of osteoarthritis and its emotional-like comorbidities

Apolinário, E.[1,2,3#]; Laranjeira, I.M.[1,2,3#]; Amorim, D.[2,3]; Pinto-Ribeiro, F.[2,3]; Dias, A.C.P.[1]
[1]CITAB—Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[2]Life and Health Sciences Research Institute (ICVS), School of Medicine, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal
[3]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
# authors contributed equally to the work being presented.

Introduction

Osteoarthritis (OA) is a debilitating form of arthritis, especially in elderly. OA is characterized by progressive degeneration of tissues leading to structural changes including cartilage erosion. It is estimated patients suffering from chronic pain also express clinically diagnosable symptoms of depression
Baccharis dracunculifolia DC (Bd) is a medicinal Brazilian shrub, known for its anti-stress and anti-inflammatory activities

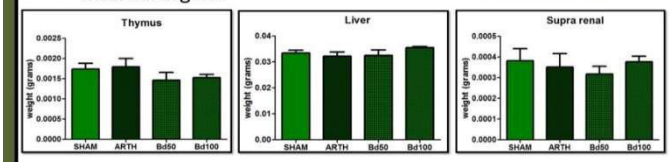
Materials and methods

OA was induced in 8-weeks old female ovariectomized Wistar rats weighting between 167-245 grams.
Animals were divided in 4 groups (n=5 each), (i) SHAM animals, (ii) OA animals, (iii) OA treated with Bd extract (50mg/Kg) and (iv) OA treated with Bd extract (100 mg/Kg).
Four weeks after OA induction, Bd extracts (50 and 100 mg/Kg) were administrated by gavage for a period of 30 days.
Statistical analysis was performed using the GraphPad Prism tool, the significant differences were indicated by * P <0.05, ** P <0.005, *** P <0.001.

Results

❖Animal welfare

- Internal organs



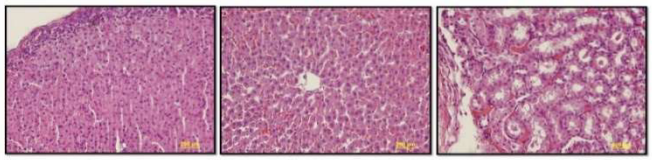
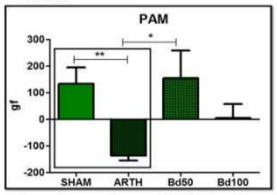


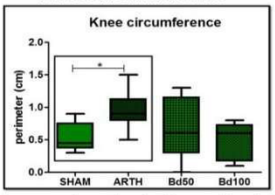
Figure 1. Photomicrophs of thymus, liver and kidney slides (400x magnification).

❖Locomotive ability

- PAM



- Knee circumference



Induction of **experimental OA** significantly **increased mechanical hyperalgesia** in OA animals ($t_{(8)}$=4.164, P=0.0031) and **altered right knee perimeters** ($t_{(8)}$=3.024, P=0.0165). Treatment with **Bd extract** significantly **decreased mechanical hyperalgesia** (ANOVAoneway: $F_{(3,19)}$=4.066, P=0.025) and **partially ameliorated knee swelling** (ANOVAone-way: $F_{(3,19)}$=2.525; P=0.0944).

- Inibition of cartilage degradation by extract treatment

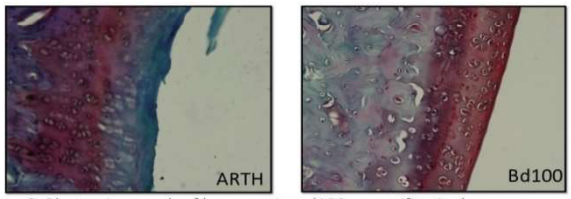
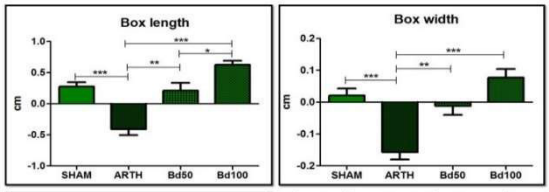
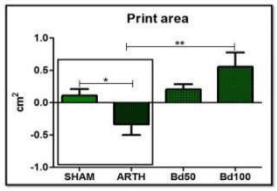


Figure 2. Photomicrograph of knee sections (100x magnification).

- Catwalk test - coordination and locomotor ability

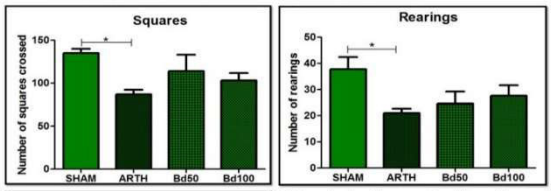


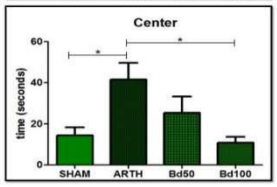


Identically, **OA induction altered** both **Box length** ($t_{(8)}$=5.794, P=0.0004; ANOVAone-way: $F_{(3,19)}$=22.09, P<0.0001), **Box width** ($t_{(8)}$=5.560, P=0.0005; ANOVAone-way: $F_{(3,19)}$=15.44; P<0,0001) and the **paw print area** between SHAM and ARTH ($t_{(8)}$=2.312, : P=0.0495; ANOVAone-way: $F_{(3,19)}$=5.705, P=0.0075). Importantly, post-hoc tests showed both **Bd extracts were able to reverse gait impairments.**

❖Emotional-like comorbidities

- Anxiety-like behaviour – Open-field test

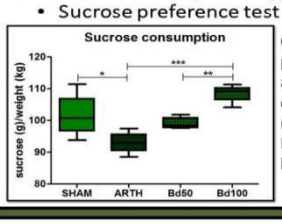




OA induction significantly altered the evaluated parameters between OA and sham animals. **Treatment partially reversed this effect** (**crossed squares**: $t_{(8)}$=6.915, P=0.0001 ANOVAone-way: $F_{(3,19)}$=3.338, P=0.0459; number of **rearings**: $t_{(8)}$=3.398, P=0.0094 ANOVAone-way: $F_{(3,19)}$=3.379, P=0.0444; **time in center**: $t_{(8)}$=3.014, P=0.0167 ANOVAone-way: $F_{(3,19)}$=5.052; P=0.0119, respectively).

❖ Anhedonic-like phenotype

- Sucrose preference test



OA induction significantly **decreased sucrose preference** ($t_{(8)}$=2.690, P=0.0275). Further analysis showed the administration of Bd extracts altered sucrose preference (ANOVAone-way: $F_{(3,19)}$=13.62; P=0.0001). Post-hoc test showed **Bd100 and Bd50**, to a lesser extend, **reversed anhedonia.**

Conclusion

- There are **no macroscopic or microscopic changes in the internal organs**, nor changes in the **weight** induced by the treatment.
- OA induction induced erosion and remodeling of cartilage, chondrocyte column disorganization and changes in the tidemark. Treatment with **Bd extract decreased cartilage degradation.**
- Treatment with **Bd extracts improved** the animals' **motor performance, inflammation** and **nociception**, especially in the group Bd 100 mg/kg.
- **Emotional-like comorbidities** were also at least **partially reversed** by treatment with Bd extracts.
- Future studies should evaluate the impact of the treatment for longer periods of administration and its impact in the progress of osteoarthritis.

Acknowledgements

This study was supported by grants from the Portuguese North Regional Operational Program (ON.2 - O Novo Norte) under the National Strategic Reference Framework (QREN), through the European Regional Development Fund (FEDER).

Annex 2:

Baccharis dracunculifolia decreases nociception, depressive-like behaviour and supraspinal activated microglia in rats with experimental monoarthritis

I. M. Laranjeira [1,2,3,#], E. Apolinário [1,2,3,#], D. Amorim [2,3], *Silva-Filho, A.A.*[4], F. Pinto-Ribeiro [2,3]; Alberto Dias [1]

1 CITAB–Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

2 Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

3 ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

4 Faculty of Pharmacy and Biochemistry, Pharmaceutical Department, Federal University of Juiz de Fora, Juiz de Fora, MG -Brazil

 # Authors contributed equally to the work

In arthritic disorders both inflammation and the progressive degeneration of joints persistently activate nociceptors, in periarticular structures, leading to the development of persistent pain and comorbid emotional impairments. Arthritis-induced peripheral sensitization leads to increased release of nociceptive molecules by primary afferents that activate neurones e glial cells in the spinal cord and supraspinal pain modulatory areas such as the amygdala (AMY) and the periaqueductal grey matter (PAG).

Baccharis dracunculifolia DC (Asteraceae) (Bd) is a medicinal shrub from the brazilian flora, popularly known as "Alecrim do Campo", considered to be an important source of active anti-inflammatory and antinociceptive compounds.

Adult 8 weeks old ovariectomized female rats (Rattus norvegicus, vr. Albinus, Wistar) weighting 210±17g were divided in four groups (n=6 per group): (i) SHAM, (ii) ARTH, (iii) ARTH treated with *B. dracunculifolia* (50mg/kg), and (iv) ARTH treated with *B. dracunculifolia* (100 mg/kg).

Mechanical hyperalgesia in ARTH animals was assessed using the pressure application measurement apparatus, anhedonia using the sucrose preference test and learned helplessness using the forced swimming test. Activated microglia was stained with IBA-I and quantified in a subset of brain slides containing the target areas, the amygdala and the periaqueductal gray

matter. A three-week oral treatment with Bd extract reversed ARTH-induced mechanical hyperalgesia and partly reserved depressive-like behaviour. Concomitantly, Bd treatment decreased the number of activated microglia in the AMY and PAG of ARTH animals.

Poster presented in:

XV Meeting of the Portuguese Society of Neurosciences (Braga, Portugal)

5th International Phytocosmetics and Phytotherapy Congress (Patras, Greece)

Oral presentation in:

65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (Basel, Switzerland)

Baccharis dracunculifolia decreases nociception, depressive-like behaviour and supraspinal activated microglia in rats with experimental monoarthritis

Laranjeira, I.M.[1,2,3]; Apolinário, E.[1,2,3]; Amorim, D.[2,3]; Silva-Filho, A.A.[4]; Pinto-Ribeiro, F.[2,3]; Dias, A.C.P.[1]

[1]CITAB—Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[2]Life and Health Sciences Research Institute (ICVS), School of Medicine, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal
[3]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[4]Faculty of Pharmacy and Biochemistry, Pharmaceutical Department, Federal University of Juiz de Fora, Juiz de Fora, MG - Brazil

Introduction

In arthritic disorders both inflammation and the **progressive degeneration of joints** persistently activate nociceptors, in periarticular structures, leading to the development of **persistent pain** and **comorbid emotional impairments**[1]. Several lines of evidence indicate arthritis-induced peripheral sensitization leads to increased release of nociceptive molecules by primary afferents that activate neurones e glial cells in the spinal cord and subsequently in supraspinal pain modulatory areas such as the amygdala (AMY) and the periaqueductal grey matter (PAG).

The first wave of glia response comprises the activation[2] and proliferation[3] of microglia. Acknowledging the role of **glial activation in pain disorders** holds significant promise for the improved management of pain disorders, and the development of novel pain control therapies[3].

A study by Santos and collaborators (2010)[4] confirmed the **anti-inflammatory properties** of ***Baccharis dracunculifolia*** (Bd) leaves. Additionally, this study also demonstrated Bd antinociceptive effect after acute and chronic oral administration in experimental models of chronic pain.

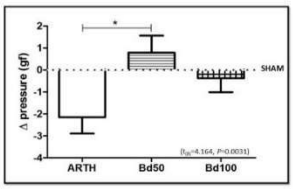
Methods

Subjects	• Adult female rats, var. Wistar han, ovariectomized
Animal model	• Kaolin/carrageenan experimental monoarthritis (ARTH)
Treatment	• Drug administration by gavage: - Bd extract - 50 mg/Kg (Bd50) - Bd extract – 100 mg/Kg (Bd100) - Vehicle solution - Phosphate buffer saline (ARTH and SHAM)
Behavior	• Forced swimming test – FST – learned helplessness • Sucrose preference test – SPT - anhedonia • Pressure application measurement – PAM – mechanical hyperalgesia
Histological confirmation	• Immunohistochemistry staining - activated microglia (IBA-I) - Amygdala (AMY) - Periaqueductal gray matter (PAG)
Statistical analysis	• GraphPad Prism software - Significant differences: * $P<0.05$, ** $P<0.005$, *** $P<0.001$

Objectives: **assessment** of the **effect** of oral administration of **Bd extract** upon **nociceptive** and comorbid **mood-like impairments** and the **activation** of supraspinal **microglia** in an experimental model of monoarthritis.

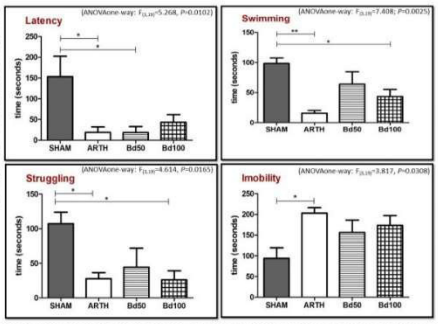
Results

PAM



ARTH significantly **increased mechanical hyperalgesia**, an effect **reversed** by treatment with **Bd extract** (ANOVAoneway: $F_{(3,16)}=4.066$, $P=0.025$)

FORCED SWIMMING TEST

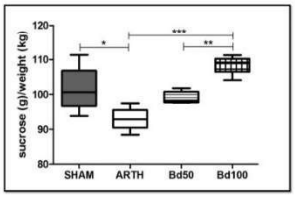


ARTH decreased:
- **latency** to immobility
- time spent **swimming**,
- time spend **struggling**

ARTH increased immobility

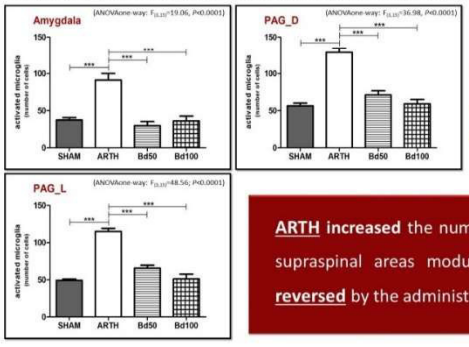
Bd extract partially reversed depression-like behaviour

SUCROSE PREFERENCE TEST



ARTH-induced **anhedonia** was **reversed** by treatment with **Bd extract** (ANOVAone-way: $F_{(3,19)}=13.62$; $P=0.0001$).

IMMUNOHISTOCHEMISTRY



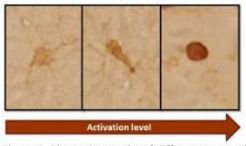


Figure 1. Photomicrographs of different stages of activated microglia (40x magnification).

ARTH increased the number of **activated microglia** in supraspinal areas modulating nociception, an effect **reversed** by the administration of **Bd extract**

Conclusions

Bd extract reversed mechanical hyperalgesia *and* ***partly reversed depressive-like behavior*** *in an experimental model of* ***monoarthritis.***

Behavioural improvements *were* ***paralleled*** *by a* ***decrease*** *in the number of* ***activated*** *supraspinal* ***microglia***

References

[1] BONNET, C. S.; WALSH, D. A. Osteoarthritis, angiogenesis and inflammation. Rheumatology, 2005, 44.1: 7-16.
[2] JI, Ru-Rong; BERTA, Temugin; NEDERGAARD, Maiken. Glia and pain: is chronic pain a gliopathy?. PAIN®, 2013, 154: S10-S28.
[3] LOGGIA, Marco L., et al. Evidence for brain glial activation in chronic pain patients. Brain, 2015, 138.3: 604-615.
[4] DOS SANTOS, Diogo A., et al. Anti-inflammatory and antinociceptive effects of Baccharis dracunculifolia DC (Asteraceae) in different experimental models. Journal of ethnopharmacology, 2010, 127.2: 543-550.

Funding

QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL
ON.2 O NOVO NORTE
European Union
European Regional Development Fund
Investing in your future

Neurosciences Research Domain
University of Minho – Portugal
www.icvs.uminho.pt